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## 3Rs-based optimization of mice behavioral testing: the habituation/dishabituation olfactory test.

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Number of Table: 0

Number of supplementary figures: 4

1 Highlights:

2

3 1) Automation of the test allows testing 10 to 20 animals in one trial

4

5 2) Our optimized version is more reliable, accurate while less time-consuming

6

7 3) Multidimensional analysis of behavior can be assessed in only one task

8

9 4) Animal distress is reduced while scientific outputs are increased

10

11

1 **Abstract**

2

3 Background: There is clear evidence that most of the paradigms that are used in the field of  
4 behavioral neuroscience suffer from a lack of reliability mainly because of oversimplification  
5 of both testing procedures and interpretations. In the present study we show how an  
6 already existing behavioral test, the olfactory habituation / dishabituation task, can be  
7 optimized in such a way that animal number and animal distress could be minimized,  
8 number/confidence of behavioral outcomes and number of explored behavioral dimensions  
9 could be increased.

10

11 New Method: We used ethologically relevant technical and procedural changes associated  
12 with videotracking-based automated quantification of sniffing behavior to validate our new  
13 setup. Mainly internal and construct validity were challenged through the implementation of  
14 a series of simple experiments.

15

16 Results: We show that the new version of the test: 1) has very good within and inter  
17 laboratory replicability, 2) is sensitive to some environmental / experimental factors while  
18 insensitive to others, 3) allows investigating hedonism, both state and trait anxiety, efficacy  
19 of anxiolytic molecules, acute stress, mental retardation-related social impairments and  
20 learning and memory. 4) We also show that interest for both nonsocial and social odors is  
21 stable over time which makes repetitive testing possible.

22

23 Conclusions: This work paves the way for future studies showing how behavioral tests /  
24 procedures may be improved by using ethologically relevant changes, in order to question  
25 laboratory animals more adequately. Refining behavioral tests may considerably increase  
26 predictivity of preclinical tests and, ultimately, help reinforcing translational research.

27

## 1) Introduction

Behavioral testing plays a central role in all areas of biomedical research. It is used not only for psychotropic drugs screening, or finding candidate genes, but also for developing valid models of neuropsychiatric disorders. However, behavioral testing procedures suffer from several issues. The first one relies on the fact that they are sometimes expensive, labor-intensive, time consuming, and require certain attention to infinite sources of artifacts. The second one is related to the strategy commonly used to achieve higher data density and explore different behavioral dimensions. Indeed, in order to understand the complexity of behavior, researchers classically confront laboratory animals to a battery of single-domain tests. Even though this approach actually provides a multidimensional evaluation of mice behavior, it is known to be associated with animal distress because of repeated exposures to adverse experimental contexts (McIlwain et al., 2001). Many behavioral tasks are based on exposure to various aversive/unpleasant stimuli in order to study mice ability to cope with the situation, and as such, may be assimilated to chronic exposure to stressful events and consequently be deleterious to animal welfare, data validity and variability (Crabbe et al., 1999; Lathe, 2004).

To resolve this, it has been proposed over the last few years, to assess more behavioral domains in a limited number of tasks i.e. to maximize the number of evaluated phenotypes per experimental manipulation (Kalueff et al., 2008). For example, it's been shown that using a modified version of the marble burying test in mice allowed researchers to explore both "emotion" and "activity" domains rather than the sole anxious dimension (Nicolas et al., 2006). Moreover, another elegantly modified version of classical behavioral tests consisted in "hybridizing" several paradigms in one, i.e. combining experimental setups in an intelligent manner allowing to assess multiple phenotypes in a single cohort (Egan, 2010). By reducing the number of tests needed to collect more behavioral endpoints, "hybrid" models would require fewer stress exposures than a combination of single-domain assays, and therefore would adhere to the Refinement process of the 3Rs principle by reducing animal distress (Russell and Burch, 1959).

The 3Rs principle was initially introduced to the biomedical field in order to set a standard for the ethical treatment of animals as sentient beings. It is particularly relevant to behavioral research as it is one of the main areas using animal models to examine resulting phenotypes, and often implies stressful and/or painful manipulations. In respect of the 3Rs principle, it is now the responsibility of all behavioral researchers to improve current protocols in behavioral testing or even create new behavioral tasks being able to efficiently optimize data gathering in a limited number of tests. One ultimate goal would be to use only one task to assess many dimensions of mice behavior.

In line with this goal, we were interested in using mice's macrosmatic ability, *i.e.* the possession of a highly developed sense of smell, in order to perform a multidimensional analysis of mice behavior. Indeed, mice use their sense of smell in many aspects of their lives relating to learning and memory, communicating with conspecifics, finding food, avoiding predators and so on. Using olfactory-based tasks is especially relevant in terms of behavioral isomorphism as humans and mice seems to express similarities in their olfactory preferences (Mandairon et al., 2009). In spite of the crucial involvement of this sensory modality in nearly all dimensions of mice behavior, few studies have dealt with olfactory-based tasks.

In this paper, we show how a well-known olfactory-based task - the habituation/dishabituation olfactory test - can be optimized in such a way that animal number and animal distress could be minimized, number/confidence of behavioral outcomes and number of explored behavioral dimensions could be increased. The original version of this test was first described in the early 1980s and consisted in evaluating the naturally occurring response of rodents to social odors (Gregg and Thiessen, 1981). Later on, the test was modified and used more largely in particular to characterize deficits in social

1 behavior in mice models of neuropsychiatric diseases (Briellmaier et al., 2012; Rabaneda et  
2 al., 2014; Yang et al., 2012). In this task, a succession of different odors, both non-social and  
3 social odors, are presented to mice through a cotton swab (Yang and Crawley, 2009a). A  
4 commonly used sequence is water, two non-social odors, and two social odors. Such a setup  
5 allowed researchers to study 3 aspects of mice behavior: odor habituation (progressive  
6 decrease in olfactory investigation towards a repeated presentation of the same odor), odor  
7 dishabituation (ability to detect new odors) and preference for social olfactory cues.  
8 Performing this test, even in its most recent version, suffers from several issues. First, testing  
9 one mouse is quite time-consuming as it takes 75 minutes (30 minutes of familiarization with  
10 the testing environment and 45 minutes of olfactory test). Thus, testing two experimental  
11 groups of 10 individuals each requires 25h *i.e.* 3-4 days of full-day testing. Second, there is  
12 high degree of animal-experimenter interaction during the test (need for experimenter to  
13 install/remove cotton swab) which is known to be disturbing for animals performing the  
14 task, potentially affecting the obtained results (Hänell and Marklund, 2014). And finally, the  
15 test is relatively labor-intensive and prone to subjectivity of experimenters as direct  
16 measures need to be live-performed by using a stopwatch.

