Behavioral and cortisol responses of shelter dogs to a cognitive bias test after olfactory enrichment with essential oils

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Abstract: A shelter environment tends to present different types of stressors dogs need to cope with. Recent work has shown that olfactory enrichment with essential oils might be able to modify the affective states of certain species (dogs, cats, horses, zoo animals…). In these studies, the welfare measurements included physiological indicators, such as corticosteroid levels, and/or behaviors related to chronic stress. The olfactory effects of 9 essential oils (*Cananga odorata,Cistus ladaniferus, Citrus aurantium, Cupressus sempervirens, Juniperus communis* var. *montana, Lavandula angustifolia, Laurus nobilis, Litsea citrata, Pelargonium graveolens*) and a blend of these oils were explored on a cognitive bias test, cortisol levels and the behaviors of 110 shelter dogs (n = 10 dogs within each group). Olfactory enrichment with the blend resulted in a reduced latency to the ambiguous cue, indicating a more optimistic bias and improved welfare. The results of this study suggest that olfactory enrichment with essential oils can have specific effects on the affective states and behaviors of shelter dogs, and could therefore be useful for shelter management. In addition, as not all of the essential oils tested individually were effective, more research should be conducted to better understand the effects of each individual essential oils on dogs.

Key Words: behavior; cognitive bias; cortisol; dogs; essential oils; olfactory enrichment; shelter; welfare.

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Introduction

A shelter environment tends to present different types of stressors dogs need to cope with: social stressors (reduced intraspecific and/or interspecific social contacts), environmental stressors (restraint for medical procedures, separation from a caretaker or handler) or psychogenic stressors (separation anxiety, use of aversive training methods by a previous owner/lack of ethological knowledge in caretakers). Moreover, stressors are known to cause activation of metabolic and endocrine responses in sheltered animals (Titulaer et al., 2013).

Recent work has shown that essential oils might be able to modify the affective states of certain species (dogs, cats, horses and zoo animals: Wells, 2004; Graham et al., 2005; Ferguson et al., 2013; Wells & Egli, 2015; Binks et al., 2018). In these studies, the welfare measurements included physiological indicators, such as corticosteroid levels (Beerda et al., 1998) or behaviors related to chronic stress, such as repetitive behaviors, nosing, paw-lifting, increased locomotion, displacement behavior or excessive drinking (Beerda et al., 1998; Haverbeke et al., 2008).

However, interpretation of these indicators can be difficult (Titulaer et al., 2013). Therefore the detection of a cognitive bias might be a complementary solution. A recent and innovative approach utilizes the influence of affective states on the interpretation of current experience. The resulting affect-induced cognitive biases can be measured (Mendl et al., 2009) through cognitive bias tests as indicators of the animal's psychological well-being (Mendl et al., 2009; Paul et al., 2005). A cognitive bias test in this context refers to the propensity of a subject to show

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behavior indicating the anticipation of either relatively positive or relatively negative outcomes in response to affectively ambiguous stimuli (Mendl et al., 2009). Changes in cognitive bias reflect an individual's experience of positive and negative events and thus its affective valence and welfare (Mendl et al., 2010). The effects of environmental enrichment have been already tested through cognitive bias test in different species such as rats (Brydges et al., 2011), pigs (Douglas et al., 2012) and European starling (Bateson & Matheson, 2007).

Several studies have found correlations between cognitive biases and affective states in a wide range of species, including mammals (Mendl et al., 2009; Doyle et al., 2010) and birds (Matheson et al., 2008; Salmeto et al., 2011). The aim of the current study was to assess whether olfactory enrichment through essential oils influences the affective states of sheltered dogs. To do that, the possible effects of 9 different of essential oils (*Cananga odorata, Cistus ladaniferus, Citrus aurantium, Cupressus sempervirens, Juniperus communis* var. *montana, Lavandula angustifolia, Laurus nobilis, Litsea citrata, Pelargonium graveolens*) and a blend of these oils on a cognitive bias test, cortisol levels and behavior of 110 shelter dogs were explored.

