Using Scalar Products to Refine the Interpretative Value of an Orientation Choice Test

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Abstract

Choice tests, which are often used to examine animal preferences, can be difficult to interpret when no clear choice has been made or when using very young animals which exclude test repetition. We present a new method to evaluate the behavior in a choice test based on the orientation of the animal and illustrate its use when facing those conditions. Using rat pups in an open field maze with a choice of odors, we obtained $x,y$ coordinates of 2 markers (head and body center) using a video-tracking freeware. Two vectors were calculated: an animal orientation vector (body to head) and a perfect orientation vector (body to odor source). The angle between the 2 vectors in each frame was converted into a scalar product ranging from 1 (pup oriented directly towards the odor source) to −1 (facing the opposite direction). A mean scalar product was calculated for each odor source, with the difference between the 2 mean scalar products indicating degree of preference for an odor. The information provided by the mean scalar product difference (MSPD) could not be obtained from other measures, such as binary choice, velocity, or distance moved. The MSPD provides a single, noncategorical value for each animal to describe degree of preference in a choice test. This variable was more effective in differentiating animals, thus allowing a reduction in the number of animals or tests necessary to reach significance.

Key words: attraction, direction of movement, immature rats, odor preference, open field maze, video tracking

Introduction

The choice test is a widely used method to examine animal behavior and is often used to investigate the ability of an animal to distinguish between odors and to reveal odor preferences (Larson and Sieprawska 2002; Dreumont-Boudreau et al. 2006). Several measures are available to quantify the result in a choice test of which the simplest is the binomial score of correct choice within a test (yes, no). However, it necessitates either a large number of animals or many tests per animal. Skinner et al. (2003) used an open field maze to train rats until they met a criterion of 18 out of 20 correct choices with more than 200 tests per animal. Repeated testing, apart from being time consuming, may result in side effects unwanted for a particular research question, such as laterality or habituation. In addition, repeated testing is not an option when using very young animals with a need for frequent rest and feeding (Al Ain et al. 2013). Other measures can be registered during a choice test, such as the latency to make a choice, but it requires that the test animal makes a choice. Non-choosing animals often leads to a censoring of data, as these subjects cannot be allocated a choice latency within the duration of the test (Faes et al. 2010). This reduces the strength of the test and the information available and may therefore require more animals to be tested.

There is therefore a need for a method, which can express the degree of preference in a choice test, even when no direct choice is made. Measurement of spatial orientation and head direction may be useful for the interpretation of the behavior observed during a test, and information on direction of movement can be obtained from various types of tracking software with different degrees of automation. However, there is no universally agreed and used method to describe degree of preference in a choice test. We describe here an original method to quantify in a non-categorical manner the relative preference of rat pups in a choice test by applying an existing mathematical technique (scalar products) to data on direction of orientation obtained from a freely available tracking program. The validity and usefulness of the method are assessed through comparisons with a variety of measures classically used in choice tests.
Material and methods

Animals

A total of 60 Wistar rat pups from 6 litters were used. Each litter (40% ≤ males ≤ 60%) were housed with their dam in a standard laboratory plastic cage with a metal grid lid. The pups were tested when 9–11 days old and had not yet opened their eyes. Each litter was separated from their dam 45–60 min before the start of the testing and placed in a plastic cage with clean sawdust as bedding and a lamp to provide heating. Feces pellets from the dam were collected in the home cage at this point in time. The pups were weighed and, using low-odor markers (UniPosca; Mitsubishi Pencil Co. Ltd, Worcester, UK), each given an individual identification number on the abdomen. Also, 2 black dots were drawn on the dorsal midline of each pup, one on the top of the head (head marker) and one on the lower back (body marker; see Figure 1).

Odorant choice test

The test arena is shown in Figure 1. It consisted of a bottomless, rectangular box ($L \times W \times H$: 29.7 × 18.7 × 5.5 cm$^3$) made of transparent solid plastic. The box was fitted with a transparent lid hinged at one end to allow easy access. A small fan (40 × 40 × 10 mm$^3$, 28 L/min capacity) was fitted in the center of the short side of the arena farthest from the odor sources and air from the box was extracted (equivalent to 9 box volumes per min). At the other end on each side of the box, 2 perpendicular arms were fitted ($L \times W \times H$: 16.8 × 6.8 × 5.5 cm$^3$) to form a T. The rat pups could not access the arms during the test, but the sides in front of each arm were perforated allowing odors to enter the box. The test arena was heated using 2 heat lamps, one on each side to ensure an even distribution of light and heat. A video camera was positioned above the center of the test arena and all tests were filmed at 15 frames per second.