17 So, the first improvement we made was to automate the setup (data collection, data  
18 analysis) by using a videotracking software. Then we carried out a series of experiment  
19 which aimed at validating and implementing our improved setup. First we assessed reliability  
20 to see the extent to which our device yields consistent results each time the measurements  
21 are performed under the same experimental conditions. Then, we evaluated replicability *i.e.*  
22 the degree of accordance between the results of the same experiment performed in the  
23 same or different laboratories. We also checked internal validity by studying potential  
24 confounding factors that might affect the independent variable (such as the composition of  
25 the urinary pool). And finally, we assessed construct and predictive validity of our setup by  
26 using physiological, pharmacological and genetic manipulations in mouse models that  
27 produce well-characterized behavioral alterations.

28 Together, these experiments establish the validity of this new setup and show the potential  
29 application of our system to multidimensional analysis of mice behavior allowing researchers  
30 to explore a wide variety of CNS disorders in one task.

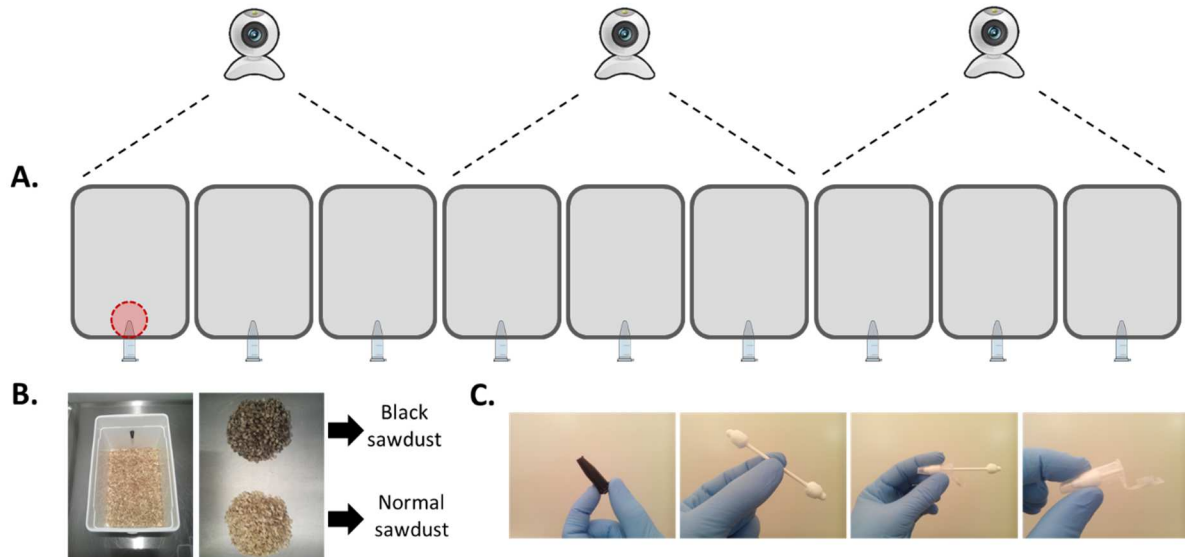
31

## 32 **2) Materials and methods**

### 33 **2.1) Experimental setup**

34 The experimental setup is depicted on Figure 1A and consisted in 9 aligned plastic boxes  
35 (Sundis; 26cm x 17.5cm x 15 cm) with a thin layer of fresh bedding. Cotton swab material  
36 was used as the medium for each odor stimulus and was inserted in a small plastic tube  
37 (Eppendorf; 1mL) cut open at the bottom (Fig 1B). Spherical gaps were made at the middle  
38 of one side of plastic boxes and were raised 8 cm above the floor level (Fig 1C). Mice  
39 behavior was captured by three USB cameras mounted on the adjacent wall and live analysis  
40 of time spent sniffing was performed automatically using the EthoVision video-tracking  
41 system (Noldus, The Netherlands). Sniffing was scored when the distance between the nose  
42 and the tube was 1.5 cm or less. To reduce novelty-induced exploratory activities, a 30 min  
43 period was set aside before testing for each subject to become habituated to the testing  
44 cage which contained a clean dry cotton swab. Classical testing consisted in a sequence of  
45 fifteen 2-min exposures to odors presented in the following order: three plain tap water,  
46 three non-social odor 1, three non-social odor 2, three social odor 1, and three social odor 2.  
47 Each inter-session interval was approximately 1 min. The almond and banana odors were  
48 made with food flavoring (Selectarôme, Cannes La Bocca, France). Social odors were made  
49 by mixing urine from a number of adult male mice (adult; C57Bl6 or other strain depending

1 the research question). Each subject was exposed to two novel social odors made from two  
2 different sources. A stock solution of each odors was prepared, aliquoted and stored at -  
3 20°C. Odors were defrosted before each experiment. All odors were made by dipping the  
4 cotton into the solution for 1 second. For each non-social odor used, adapted concentration  
5 was determined in respect of its initial intensity (banana: 1/1000; almond: 1/25; raspberry:  
6 1/100, orange: 1/10, toffee 1/600, chicory 1/100).