Materials and Methods

Participants

One hundred ten dogs ranging in age from 1 to 10 years, of both sexes, and of either pure or mixed breed, were enrolled in the study and randomly allocated to one of 11 different groups (Table 1). The dogs lived in groups of three in kennels with an indoor section measuring 1.5 meters x1.5 meters and an outdoor run measuring 1.5 meters x 2 meters, joined by a metal door operated by staff. Water was available *ad libitum*, and food was provided twice per day, at 8 am and 6 pm.

Dogs were selected based on the following criteria: (a) success at the training phase, (b) no previous diagnosis of anxiety or aggressive behavior, (c) some socialization prerequisites, such as the ability to deal with people without fear, (d) the veterinarian's agreement and (e) ability to walk on leash (f) good medical health.

Table 1. Description of the study protocol.

Group	Number of dogs	Pre-test training	Cognitive test 1	Exposure to collar for 3 hours	Cognitive test 2
1	10	Yes	Before exposure to collar and after collection of saliva at T0	No addition (control group)	After exposure to collar and after collection of saliva at T1
2	10	Yes	Before exposure to collar and after collection of saliva at T0	The blend	After exposure to collar and after collection of saliva at T1
3	10	Yes	Before exposure to collar and after collection of saliva at T0	*Litsea citrata*	After exposure to collar and after collection of saliva at T1
4	10	Yes	Before exposure to collar and after collection of saliva at T0	*Cupressus sempervirens*	After exposure to collar and after collection of saliva at T1
5	10	Yes	Before exposure to collar and after collection of saliva at T0	*Citrus aurantium*	After exposure to collar and after collection of saliva at T1
6	10	Yes	Before exposure to collar and after collection of saliva at T0	*Pelargonium graveolens*	After exposure to collar and after collection of saliva at T1

7	10	Yes	Before exposure to collar and after collection of saliva at T0	*Lavandula angustifolia*	After exposure to collar and after collection of saliva at T1
8	10	Yes	Before exposure to collar and after collection of saliva at T0	*Cananga odorata*	After exposure to collar and after collection of saliva at T1
9	10	Yes	Before exposure to collar and after collection of saliva at T0	*Juniperus communis* var. Montana	After exposure to collar and after collection of saliva at T1
10	10	Yes	Before exposure to collar and after collection of saliva at T0	*Cistus ladaniferus*	After exposure to collar and after collection of saliva at T1
11	10	Yes	Before exposure to collar and after collection of saliva at T0	*Laurus nobilis*	After exposure to collar and after collection of saliva at T1

Olfactory enrichment

A blend of 9 essential oils (Arhomani, Belgium) and each separate oil of the blend (Flora s.r.l., Pisa, Italy) were tested, for a total of 10 treatments and a control group (Table 1). Essential oils were diffused through a cotton collar worn by the dogs for 3 hours before starting the second cognitive test procedure (see below). The collar, just before being applied to the dog, had 1 drop of an individual oil or of the blend added to it. The control group, as the experimental groups, wore a cotton collar for 3 hours but without any oils or other addition.

During this part of the experiment, the dogs stayed in the pen where they routinely spent time. Dogs were allowed to mix in the same pen only if they were allocated to the same essential oil group. In order to avoid odour contamination, there was a distance of 500m between the different pens.

Test protocol

Cognitive test

All dogs of the 11 groups were subjected to two cognitive bias (CB) tests (modified from (Mendl et al., 2010), one prior to (CB 1) and one after essential oil exposure (CB 2). CB 2 was performed 3 hours after T0. To avoid more stress caused by a different routine in the shelter, we could not control for order effects. All the dogs were tested on the second cognitive bias test following olfactory enrichment.The protocol of (Mendl et al., 2010) and (Owczarczak-Garstecka & Burman, 2016) was modified in this study based on a pilot study we carried out, in which we observed that dogs in the shelter were unable to maintain attention during the original cognitive test as proposed in (Mendl et al., 2010) and (Owczarczak-Garstecka & Burman, 2016). For this reason, we used a shortened version. During the training session, all dogs received a minimum of 8 training trials instead of 15. During the test, we used just one ambiguous location instead of three ambiguous locations. The test phase involved 6 trials (instead of the 32 proposed by (Mendl et al., 2010).