Two different odor sources were used for testing (see below), one in each of the 2 arms of the test arena. The odorants were drawn into the arena by the fan, creating a Y-shaped odor trail (which we confirmed prior to the tests by observing the vapor trail released by dry ice in place of odors). Before the start of each test, a sheet of A4 paper was placed underneath the test box, covering the entire floor. Each sheet had a circle printed at the center (see Figure 1) to ensure the same starting position within the arena for all test animals.

For the test, each rat pup was gently placed in the center of the circle and allowed to move freely within the box. The test ended when the nose of the pup touched one of the perforated areas on the side of the box (henceforth called a choice) or after 1 min, if no choice was made. The reason for stopping the test as soon as a choice had been made was to prevent the inclusion of tracking data from movements made by the pup after choosing. The test order was random within litter, and when all pups in a litter had been tested, they were returned to their dam.

Different combinations of odors were used. In Exp. 1, 4 litters were tested with feces from their own dam in one arm,
and either clean saw dust (litters 1 and 2; \( n = 24 \)) or feces from another dam (litters 3 and 4; \( n = 20 \)) in the other arm, as rat pups have been found to show a preference for the feces of their mothers (Brown and Elrick 1983). In previous trials using the open field maze, we sometimes (François et al. 2013) but not always obtained clear-cut preferences for maternal feces, the latter type of data being exactly what we needed to develop and validate our method. Hence, the data from Exp. 1 were used to develop the scalar product method, and in order to test the method with different, but still biologically relevant odors, 2 additional experiments were carried out. In Exp. 2, litters 1 and 2 were tested twice on 2 consecutive days and given a choice of 2 single-molecule odors (6-methyl-5-hepten-2-one versus 1-hexanol; compounds purchased from Sigma-Aldrich) and a choice of 2 types of female rat feces (estrus or di-estrus determined from vaginal smears; Yener et al. 2007) harvested from adult, nulliparous Brown Norway females (see Nielsen et al. 2011 for details). Defrosted pellets from 3 or 4 females were used in the test of the pups. Estrus and di-estrus rat feces were also used as odors in Exp. 3 (litters 5 and 6; \( n = 16 \)). These odor sources were chosen as rat pups have been found to be attracted to the smell of estrus but not di-estrus females (Fillion and Blass 1986). Furthermore, 6-methyl-5-hepten-2-one (methylheptenone) has been shown to be a significant part of the estrus smell (Nielsen et al. 2013), and both this molecule and 1-hexanol (herb odor) are present in rat feces (Nielsen et al. 2011). To our knowledge, these odors had never before been used in a choice test with rat pups, and we did not expect all of the pups to make a choice, which would leave the data suitable for assessing the value of our method.

All procedures carried out in these experiments were approved by the local ethics committee (COMETHEA; Comité d’éthique appliqué à l’expérimentation animale; Avis 12/069) and carried out in accordance with current European legislation (EU Directive 2010) and ethical guidelines (Sherwin et al. 2003).

**Video tracking**

The movement of each rat during the test was traced using a freely available tracking program (Kinovea 0.8.15; www.kinovea.org). The 2 dorsal marker points were used to track 2 separate trajectories for the head and the body of the pup; an example is shown in Figure 2a in red and blue, respectively. The data consisted of frame-by-frame coordinates (15 frames/s) of the 2 marker points in pixels relative to the bottom left hand corner of the test arena designated as the origin.

**Data collection and analysis**

For all test videos, it was determined whether a choice had been made and, if so, which one as well as choice latency. For each video tracking, the coordinates of the center of each odor source (the midpoint of the perforated part of the box side leading to the odor source) were registered, together with the coordinates of the head and body marker of the animal in each video frame. Based on the tracking data from the head marker, we calculated the time spent in each quadrant of the box, the distance moved (14 pixels = 1 cm), and the mean velocity during the test.