7

8 **Figure 1: Experimental setup.** (A) The setup consisted in 9 aligned plastic boxes  
9 (26x17.5x15cm) with a thin layer of fresh bedding. Three USB cameras were mounted on the  
10 adjacent wall and live analysis of mice behavior was performed. Spherical gaps were made at  
11 the middle of one side of plastic boxes and were raised 8 cm above the floor level. Cotton  
12 swab material was used as the medium for each odor stimulus and was inserted in a small  
13 plastic tube (Eppendorf; 1.5mL) cut open at the bottom. (B) White boxes containing light  
14 sawdust was used for experiments using dark mice (CBA/JRj, C57BL/6JRj, DBA/2) and black  
15 boxes containing homemade dark sawdust was used for experiments using dark mice (CD1,  
16 Balb/cJRj) . (C) Pictures showing how small plastic tubes containing all the odors were  
17 prepared: cotton swab was inserted inside the tube and then cut. Black or transparent tubes  
18 were used depending on the colour of mice.

19

## 20 2.2) Animals

21 Experimental procedures involved adult male C57BL/6JRj mice, 2-3 month old, purchased  
22 from Janvier Labs (Le Genest St Isle, France). In the “breeding zone” experiment, adult male  
23 C57BL/6JRj mice, 2-3 month old, were from Janvier Labs and from two different breeding  
24 areas from our facility. Adult CBA/JRj mice (3 month old) from Janvier Labs were used in the  
25 assessment of internal validity. Adult Balb/cJRj (3 month old) were purchased from Janvier  
26 Labs and were used as a model of trait anxiety and in the assessment of the pharmacological  
27 relief of anxiety. Fmr1 knockout (Fmr1-ko) mice, 3 month old, were obtained from our  
28 facility. All mice were bred and maintained on a 12-h light/dark cycle (lights on from 7:00am  
29 to 7:00pm) with food and water ad libitum in a temperature controlled (21 +/- 1 degree)  
30 room in the animal resource facility (UPS44, CNRS Orléans - France). They were reared in  
31 standard cages (dimensions). All aspects of animal care and experimentation were in  
32 accordance with the European Parliament and Council Directive (2010/63/EU).

33

## 2.3) Urine collection and urinary mixtures preparation

A urine sample was obtained by mixing urine from multiple unknown mice. (10 to 30 gonadally intact males). The urine was collected by holding one mouse by the scruff of the neck over a funnel and gently applying pressure to the abdomen. One animal typically discharges 0.2-0.3 ml of urine. Pooled samples were then aliquoted (0.5 or 1mL vials) and stored at -20°C until use. Aliquots were thawed shortly before experiments and not refreeze afterwards.

## Drugs

Diazepam was purchased from Sigma-Aldrich and was used to assess predictive validity. The drug was administered through intraperitoneal injection at 1mg/kg 30 minutes before testing. The dose was chosen based on previous data obtained in our lab showing anxiolytic-like effects with no sedative effects.

## 2.4) Procedure

A total of 10 experiments have been carried out to validate our setup. Details on the number of animals involved in each experiment are provided in Supplementary Table S1.

### 2.5.1) Internal validity

Internal validity refers to how accurate is the behavioral measure, *i.e.* to which extent it is influenced by putative confounding factors. High reliability and replicability are the foundation of good internal validity. We thus investigated these criteria as described below.

#### 2.5.1.1) Experiment 1 (Within & inter laboratory replicability)

At their arrival, mice were accommodated in our breeding facility during a minimum of two weeks. Within-lab replicability was assessed by exposing two groups of adult C57BL/6JRj male mice to the following odorants: almond 1/25, banana 1/1000, social 1 and social 2 (social odors being two different mixtures of urine collected from unknown age & sex matched conspecifics). To assess inter-lab replicability, two other groups of male C57BL/6JRj mice were transferred to another breeding facility, and after two weeks of habituation, they were tested in the setup. The experiment was run by different experimenters and mice were exposed to the same odorants.

#### 2.5.1.2) Potential confounding factors

##### - Experiment 2 (Effect of social odors constitution)

To verify whether the composition of the urinary mixtures, we exposed adult C57BL/6JRj male mice to various mixtures of urine obtained from strain, age & sex matched conspecifics.

We first tested whether the urinary mixtures of C57BL/6JRj mice, containing different individuals of the same strain, could affect obtained results. In this way, two groups of mice were respectively exposed to mixtures A and B for the first one and to mixtures C and D for the other. Each mixture being composed of the urine collected from adult C57BL/6JRj male mice. Non-social odors were the same as mentioned above *i.e.* almond (1/25) and banana (1/1000). We were also interested in studying the effect of changing the ethological meaning of social odors by presenting to C57BL/6JRj mice urinary mixtures collected from other



1 strains: CBA/JRj and CD1 mice (age & sex matched). With this aim, two urinary mixtures  
2 collected from either adult CBA/JRj or CD1 male mice were presented to tested C57BL/6JRj  
3 mice.

4

5 **- Experiment 3 (Influence of ethological meaning of social odor 1 over social**  
6 **odor 2 interest)**

7 To explore the putative influence of social odor 1 over social odor 2 we exposed one cohort  
8 of adult C57BL/6JRj male mice to a social odor 1 of different ethological meaning as social  
9 odor 2. In this way, we presented to only one group of mice a urinary mixtures collected  
10 from juvenile male mice of the same strain and a mixture collected from adult odor as social  
11 odor 2. The other group was exposed to two odors collected from adult C57BL/6JRj male  
12 mice. Behavioral response of adult male mice has been shown to be qualitatively different in  
13 response to juvenile compared to adult conspecifics: social/aggressive contacts are of lesser  
14 importance (Winslow, 2003). Such a procedure has also the benefit to inform about the  
15 reaction of adult male mice in response to juvenile odor.