In addition, during the pilot study, we realized that shelter dogs were much more interested in humans than in food, so we changed the original protocol by having the researcher behind the camera rather than behind the bowl put on the ground, in order to avoid the dog choosing that bowl for its closeness with a person (the researcher).

Training and cognitive tests were performed with each dog enrolled in the experiment individually led to a test area (6 meters x 6 meters) within the shelter, the same for all sessions and all dogs. The setting is described in figure 1. The bowl was placed at one of three predetermined locations (two during the training) 4 meters in front of the dog's fixed starting position. The latency to reach the bowl, was defined as the time elapsed between release from the lead and the dog putting its head into the bowl, or touching the rim of the bowl with its nose (Mendl et al., 2010). CB tests were video recorded and then analysed as described below.

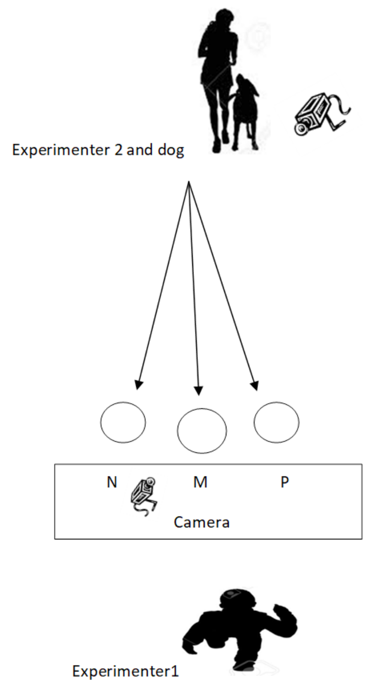

Figure 1. Experimental setting.

Training

Dogs were first trained to associate a certain location with a reward. During the training, the distance between the two bowls (Positive and Negative) was 1meter.When the bowl was placed at the 'positive' location (P) on one side of the test area, it contained food, and when it was placed at the 'negative' location (N) on the opposite side of the test area, it was empty. Two visually identical bowls were used for rewarded (P) and non-rewarded (N) locations, and both bowls had a piece of food taped to their bottom sides that were inaccessible to the dogs to control for odour cues. Training was complete when the dogs reached a pre-set criterion, that is, when the dog ran to the positive location faster than to the negative one twice consecutively.

Each training session started after a 10-minute period of habituation with the researchers in the experimental area (Figure 1). The dog was put on a lead and held by one of the researchers behind a barrier, while the other researcher stood at the far end of the room and baited (or did not bait, depending on trial type) a food bowl with 50 gr of commercial dog food. The dog was released to approach the bowl. Each dog received at least 8 training trials conducted so that no location was repeated more than twice. Each training session started with two positive (rewarded) trials to encourage participation, followed by two negative (non-rewarded) trials. The remaining trials were randomly assigned to be rewarded or non-rewarded. The latency to reach the bowl, defined as the time elapsed between release from the lead and the dog putting its head into the bowl or touching the rim of the bowl with its nose, was recorded for each trial using a stopwatch. The maximum time allowed per trial was 30 seconds. If the dog did not reach the food bowl within that time, the maximum time was scored.

Test

When the training was completed, the test started. Each dog was presented a food bowl in three locations, positive (P), negative (N) and intermediate (M).The Middle bowl was located between Positive and Negative bowl. The distance between Positive (or Negative) and Middle Bowl was 50 cm.The bowl was presented in each location twice (P1, M1, N1 and P2, M2, N2) but in different order. The accessible food was only present in the positive location (P). Negative (N) and Intermediate (M) locations remained empty but with olfactory control cues. All the tests were videorecorded. Since in each CB the locations were tested twice, we used mean values for each location in CB 1 and in CB 2 in further analyses.

Behavioral observations

The observations of dog behaviors were carried out on the videos recorded during the whole test. Each dog was observed using a continuous sampling method.

The behavioral analysis was conducted using the ethogram reported in Table 2 and 3 (Haverbeke et al., 2008). Depending on the type of behavior, either the duration (in seconds) or the number of occurrences was recorded.

Table 2. Behaviors scored in terms of number of occurrences.