We used the body marker coordinates to calculate (for each video frame) 2 types of vectors: an animal orientation vector (AOV), which takes its origin at the body marker and goes through the head marker (Figure 1; eq. 1), and a perfect orientation vector (POV), which takes its origin at the body marker, and is oriented toward the center of one of the 2 odor sources (Figure 1; eq. 2). One POV was calculated for each odor source. If, for any given frame, the AOV is equal to one of the POVs, the animal is at that moment oriented directly toward the corresponding odor, and the angle between these 2 vectors is 0. The angle between each POV and the AOV in each video frame was converted into a scalar product (equal to the cosine of the angle between the 2 vectors of unit length; eq. 3; Figures 1 and 2b,c) ranging from 1 when the pup was oriented directly toward the odor and to −1 when facing the opposite direction.

\[
\text{AOVangle} = \text{atan2}(X_h - X_b, Y_h - Y_b) \\
\text{POVangle} = \text{atan2}(X_t - X_b, Y_t - Y_b) \\
\text{Scalar product} = \cos(\text{POVangle} - \text{AOVangle})
\]

where AOVangle is the angle in radians of the AOV relative to the positive x axis; POVangle is the angle of the POV relative to the positive x axis; \( X_h \) and \( Y_h \) are the coordinates of the head marker; \( X_b \) and \( Y_b \) are the coordinates of the body marker; and \( X_t \) and \( Y_t \) are the coordinates of the odor origin. The function atan2 is the arctangent with 2 arguments, where atan2(\( x, y \)) is the angle in radians between the positive x axis of a plane and the point given by the coordinates (\( x, y \)) on it.

If the pup made a choice within 1 min, the remaining frames were allocated a scalar product of 1 for the odor chosen and −1 for the other odor. This was done for 2 reasons: 1) to obtain the scalar products from the same number of frames for all rats independent of whether a choice was made or not and 2) to integrate choice latency into the scalar product in a way which did not punish slow animals moving in the direction of an odor source and, at the same time, rewarded animals that made a fast choice. For each frame, the difference between the scalar products for each odor was calculated and the mean scalar product difference (MSPD) was calculated for each pup (this is equivalent to the difference between the means of the 2 scalar products). It is important to note that this method does not require one of the odors to be appointed status as the correct choice. The MSPD ranges from −2 to 2 and if its value is significantly different from 0, it will indicate a greater orientation
of a pup toward one of the 2 odor sources (positive or negative according to the preferred odor; Figure 2d).

Data from the 3 experiments were analyzed in Minitab (Minitab ver. 12.2) using one-sample $T$-tests and, when assumptions for parametric testing were not met, one-sample Wilcoxon signed rank test and chi-square. Normal distributions of data were tested using the Anderson–Darling normality test. For Exp. 1, correlation and regression
analyses were carried out between the MSPD and other variables, fitting different intercepts for each litter in the analyses to account for any between-litter variation.

Results

Relationships between the MSPD and classical choice test measures

In Exp. 1, we tested the attractiveness of maternal fecal odors to the rat pups when given a choice between maternal fecal pellets and another odor. Based on the choices made by the pups, no overall preference of maternal odor was found as of the 44 pups tested, 17 chose the maternal odor source, 14 chose the other odor, and the remaining 13 moved about the arena but did not make a choice. Across litters, the choice was not dependent on the sex of the pup ($\chi^2 = 0.421; df = 2; P = 0.810$).

The MSPD was calculated for each pup and is shown for each litter in Figure 3. Overall, the data were normally distributed ($A^2 = 0.603; P = 0.110$) and no significant preference for maternal odor was found (MSPD mean ± SE: median $= 0.172; P = 0.127; T = 0.75; P = 0.460$). Within-litter tests of MSPD medians showed one of the 4 litters (litter 2) to have a median significantly different from 0 (Wilcoxon MSPD median = 0.172; $P = 0.021$). However, it is clear from the figure that the degree of preference for maternal odor differs between rat pups, with some non-choosing pups achieving higher MSPD values than pups choosing the maternal odor, both within and across litters. The MSPD thus allows a more detailed differentiation between pups than a crude division into choosers (maternal or other) and non-choosers.