16

**- Experiment 4 (Effects of breeding conditions)**

17 Three groups of adult C57BL/6JRj male mice were exposed to the setup as described above  
18 (with the same odorants). Each group of mice was reared in 3 breeding facilities differing by  
19 their health status, housing conditions and diet. One group was from Janvier Labs (Breeding  
20 3), bred in Specific Pathogen Free (SPF) conditions and socially enriched environments. The  
21 two other groups were bred in less enriched environment of our own facilities -Transgenesis,  
22 Archiving and Animal Models, TAAM, Orléans, France. In one of these facilities, mice were  
23 bred and maintained in SPF conditions (Breeding 2) while in the other, they were bred in  
24 conventional conditions (Breeding 1). Two weeks before testing, all mice were transferred in  
25 our research facility in order to habituate to local housing conditions.

26

**- Experiment 5 (Non-social odor aversion vs. salience)**

27 To assess whether non-social odors intensity is indicative of the pleasantness / aversion of  
28 the olfactory experience, we exposed two groups of adult C57BL/6JRj male mice to the same  
29 non-social odors at various concentration. With this aim one group was exposed to “diluted”  
30 odors of almond (1/25), banana (1/1000), raspberry (1/100), orange (1/100), toffee (1/600)  
31 and chicory (1/100). The other group was exposed to the same pure odors.

32

**2.5.2) Construct validity**

33

**2.5.2.1) Experiment 6 (Trait and state anxiety)**

34 To check whether differences in trait anxiety levels can be highlighted in our setup we ran  
35 adult C57BL/6JRj male mice and adult Balb/cJRj mice in the olfactory task. All mice were  
36 obtained from Janvier Labs and were reared during two weeks in our breeding facility before  
37 testing. Non-social odors were water, almond (1/25) and banana (1/1000) while social odors  
38 consisted of two mixtures of adult C57BL/6JRj male mice. Moreover, the effect of  
39 environmental stress was first assessed by testing adult C57BL/6JRj male mice in two  
40 conditions, with and without sawdust in the testing box. Sawdust removal is known to be  
41 stressful for rodents, inducing both behavioral and physiological changes (Laviola et al.,  
42 2002) . Non-social odors were water, almond (1/25) and banana (1/1000), and social odors  
43 were two different urinary mixtures collected from adult C57BL/6JRj male mice. Given that  
44 Balb/cJRj mice are known to be more stress-responsive (Griebel et al., 2000), we also tested  
45 adult Balb/cJRj male mice in these two conditions.

### 2.5.2.2) Experiment 7 (Mental retardation model: FmR1 KO mice)

To further establish construct validity we used FmR1-KO mice, known to display abnormal behavioral response towards social stimuli (Hébert et al., 2014).

Adult C57BL/6JRj J and Fmr1 knockout male mice were first assessed in their interest to non-social and social odors. The former were water, almond (1/25) and banana (1/1000), and the latter were urinary mixtures collected from adult C57BL/6JRj male mice. The two mixtures were different in their composition. According to the literature, FmR1 knockout mice also display abnormal social behavior in response to juvenile conspecifics (Gkogkas et al., 2014; Pietropaolo et al., 2011). So, to go further in details, we evaluated the interest of adult C57BL/6JRj vs. Fmr1 knockout male mice for social odors constituted of urines collected from C57BL/6JRj juveniles. Non-social odors were the same as previously described.

### 2.5.2.4) Experiment 8 (Memory)

We also wanted to verify whether our setup could be used to address memory-related behavior. With this aim, we explored phases of memory processes: acquisition and retention. In a first bout of experiment we were interested in a test – retest approach but we changes the delay between the two in order to assess the duration of olfactory memory.

#### - Mid-term memory: 24h inter trial with the same odors

First, adult C57BL/6JRj male mice were run twice in our setup with a 24h delay between test and retest trials. Non-social odors were water, almond (1/25) and banana (1/1000), and social ones were two urinary mixtures collected from unknown adult C57BL/6JRj male mice. Retest trial was performed the day after and the same odors were used.

#### - Long-term memory: 5 days inter trial with the same odors

With the same protocol, two other groups of adult C57BL/6JRj male mice were tested according to the same protocol as described above but the inter trial delay was 5 days.

### 2.5.3) Predictive validity

#### - Experiment 9 (Pharmacological relief of anxiety)

Adult Balb/cJRj male mice were injected i.p. with 1 mg/kg diazepam (Sigma-Aldrich) diluted in propyleneglycol mixed with distilled water (ratio 1:1) for a final injection volume of 0.1 mL for 10 g of body weight. All injections were given 30 min before olfactory testing. Control mice were injected with vehicle. Non-social odors were water, almond (1/25) and banana (1/1000) while social odors were two urinary mixtures collected from unknown adult C57BL/6JRj male mice.

### 2.5.4) Experiment 10 (Test-Retest strategy: repeated testing)

To assess whether the setup could be used in a repeated testing approach, we tested two groups of adult C57BL/6JRj male mice through 6 sessions with 5 days of inter trial delay. To avoid odor habituation as a confounding factor we used novel non-social and social odors within each session. Thus, all odors were used as follow: first session, water, almond (1/25), banana (1/1000) and two social odors; session 2: water, orange (1/10), toffee (1/600) and two social odors; session 3: water, cotton candy (1/400), exotic fruits (1/200) and two social odors; session 4: water, pineapple (1/100), peach (1/100) and two social odors; session 5: water, red fruits (1/100), vanilla (1/100) and two social odors; session 6: water, chicory (1/100), lemon (1/100) and two social odors. All social odors were collected from unknown

1 adult C57BL/6JRj male mice and were different in their composition from one session to  
2 another. For non-social odor, respective dilution factors were determined according to their  
3 intensity rated by Human experimenters.