Behavior	Description
Oral behaviors: Yawning	Mouth open to apparent fullest extent while eyes are closed
Non-directed licking Snout licking	Tongue out, the tip of the tongue is briefly extended Part of the tongue is shown and moved along the upper lip
No oral behaviors Paw lifting	Fore paw lifted into a position of approximately 45°
Urinatingsquat	Urinating by squatting while keeping both hind limbs on cage floor
Urinating, limbraised	Urinating while raising one hind limb
Defecating	Excreting the contents of the bowels

Table 3. Behaviors analysed in terms of duration (seconds).

Behaviors	
Repetitive or stereotypicbehavior	
Pacing	Immediately repeating a path just taken and continuing in the repetition in circles, in a figure eight pattern or fence/wall-line running
Circling	Continuous walking in short circles, apparently chasing its tail or hind limbs
Other behaviors	Manipulating environment (Stereotypic interactions with elements from the environment, such as digging (scratching the floor with the forepaws in a way that is similar to how dogs dig holes), floor licking (licking the floor with the tongue)), Auto grooming
Notseen	Unable to determine behavior of the dog owing to darkness or the position of the dog
Miscellaneousoralbehaviors	
Barking	loud, rough noise
Roaring	loud, deep sound
Growling	low, rough sound
Whining	long, high sound
Yelping	sudden, short, high sound
Panting	Increased frequency of inhalation and exhalation often in combination with the opening of the mouth
Teethclapping	Making short loud noise by hitting teeth together

Notseen	Unable to determine the behavior of the dog owing to darkness or the position of the dog
Locomotive states	
Prone, head down	Trunk of body on floor, chin or side of head in contact with the floor, paws or limbs
Prone, head up	Trunk of body on the floor, no part of the head in contact with the paws
Sitting	Only hindquarters and front paws in contact with the floor
Standing	Upright with at least three paws in contact with the floor without any walk
Walking	Takes at least one step, shifting body position
Highly active	Any motion across floor faster than a walk, including trotting and jumping
Changing from one state of locomotion to another	
Notseen	Unable to determine behavior of dog owing to darkness or the position of the dog
Postures	
High	The breed specific posture as shown by dogs under neutral conditions, but in addition the tail is positioned higher or the position of the head is elevated, and the ears are pointed forwards, or the animal is standing extremely erect
Neutral	The breed posture shown by dogs under neutral conditions
Halflow	Two or more of the following three features are displayed: a lowered position of the tail (compared to the neutral posture), a backward position of the ears and bent legs
Low	The position of the tail is lowered, the ears are positioned backwards, and the legs are bent
Verylow	Low posture, but now the tail is curled forward between the hind legs
Notseen	Unable to determine the behavior of the dog owing to darkness or the position of the dog

Cortisol

Saliva samples for the assessment of plasma cortisol concentrations were collected, at the same time in the day, before the addition of the oils at T0 (to identify the basal cortisol levels) and at T1, i.e. after 3 hours exposure to the collar for all groups, including the control one. Collection was always carried out before the cognitive bias tests at T0 and T1. Saliva samples were collected using Salivette Cortisol code blue(Sarstedt, Nümbrecht, Germany) and stored at-20 °C until they were further processed using a commercial ELISA kit (Diametra, Milano, Italy).

Statistical analysis

The statistical analysis was performed using IBM SPSS Statistics for Windows, version 22.0 (Armonk, NY: IBM Corp). For each of the oils under study, the difference in the variables measured before and after exposure was tested using a Wilcoxon signed-rank test. This paired difference test was used because each subject is measured twice, resulting in *pairs* of observations. This reduces the effect of confounders like individual differences (e.g. in pace length or in interest in food) between dogs. The test statistics (sum of positive ranks) as well as the two-sided p-values are reported in the results below. P values ≤ 0.05 were deemed statistically significant.

We additionally tested for T0 as well as T1 whether the dogs' responses during the cognitive bias tests were appropriate (i.e. dogs were slower to approach the 'negative' location N when compared to the 'positive' location P) by using a one-sided paired t-test comparing latency to approach N versus latency to approach P. Statistical p values ≤ 0.05 were deemed statistically significant.

Results

Cognitive test

We explored the dogs' latency to approach P and N, just to make sure dogs' response to the CB test 1 (before exposure) and CB test 2 (after exposure) was appropriate (i.e. animals were slower to approach N than P). The results are reported in Table 4.