Figures 4a–d show the relationship between the MSPD and other measures commonly used to quantify the results of a choice test, as well as the live weight of the pups. The latter was included as a proxy for the vigor of each rat pup. These relationships were analyzed not to imply a biological relationship between them but to investigate if the MSPD was merely a different way to describe these other variables. However, no systematic or significant relationships were found between the MSPD and any of these 4 measures (distance moved: $R^2 = 7.8\%; F_{4,39} = 0.83; P = 0.516$; velocity: $R^2 = 9.5\%; F_{4,39} = 1.02; P = 0.408$; percentage of test duration spent in arena quadrant nearest maternal odor: $R^2 = 9.8\%; F_{4,39} = 1.06; P = 0.388$; live weight: $R^2 = 6.0\%; F_{4,39} = 0.62; P = 0.650$). These results can be compared to the significant, positive relationship found between velocity and live weight (with a velocity increase of 5.2 cm/min for each 1 g increment in live weight), confirming the latter as a proxy for pup vivacity ($R^2 = 75.5\%; F_{4,39} = 30.0; P < 0.001$).

For most of the choosing pups, the sign of the MSPD values (positive or negative) correspond to the choice of maternal or other odor, respectively. However, one of the pups choosing the maternal odor achieved a negative MSPD (litter 2; Figure 3). To investigate this further, the scalar product for the maternal odor for this particular pup is shown in Figure 5a,b. From these figures, it is apparent that the pup spent a large proportion of the test facing away from the maternal odor, while making weaving movements with the head before eventually picking up the scent trail and moving toward the maternal odor. This illustrates how the MSPD takes into account the length of time taken to orientate toward a stimulus: the fast approach toward the maternal odor. This illustrates how the MSPD takes into account the length of time taken to orientate toward a stimulus: the fast approach toward the maternal odor at the end of the test did not make up for the time taken to pick up the trail for this pup.

The MSPD differentiates animals in a choice test better than other measures

In the second set of experiments, we aimed at making the choice test as hard as possible for pups. If our method improves the extraction of information from the tests, the results should be at the limit of detection with classical methods. We therefore tested the rat pups using nonmaternal odors with biological relevance for rats in Exp 2 and 3.

In Exp. 2, no differences in MSPD were found between the 2 litters within each odor pair and the data were pooled across litters. When tested with methylheptenone and herb odor, 9 pups out of 24 chose methylheptenone (38%) and only one pup chose herb odor, with 14 pups not choosing any odor. A significant preference for methylheptenone would only be seen...
if non-choosing individuals were excluded (9 out of 10; 90%). In contrast, the MSPD for these pups are shown in Figure 6, and a significant preference for methylheptenone was found (Wilcoxon MSPD median = 0.255; $P = 0.018$). Also, the MSPD enables us to distinguish between the pups within each choice category, with a large spread in MSPD values both within the methylheptenone choosing pups and within the non-choosers.

When the same rat pups were tested with estrus or di-estrus feces from female rats, no significant preference was found for any of these odors (Wilcoxon MSPD median = −0.072; $P = 0.420$).
Figure 5  (a) Orientation plot of head tracking for the rat pup, which chose the maternal odor, but had a negative MSPD; note the weaving head movements visible in the bottom half of the figure, the blue color indicating that the pup is facing away from the target odor; (b) scalar product for the maternal odor over the test for the same pup as shown in (a); the maternal odor was reached after 48.7 s, and the remaining time was allocated a scalar product of 1, indicated by the stippled continuation of the curve.
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P = 0.520). Seven pups chose estrus and 7 chose di-estrus odor, with 10 pups not choosing any odor (Exp. 2; Figure 6).

In Exp. 3, we repeated the test with different samples of feces from female rats in either estrus or di-estrus using 16 rat pups from 2 litters. Again, no differences in MSPD were found between the 2 litters and the data were pooled. Four pups chose estrus odor and 12 pups did not make a choice. The MSPD for these pups showed a significant preference for estrus odor (Wilcoxon MSPD median = 0.274; P = 0.001; Figure 6). If we included only the 12 non-choosing pups in the MSPD analysis, a significant preference for estrus odor was still apparent (Wilcoxon MSPD median = 0.131; P = 0.011). The MSPD thus performs better than methods based on categorical choices by revealing latent preferences even when no direct choice is made.