## 4 **2.5) Data analyses and statistics**

5 Data were first computed as the total time spent sniffing over the three presentations of  
6 each odor. This cumulative duration has the benefit of highlighting robust changes in  
7 independent variables of interest but can be misleading when subtle effects are at work. In  
8 this case, we analyzed and depicted data as originally described in the literature (**Brielmaier**  
9 **et al., 2012**). This more detailed analysis has the advantage to emphasize discrete processes  
10 such as odor habituation and response to novel odors which could be very informative  
11 depending on the issue raised by the researcher. Such an analysis strategy is completely in  
12 accordance with our goal to not only achieve higher data density but also explore different  
13 behavioral dimensions from a single behavioral test. Then, this approach has to be  
14 considered as another way to improve behavioral testing procedures in respect of the 3Rs  
15 principle.

16 Data were analyzed using non-parametric procedures especially adapted for statistical  
17 analysis of small samples ( $n < 30$ ). Moreover, our data did not meet the prerequisite criteria  
18 to apply parametric statistics i.e. homoscedasticity and normality of distributions (Siegel,  
19 1988). In addition, these statistical procedures have the benefit to have better relative  
20 power-efficiency for small samples (Bridge and Sawilowsky, 1999). Independent two-group  
21 comparisons were analyzed using the Mann–Whitney U test. When considering more than  
22 two groups, a global analysis was done first using the Kruskal–Wallis “ANOVA-on-ranks”  
23 procedure, then, when necessary ( $p < 0.05$ ), by Bonferroni post hoc analyses (Siegel, 1988)  
24 including the correction for multiple comparisons. Dependent two-group comparisons were  
25 performed by using Wilcoxon test. When considering more than two groups, the Friedman  
26 ANOVA was computed and Dunn post hoc analyses were done for multiple comparisons.

## 27 **3) Results**

### 28 **3.1) Internal validity**

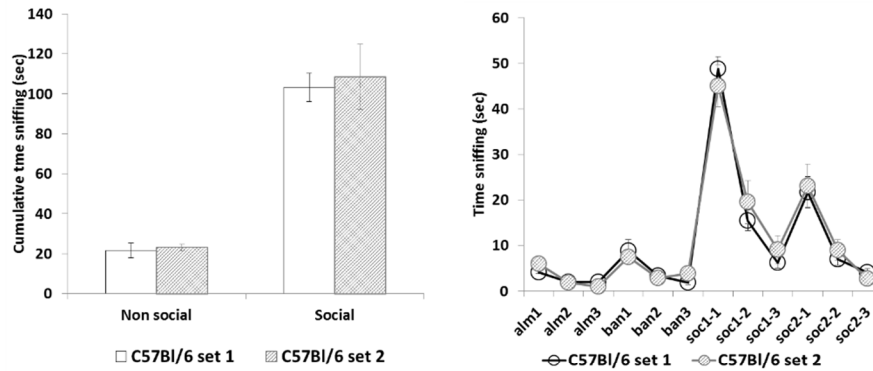
#### 29 **3.1.1) Experiment 1 (within & inter laboratory replicability)**

30 With the aim of evaluating the accuracy of our behavioral measures we first investigated  
31 how consistent was the interest of mice for social vs. non-social odors in our new setup. We  
32 first exposed 2 groups of mice to the same set of odors. We did not observed any differences  
33 in the time spent sniffing regardless of the odor (Figure 2A; **Mann-Whitney test:  $U=54$ ,**  
34  **$p=0.51$  for non-social odors and  $U=92$ ,  $p=0.87$  for social odors**). Then we wanted to verify  
35 whether the replicability of our result could be extended to different testing laboratories.  
36 Two groups of mice, exposed to the same set of odors, but reared and tested in two  
37 different facilities and by different experimenters, displayed similar interest towards non-  
38 social (**Mann-Whitney test:  $U=133$ ,  $p=0.14$** ) and social odors (**Mann-Whitney test:  $U=103$ ,**  
39  **$p=0.91$** ) (Figure 2B).

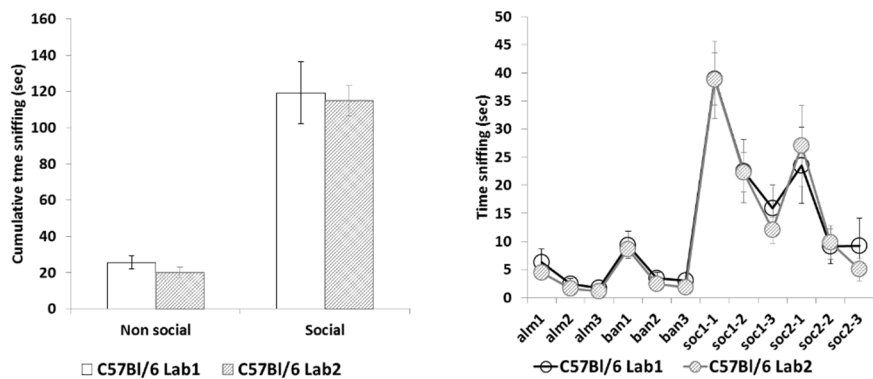
40 These data indicate that the results are reliable in replications across cohorts of animals in  
41 the same laboratory, and in different laboratories across geographic locations.

42

### A. Within lab replicability



### B. Inter lab replicability



1

2 **Figure 2: Internal validity analysis. (A)** Time spent investigating non-social (almond, banana)  
3 and social odors (two urinary mixtures of adult C57BL/6JRj male mice) by two groups of  
4 C57BL/6JRj mice within the same laboratory. Set 1: n=16 and set 2: n=14. **(B)** Time spent  
5 investigating non-social (almond, banana) and social odors (two urinary mixtures of adult  
6 C57BL/6JRj male mice) by two groups of C57BL/6JRj mice within two different laboratories.  
7 Lab1: n=20 and lab2: n=16. Left panel: cumulative time spent exploring and right panel: time  
8 spent exploring each presentation of odors.

9

10

#### 3.1.2) Potential confounding factors

11

We were then interested in improving our understanding of mice behavioral response during  
12 the test. In particular, we studied the extent to which putative confounding variables may  
13 influence the independent variables of interest.