Table 4. Statistical results of the comparison between latency to reach positive and negative locations before exposure and after exposure to essential oils (CB 1: Cognitive test before exposure; CB 2: Cognitive test after exposure).

		Mean (seconds)	N	Standard. Deviation	Standard Error Mean
CB 1	Latency before exposure P location	19.32	110	17.01	1.62
	Latency before exposure N location	24.18	110	27.28	2.60
CB 2	Latency after exposure P location	2.64	110	0.48	0.05
	Latency after exposure N location	13.81	110	13.53	1.29

The analysis revealed a significant effect of the blend "The blend" in reducing the latency to reach the intermediate position (test statistic=3; n=10; p=0.039). We also observed a trend towards reducing the latency to reach the intermediate position (test statistic=5; n=10; p=0.078) for *Litsea citrata* oil (Table 5).

Table 5. Latency (mean ± Standard Deviation in seconds before and after 3 hours of exposure) and cortisol values (mean ± Standard Deviation in ng/ml before and after 3 hours of exposure) to each essential oil or after 3 hours without any exposure in the control group (P < 0.05, *).

	Before exposure	After 3 hours of exposure (T1)	Statistical results	Before exposure	After 3 hours of exposure (T1)	Statistical results
	Latency value (seconds)			Cortisol value (ng/ml)		
Control group (no exposure)	20.60±11.00	15.95±10.98	P=0.38	2.406 ± 0.30*	1.762 ± 0.435	P=0.03*
Cananga odorata	18.65±7.84	16.92±9.35	P=0.84	1.923 ± 0.70	1.512 ± 0.111	P=0.08
Cistus ladaniferus	18.77±11.78	14.98±11.93	P=0.54	1.538 ± 0.22	1.424 ± 0.132	P=0.18
Citrus aurantium	17.22±12.48	11.21±11.98	P=0.35	1.642 ± 0.21	1.507 ± 0.196	P=0.43
Cupres sussempervirens	24.47±7.14	18.46±11.32	P=0.19	1.766 ± 0.58	2.175 ± 0.424	P=0.12
Juniperus communis var. Montana	21.93±9.30	14.06±13.71	P=0.20	1.397 ± 0.30	1.497 ± 0.364	P=0.74
Laurus nobilis	20.80 ±11.35	15.45±9.59	P=0.10	1.082 ± 0.45	1.435 ± 0.198	P=0.14
Lavandula angustifolia	22.19±9.60	16.70±14.08	P=0.29	1.821 ± 0.39*	1.549 ± 0.245	P=0.03*
Litsea citrate	21.97±9.34	14.70±10.39	P=0.078	1.467 ± 0.30	1.919 ± 0.313	P=0.078
Pelargonium graveolens	20.74±9.58	15.48±10.70	P=0.10	1.287 ± 0.33	1.596 ± 0.504	P=0.10
The blend	23.83±9.80	13.46±11.28	P=0.039*	1.557 ± 0.49	1.316 ± 0.119	P=0.25

Behavioral observations

Only the olfactory enrichment with *Laurus nobilis* induced a significantly longer duration of high posture among these dogs (test statistic=26.5; n=10; p=0.047).

The analysis revealed non-significant trends for different oils: *Cananga odorata* reduced the "nosing" time (test statistic=9; n=10; p=0.064), *Citrus aurantium* (test statistic=46; n=10; p=0.064) and *Cupressus sempervirens* (test statistic=39; n=10; p=0.055) increased the time spent in "tail wagging", and *Pelargonium graveolens* (test statistic=3; n=10; p=0.078) reduced the time spent in "non-oral stress behaviors" (circling, pacing, manipulation of environment, autogrooming).

Cortisol

Olfactory enrichment with *Lavandula angustifolia* induced a significant reduction in saliva cortisol levels (test statistic=3; n=8; p=0.039). A similar significant reduction was also found in the control group (test statistic= 0; n=6; p=0.031) (Table 5).