Discussion

In behavioral choice tests, the orientation of the animal toward a given stimulus is likely to reflect the attractiveness of this stimulus relative to other stimuli presented at the same time. This is particularly relevant when the stimuli are different odors but may also apply to visual stimuli. We wanted to develop a method that could quantify this orientation, preferably as an accumulated measure over the test period expressed as a single, noncategorical value, but without a priori assigning one of the possible choices as correct. To achieve this, we applied a well-known mathematical method—the scalar product—to data obtained from the tracking of 2 body markers on a frame-by-frame basis. This allowed us to calculate the mean scalar product to describe the average direction of orientation relative to each odor source and to express the relative orientation between 2 odors as one, noncategorical value, the MSPD, which by inference describe the relative preference of the 2 odors presented.

Properties of the MSPD

The first thing to note about the scalar product is that it is not linearly related to the angle between the 2 vectors (Figure 2b). This means that a higher scalar product value is reached when the animal is orientated even remotely toward one of the stimulus choices than if a straight linear conversion of the angle had been used. Similarly, any orientation away from the stimulus (more than 90° in any direction) is penalized by a larger negative value. The use of an open field maze was chosen to allow the experimental animals to move freely within the experimental set-up, thus enabling us to study their movements in more detail than is usually the case in the constrained environment of the standard T-maze. Placing the pup at the cross-point of the Y-shaped odor plume at the start of the test maximizes the likelihood that the pup is exposed to a mixture of the 2 odors, with each arm of the Y leaving a single-odor trail to the source. If the pup moves away from the odor sources toward the fan-end of the maze, the odor trail would be highly diluted, and orientation toward the stem of the Y-shaped plume comprising a diluted mixture of odorants would not be an expression of preference for either odor.

The second thing to note is that the MSPD can be obtained relatively easy. Tracking the movements of the test animal using dedicated computer software usually involves registering the x,y coordinates of one point on the animal; these data are often used to calculate distance moved and mean velocity during the test. In the present study, the coordinates over time of 2 points were obtained simultaneously using freely available software. Tracking 2 markers on the test animal is as quick as tracking one, and allows the data to be used to calculate the scalar products over time. Other tracking programs may yield the same type of data without the need to mark the animal (de Chaumont et al. 2012); this may be useful when using pigmented rodent strains or other animal species.

When using a behavioral test involving olfaction, the result may reflect 1) the animal’s ability to detect and discriminate odors, 2) the relative attraction of (preference for) the odors, 3) differences between animals in their motivation to search for odors. As we tested all rat pups under similar conditions,
the motivation and ability to detect and discriminate by the pups can be assumed equal. Orientation toward one odor thus reflects its attractiveness relative to the other odor, and calculating a scalar product for each odor therefore allows us to quantify the relative preference of the pup for the 2 odors, expressed as the difference (MSPD). The MSPD takes into account the speed with which an odor is located by adding for the remainder of the test a bonus of 1 to the scalar product of the chosen odor, and a penalty of $-1$ to the scalar product of the non-chosen odor. At the same time, it does not punish slower moving individuals as long as they are facing in the right direction. This is particularly important when using very young animals (Lee et al. 2011; Hammock et al. 2013). The addition of bonus and penalty points could potentially push the data toward a bimodal distribution; however, this would only be the case when a large proportion of the pups make a choice (as nothing is added when no choice is made and the test lasts 1 min), and more so if the choice is made quickly (as a larger number of frames are allocated bonus and penalty points). In experiments where this is happening, there will be no need to apply the MSPD as a method, because the pups tested will have split into 2 easily distinguishable groups based on their choice of odors. Thus, when relatively clear choices are seen, a simpler method than the MSPD may suffice.

Finally, when we compared the MSPD with 4 other measures obtained before or during testing, (distance moved, velocity, % time spent near maternal odor, and live weight), no relationship was found between the MSPD and any of these variables. It is important to keep in mind that the regression analyses were performed not to indicate a biological relationship between the MSPD and these 4 measures but to demonstrate that the MSPD is not simply another way of quantifying a characteristic that could have been extracted using a simpler method. We are thus not implying that, say, mean velocity can be used to indicate odor preference, but simply showing that MSPD is independent of commonly used measures obtained when using choice tests. The MSPD therefore provides additional information to be included in the analyses of choice tests.