14

15

#### - Experiment 2 (Effects of social odors constitution)

16

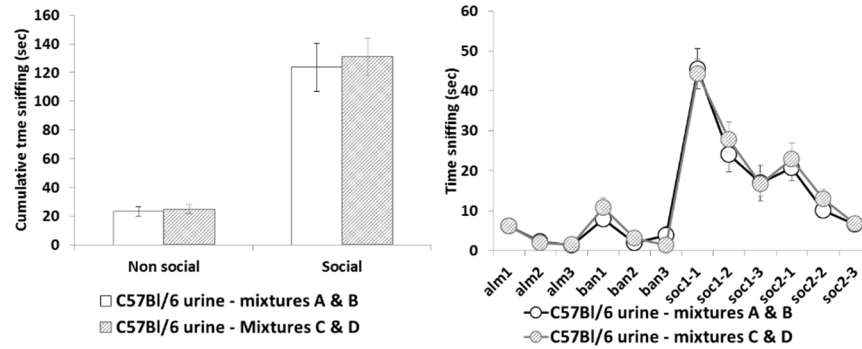
Mice have the ability to discriminate between individuals within the same strain, and  
17 dominant vs. subordinate males (Drickamer, 1992). We first wondered if mice interest in  
18 social odors could vary depending on the composition of the 2 urinary mixtures to which  
19 they were exposed. In this way, 2 groups of mice were exposed to social odors differing in  
20 their composition. We found the 2 groups of mice to be equally interested in social odors  
21 irrespective of their composition (Figure 3A; Mann-Whitney test; U=146; p=0.67).

22

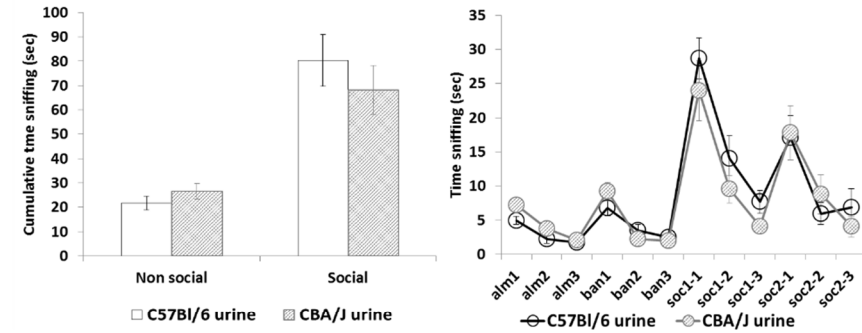
It was also reported that mice could discriminate between urine odors of different strains.  
23 In respect of this, we exposed 2 cohorts of C57BL/6JRj mice to either related (same strain as  
24 tested mice) or unrelated strain odors. In a first experiment, we observed that C57BL/6JRj  
25 mice displayed no significant preference for social odors collected from its own strain  
26 compared to unrelated strain odors (urinary mixtures from male CBA/JRj mice) (Figure 3B;  
27 Mann-Whitney test; U=236; p=0.49). This was also true when C57BL/6JRj mice were exposed  
28 to CD1 urinary mixtures (Figure 3C; Mann-Whitney test; U=145; p=0.61).

29

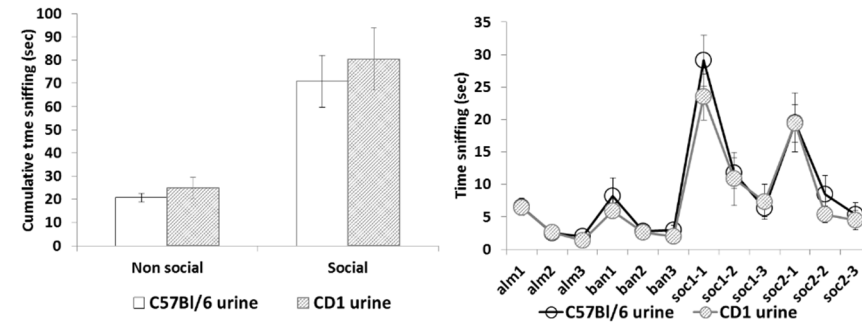
### A. C57Bl/6 mice exposed to different C57Bl/6 urinary mixtures



### B. C57Bl/6 mice exposed to C57Bl/6 vs. CBA/J urinary mixtures



### C. C57Bl/6 mice exposed to C57Bl/6 vs. CD1 urinary mixtures



1

2 **Figure 3: Effect of social odors constitution. (A)** Time spent investigating non-social (almond,  
 3 banana) and social odors originated from the same strain (urinary mixtures of adult  
 4 C57BL/6JRj male mice) by two groups of C57BL/6JRj mice. The four mixtures all differed in  
 5 their composition (urines of individuals contained in odor A were different from that of odor  
 6 B, C and D). n=15-16 in each group. **(B)** Time spent investigating non-social (almond,  
 7 banana) and social odors originated from an unrelated strain (urinary mixtures of adult CBA/JRj  
 8 male mice) or from the same strain (adult C57BL/6JRj male mice) by two groups of C57BL/6JRj  
 9 mice. n=13-15- in respective groups. **(C)** Time spent investigating non-social (almond,  
 10 banana) and social odors originated from an unrelated strain (urinary mixtures of adult CD1  
 11 male mice) or from the same strain (adult C57BL/6JRj male mice) by two groups of  
 12 C57BL/6JRj mice. n=18 in each group. Left panel: cumulative time spent exploring and right  
 13 panel: time spent exploring each presentation of odors.