Discussion

Cognitive test

In the present study, authors applied a cognitive test to evaluate the effectiveness of olfactory enrichment with essential oils in reducing the level of stress in sheltered dogs. Olfactory enrichment with the blend of oils resulted in a reduced latency to the ambiguous cue in the cognitive test, indicating a more optimistic bias and, consequently, an improved welfare (Mendl et al., 2010). These results provide support for the idea that the interactions between compounds often result in biological activity that is greater than the activity of the isolated compounds(Galindo et al., 2010).

Many domestic dogs are kept in rescue and rehoming shelters which are frequently stressful and impoverished environments. Dog's welfare is often compromised within these environments and there is a need to determine new practical and effective methods to improve the welfare of these kenneled dogs (Binks et al., 2018).The development of objective methods to assess the affective states of non-human animals is a crucial step in improving animal welfare (e.g. Dawkins, 2008.). Mendl (Mendl et al., 2009) enumerated several potential advantages of the cognitive bias test, including the ability to make *a priori* predictions for different species: mammals (Mendl et al., 2009; Doyle et al., 2010), birds (Matheson et al., 2008; Salmeto et al., 2011) and insects (Salmeto et al., 2011). Douglas et al. (2012) support the hypothesis that an enriched environment induces a more optimistic cognitive bias indicative of a more positive affective state and better welfare in pigs.

Negative effects from inadequate environmental manipulations have been investigated by several researchers. Environmental manipulations chosen to induce negative effect produce pessimistic cognitive biases in animals' responses to ambiguous stimuli (reviewed in (Mendl et al., 2009)). Rats show pessimistic responses when housed in impoverished cages but switch to optimistic responses when moved to enriched cages (Brydges et al., 2011).

To our knowledge, this is the first time that a cognitive bias test has been applied to assess the effect of olfactory enrichment with essential oils. Although this is a first study on this topic and the number of dogs tested in each experimental group was relatively low, it is remarkable that the statistical analysis revealed some significant differences. In particular, the results regarding the blend of oils are in line with previous studies that reported improved optimism through environmental enrichment (Douglas et al., 2012) in pigs.

However, we should take into account that the medium latency for reaching the positive location in the cognitive bias test 2 (P2- after exposure) is significantly lower than the medium latency for reaching the positive location in test 1 (P1- before exposure). So although the dogs respond appropriate to each CB-test (i.e. approaching N significantly slower than P), they approach both P and N significantly faster during CB2 than during CB1, which might suggest some eagerness to participate in the test.

Behavioral observations

The results of the present study indicate that olfactory enrichment with *Laurus nobilis* induced high posture among dogs. In volatiles, *Laurus nobilis* has been reported to improve vigilance performance in a discrimination task (Matsubara et al., 2011), which could be interpreted positively as increased self-confidence (Fatjó et al., 2007) or alternatively as a heightened alertness due to a negative state. In humans, a high individual variability in response to olfactory exposure to 1,8-cineol (major component of *Laurus nobilis*), jasmine absolute ether, linalyl acetate and peppermint essential oil has been observed on vigilance (Heuberger & Ilmberger, 2010). In the present study, dog's high posture after olfactory exposure with *Laurus nobilis*is not accompanied by other signs of alertness and can therefore be interpreted as a sign of improved self-confidence in line with Haverbeke et al. (2008).

Some essential oils had a slight effect on behavior. Olfactory enrichment with *Cananga odorata* reduced the "nosing" time. This could indicate a decrease of stress among the dogs (Beerda et al., 1998). In line with these interpretations, Hongratanaworakit and Buchbauer (2004) showed that in humans *Cananga odorata* decreases blood pressure and pulse rate and increases subjective attentiveness and alertness. Olfactory enrichment with *Citrus aurantium* and *Cupressus sempervirens* increased the time spent in "tail wagging". Tail wagging can be seen in the interactive social context or to facilitate interaction and could have ambivalent interpretations going from an increase to a decrease in confidence in dogs (Gasci et al., 2005). In the present study, the exposure of dogs to *Citrus aurantium* and *Cupressus sempervirens* are not accompanied by other changes. Therefore it is likely that in this study tail wagging is a sign of relaxation. This is in line with previous results that have demonstrated anti-anxiety effects of both *Citrus aurantium* (in humans (Akhlaghi et al., 2011; Carvalho-Freitas et al., 2002; De Moraes Pultrini et al., 2006) and rats (Leite et al., 2008) and *Cupressus sempervirens*(in humans Bouguenoun et al., 2006).