What does the MSPD method add compared to other analyses of choice tests?

Other measures are available to quantify the result of a choice test. The most widely used is the binomial score of choice (A or B) within a test, which requires a choice to be made (Martin and Bateson 2007). Some of the problems associated with binomial and other categorical variables can be overcome by using continuous variables, which are easily obtained such as time taken to reach target. However, in the case where only one or a few tests are being carried out per animal, the likelihood remains that a test animal does not make a choice. These animals are often allocated a fixed (and nonsensical) time to reach target of “test duration + 1 s,” causing bias in the analysis, or the data are right-censored (non-choosers excluded), which reduces the information available (Faes et al. 2010). The MSPD was able to reveal latent preferences even when no clear choices were made. This indicates that the use of scalar products could reduce the number of animals necessary in choice tests. The reduction in the use of experimental animals is one of the 3 milestones in the 3R (replacement, reduction, refinement) principle of animal experimentation (Russel and Burch 1959) and use of the MSPD may enable researchers to obtain comparable levels of information from fewer animals as well as more information from the animals used.

Scalar products (as opposed to their difference) may themselves be used in single stimulus tests, such as a novel object test. In combination with distance from stimulus, which is easily calculated from tracking data, the scalar product could be used to determine the attractiveness, aversiveness, or fearfulness associated with a single stimulus placed within a test arena. Keeping a great distance from the stimulus while facing it may indicate fear, whereas keeping away from the stimulus with the back turned could reflect aversion or dislike. Also, the mean scalar product can be used in other types of open mazes to provide a noncategorical overall value for the orientation of the animal toward speed of finding various targets such as the platform in a Morris water maze (Yim and Skelton 2013), the maternal litter in a homing test (Scattoni et al. 2008) or specific arms in radial arenas (Wallace et al. 2002; Catania 2013).

Using the MSPD to measure relative preference between 2 odors

We used a test of the response of rat pups to maternal odors to develop and refine the MSPD method. Previous tests had shown a large variation between litters in the number of rat pups choosing the fecal odor from their own dam. Although only one of the 4 litters tested showed a significant preference for the maternal odor, the MSPD revealed a large variation both within and between litters. It also became clear that ordering the pups according to the MSPD would rank some non-choosing pups higher than choosing pups. This is because the MSPD arise from the relative orientation of the pup toward the 2 odors presented and can therefore discriminate more subtle differences between test animals. As such, MSPD values can be used to preselect test subjects for olfactory trials, to balance subsequent treatments by or as a covariate in statistical analyses of treatment effects.

We also used the MSPD method in a choice test where pups were exposed to odors associated with estrus. Methylheptenone, previously found to be a significant part of estrus odor (Nielsen et al. 2013), was preferred to herb odor, but the same litters showed no preference for estrus odor over di-estrus odor, as found by Fillion and Blass (1986). A repetition of the test with different litters and different odor samples showed a preference of the rat pups for female estrus feces. As predicted, a large number of pups made no direct odor choice and the MSPD method proved useful as a way to rank the
pups according to their attraction to and degree of preference for odors associated with estrus. These preliminary results show that the attraction of rat pups to estrus odors and components thereof is not a conditioned response, as it is established very early in life, suggesting that it may even be innate.

Conclusions
Scalar products can be calculated from tracking data of 2 body markers during a choice test, in this case between odors. The mean scalar product for an odor source is a way to express the orientation of an animal relative to this odor source in a single, noncategorical value across a test. The mean difference between the 2 scalar products (MSPD) can be used to quantify the relative preference of an animal for the 2 odors even when no direct choice is made, and without a priori assumptions about a correct choice. The MSPD is more than a proxy for other measures obtainable before or during testing. It can be used instead of or in conjunction with existing methods and results in a more refined description of the response of individual animals in a choice test situation. Using the MSPD will reduce the number of animals and the number of test necessary to interpret behavioral tests of preference and relative attractiveness of stimuli.

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