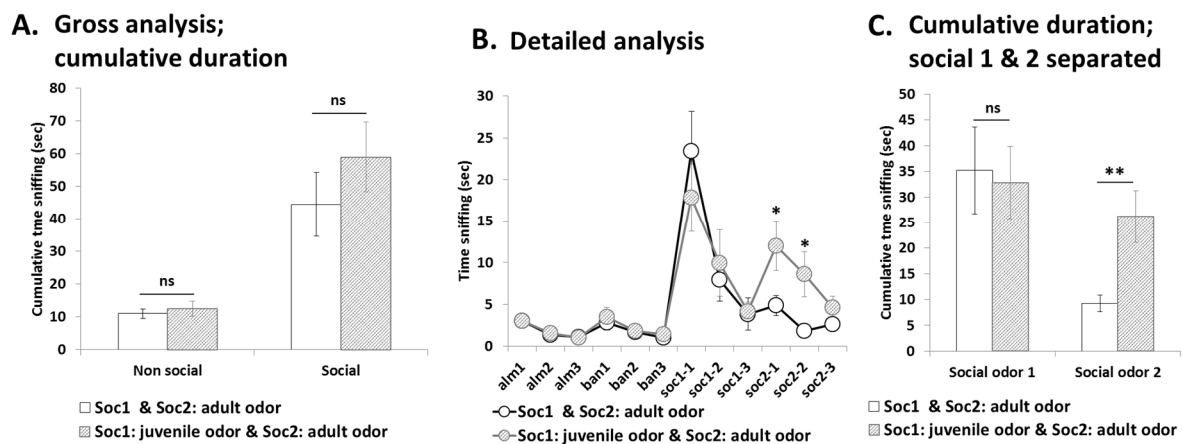
14

15 **- Experiment 3 (Influence of ethological meaning of social odor 1 over social**  
 16 **odor 2 interest)**

17 To further understand what is measured during the test, we decided to check whether  
 18 exposure to social odor 1 may influence mice interest for social odor 2. With this aim, we  
 19 exposed one cohort of adult C57BL/6JRj male mice to juvenile odor as social odor 1 and  
 20 adult odor as social odor 2 while the other group was exposed to 2 odors collected from  
 21 adult C57BL/6JRj male mice. Behavioral response of adult male mice has been shown to be

1 qualitatively different in response to juvenile compared to adult conspecifics:  
 2 social/aggressive contacts are of lesser importance during adult / juvenile encounters  
 3 (Winslow, 2003). Such a procedure has also the benefit to inform about the reaction of adult  
 4 male mice in response to juvenile odor. We found the 2 groups of mice to be equally  
 5 interested in both non-social and social odors when gross analysis was performed (Figure 4A;  
 6 **Mann-Whitney test; U=110; p=0.20**). However, significant differences were revealed in more  
 7 detailed analyses: mice exposed to juvenile odor as social odor 1 displayed increased  
 8 interest for social odor 2 (urinary mixture from adult C56Bl/6JrJ male mice) (Figure 4B;  
 9 **Mann-Whitney test: U=128.5; p=0.045 and U=136.5; p=0.026 respectively**, and Figure 4C;  
 10 **Mann-Whitney test: U=143.5; p=0.0016**).

11



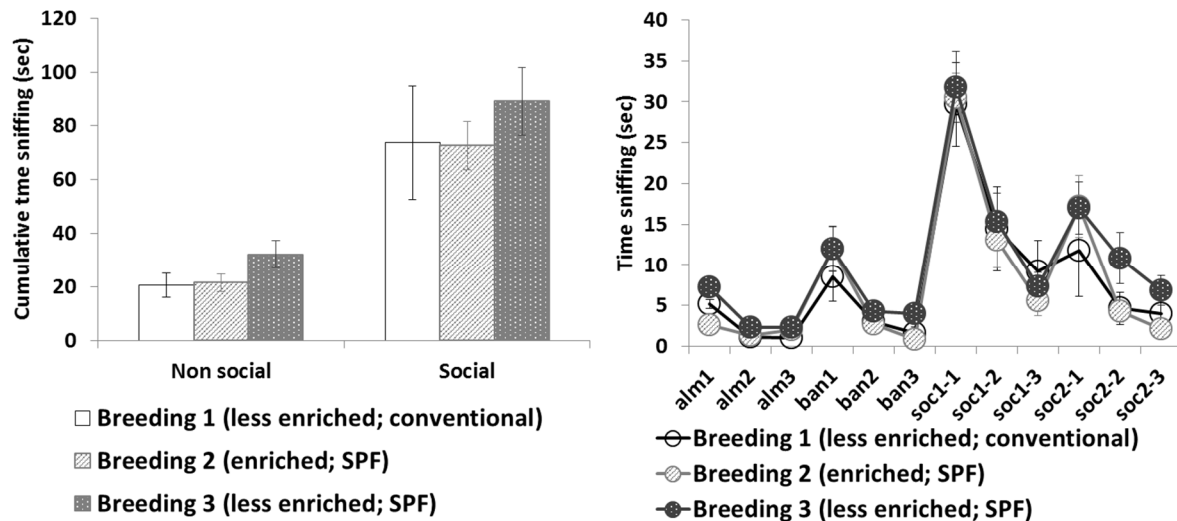
12

13 **Figure 4: Effect of social odor 1 ethological meaning over social odor 2 interest. (A)**  
 14 *Cumulative time spent investigating non-social and social odors. (B) Time spent investigating*  
 15 *each presentation of non-social (almond, banana) and social odors. Social odors were from*  
 16 *the same strain but varied in their ethological significance depending on the experimental*  
 17 *group: the “Soc1 & Soc2 adult odor” group was exposed to 2 urinary mixtures collected from*  
 18 *adult C57BL/6JrJ male mice while the “Soc1: juvenile group & Soc2: adult odor” group was*  
 19 *consecutively exposed to juvenile C57BL/6JrJ male mice odor (as social odor 1) and adult*  
 20 *C57BL/6JrJ male mice odor (as social odor 2). Social odor 2 was the same between the 2*  
 21 *experimental groups. (C) Cumulative time spent investigating social 1 and social odor 2.*  
 22 *n=13-14 in each group. \* p<0.05; \*\* p<0.01 compared to Soc1 & 2: adult odor.*

23

#### - Experiment 4 (Effects of breeding conditions)

1  
2 Then, we assessed the effect of environmental enrichment during breeding, a variable  
3 known to be a potent modifier of mice emotional and cognitive behavior (Coke-Murphy et  
4 al., 2014). With this aim we exposed 3 cohorts of C57BL/6JRj male mice, differing in their  
5 breeding conditions (health status, diet, enrichment in cage, human handling), to the same  
6 non-social and social odors. Mice bred in less enriched zones (zone 1: less enriched -  
7 conventional zone; and zone 3: less enriched - SPF zone) were not significantly different from  
8 mice reared in enriched zone (zone 2: environmentally enriched - human handling - SPF)  
9 (Figure 5; Kruskal-Wallis ANOVA:  $K=3.79$ ;  $df=2$ ;  $p=0.15$  for non-social odors and  $K=1.39$ ;  $df=2$ ;  
10  $p=0.50$  for social odors).