Olfactory enrichment with *Pelargonium graveolens* reduced the time spent in "non-oral stress behaviors" (circling, pacing, manipulation of environment, autogrooming). This observed reduction of stress behaviors (Haverbeke et al., 2008) is in line with the findings of Rashidi Fakari et al., 2015, who observed an anxiolytic and sedative effect of *Pelargonium graveolens* in humans.

Cortisol

The observed reduction in saliva cortisol with *Lavandula angustifolia*is in line with Atsumi & Tonosaki who have observed a decrease of salivary cortisol level on humans after smelling lavender essential oil (Atsumi & Tonosaki, 2007). In addition, a previous study using olfactory enrichment with *Lavandula angustifolia* on sheltered dogs showed a change in dogs' activities (resting time) suggestive of relaxation (Graham et al., 2005).

We also observed a reduction of cortisol levels in the control group. This finding is in line with previous research in dogs (Shiverdecker et al., 2013; Cobb et al., 2016). One possible explanation is that, the mere application of a cognitive test can result in a stress relieving factor, being a sort of cognitive enrichment for sheltered dogs. However, this does not explain why the cognitive test with essential oil exposure had no effect on cortisol levels except in the *Lavandula angustifolia* group. Another explanation is that essential oils (except *Lavandula angustifolia*) has increased neophobia (i.e. the fear of novelty, which can be sometimes observed in captive animals that have received little or no previous novel sensory stimulation (Mason et al., 1991) as observed in Goeldi's monkeys exposed to peppermint oil (Boon, 2003) and in a young tiger exposed to catnip

(Todd, 2015). However, as dogs belong to a species who tends to be very neophilic (Kaulfuss & Mills, 2008) this explanation might probably not be considered for the canine species.As the interpretation remains open, further studies are required in order to demonstrate through a more detailed and rigorous analysis the effects of *Lavandula angustifolia* essential oil on cortisol levels versus the effects of the other essential oils.

Although saliva collection was carried out at different times of the day, it is unlikely that the differences we observed were influenced by this. In fact, previous research has not found a circadian rhythm in the HPA (Hypothalamic Pituitary Adrenal) activity of dogs: neither in laboratory dogs at 30 minutes intervals over a period of 28 hours (Takahashi et al., 1981) nor at 20 minutes intervals over a period of 25 hours (Kemppainen & Sartin, 1994), nor in working dogs exposed to defense training and trailing tasks at 90-180 minutes intervals over a period of 24 hours (Kolevska et al., 2003).

The saliva cortisol collected after T1 could not have reflected an earlier emotional state (pre-olfactory enrichment), because cortisol concentrations rise approximately 20 minutes after a dog encounters a stressor (Vincent & Michell, 1992). Moreover, previous authors (Kirschbaum & Hellhammer, 2000) have shown that changes in plasma and salivary cortisol levels are closely synchronized: after injections of cortisol, salivary levels increase within 1 minute and peak concentrations in blood are seen 2-3 minutes later in saliva.

Some methodological limitations have been encountered during this study. Firstly, we used a short version of the cognitive bias test because the sheltered dogs were not able to perform the longer test (author's observations in an unpublished, pilot study). Their limited performance might be due to the fact that these dogs were not accustomed to be involved in cognitive activities in their actual environment (presence of physical and social stimuli).

Secondly, being a study carried out in the field and not in a laboratory setting, many factors could not be controlled. For instance, there is a potential risk of olfactory confounding effects. However, in order to reduce the risk of crossed stimulation among different essential oils groups, a distance of 500 meters from one pen to another was set. Further, in a shelter environment the quantity of olfactory stimuli is high and similar for all dogs. Thus a possible effect of odours other than essential oils should be equally distributed for all dogs, which is not the case in the present study. Lastly, even if we might consider any olfactory confounding effect, the main olfactory effect should still remain the one obtained by the tested essential oil as it is the most proximate odour from the dog's nose. In order to confirm our results, further research should investigate the maximum or minimum distance necessary to create an olfactory effect with essential oils.

Thirdly, the findings should be interpreted with caution because it is possible that the dogs' behavior was influenced by a learning effect and a decreased interest because the cognitive bias test was repeated twice. However, each time that a cognitive bias test is being used, information processing, including attention, learning, memory and decision-making is being addressed (Mendl et al., 2009).

Fourthly, in our study all the dogs were tested on the second cognitive bias test following olfactory enrichment. Unfortunately, we could not control for any order effects, because a different protocol would have caused changes in the shelter routine and therefore additional stress for the shelter dogs and the staff. Nevertheless, as the order was the same for the tested dogs, the results of a potential order effect should be the same for all groups. The different findings observed in sub samples suggest that essential oils have different effects: this could be a combination of essential oils' stimulation and repetition of the test. Further research should investigate the effects of single essential oils in different conditions.The tendencies or significant decreases that are found in different behaviors in various groups could be caused by an increase in confidence the dogs experienced in the second CB (they were familiar with the CB and might have been eager to participate and enjoy human contact or the enrichment).If a design would be applied in which 50% of the dogs start with essential oils (group 1) and 50% of the dogs without essential oils (group 2), it is not possible to conduct the control-CB within the same day as the essential oil-CB for the first group. It is quite likely that after 3 hours of exposure to essential oils, an effect of essential oils would still be present during the control-CB. Conducting the control-CB at another day would generate a confounding effect of day.

Lastly, we should take into account that Galindo (Galindo et al., 2010) affirmed that effects of essential oils can vary considerably depending on the dosage. In our study, we used the same dosage for each oil. Further studies will need to focus on the effects which obtained by diffusing different concentrations of essential oils.

Conclusions

These preliminary results suggest that olfactory enrichment with essential oils can influence the affective states and behaviors of shelter dogs. More research is needed to understand the impact of each individual essential oil and its effect on dog's welfare, considering possible factors affecting their influence, including individual factors or different concentrations of the essential oils.

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Author Contributions

The idea for the paper was conceived by Haverbeke A. and Uccheddu S. The experimental protocol was designed by Uccheddu S., Haverbeke A. and Mariti C. The data were statistically analysed by Arnouts H. and Sannen A. and discussed by all authors. The videos were analysed by Gutierrez Rufo J. The cortisol concentration in the saliva was analysed by Mariti C. and Gazzano A.. The paper was written by Uccheddu S. and Haverbeke A. and discussed by all authors.

Conflicts of Interest

There could be a potential conflict of interest because Haverbeke A. has selected the composition of the oils of the blend. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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Risposte comportamentali e del cortisolo di cani di canile sottoposti ad un "Cognitive bias test" dopo arricchimento olfattivo con olii essenziali

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Sintesi

L'ambiente di canile comporta per gli animali diverse forme di stress a cui i cani devono adattarsi. Recenti ricerche hanno dimostrato che l'arricchimento con olii essenziali potrebbe essere in grado di modificare lo stato emozionale di certe specie animali (cani, gatti, animali di zoo..). In questi studi la valutazione del welfare includeva indicatori fisiologici, come ad esempio le concentrazioni di corticosteroidi e/o comportamenti correlati allo stress cronico.

L'effetto olfattorio di 9 olii essenziali (*Cananga odorata, Cistus ladaniferus, Citrus aurantium, Cupressus sempervirens, Juniperus communis* var. *montana, Lavandula angustifolia, Laurus nobilis, Litsea citrata, Pelargonium graveolens*) e di una miscela di questi olii è stato valutato sui risultati di un "Cognitive bias test", sui livelli di cortisolo e sul comportamento di 110 cani di canile (n= 10 cani per ogni gruppo).

L'arricchimento olfattivo con la miscela di olii ha ridotto la latenza della scelta dello stimolo ambiguo, indicando un pregiudizio ottimistico ed un miglioramento del welfare.

I risultati di questo studio suggeriscono che l'arricchimento olfattivo con olii essenziali può avere un effetto specifico sullo stato emozionale e sul comportamento dei cani di canile e potrebbe perciò essere utile nel management di queste strutture.

Inoltre, poiché non tutti gli olii testati singolarmente si sono dimostrati efficaci, ulteriori ricerche dovrebbero essere effettuate per comprendere meglio gli effetti dei singoli olii sul cane.