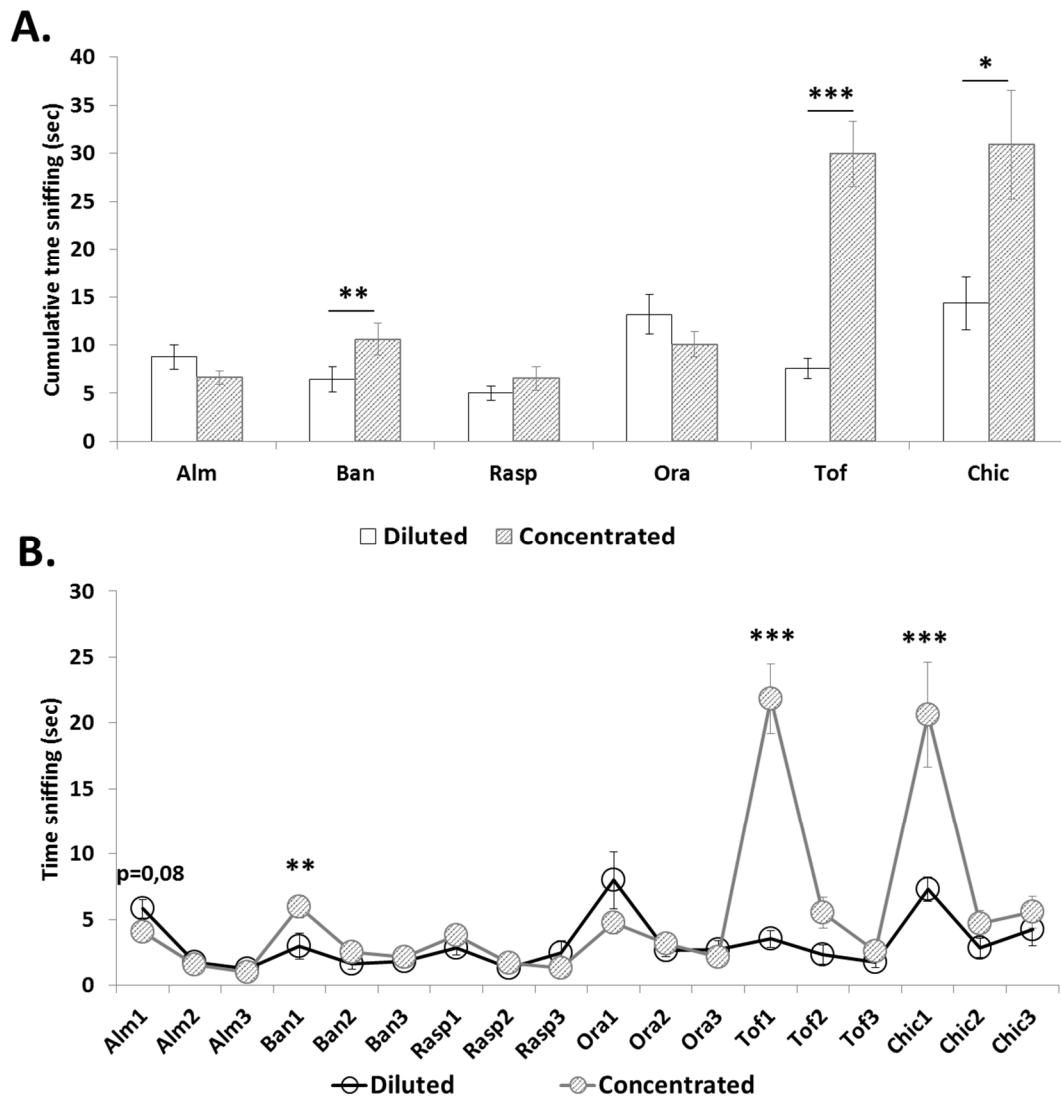


11  
12 **Figure 5: Effect of breeding conditions.** Cumulative time spent investigating non-social  
13 (almond, banana) and social odors. The two social odors were from adult C57BL/6JRj  
14 mice and were different in composition.  $n=10$  for Breeding 2,  $n=11$  Breeding 3 and  $n=14$  for  
15 Breeding 3. Left panel: cumulative time spent exploring and right panel: time spent exploring  
16 each presentation of odors.

#### - Experiment 5 (Non-social odor aversion vs. salience)

19 It was recently shown that Humans and mice express similar olfactory preferences, both of  
20 them spending more time sniffing odorants rated as pleasant by humans (Mandairon et al.,  
21 2009). This finding indicates that mice display olfactory preferences for some non-social  
22 odors and that time spent sniffing could inform about odor salience and odor aversion. We  
23 were thus interested in verifying that our setup could be used to detect differences in non-  
24 social odors hedonic values, allowing to study the field of positive emotion / motivation.  
25 Given that odor hedonic valence might differ depending on odor concentration (Nunez-Parra  
26 et al., 2014) we used two concentrations for each odorants and assessed if investigation  
27 time was increased or decreased. Odor was considered to be salient when investigation time  
28 and concentration concomitantly increased. And odor was considered to be aversive when  
29 investigation time decreased and concentration increased. All odor tested - almond, banana,  
30 raspberry, orange, toffee and chicory – were not known to be of particular ethological value  
31 for rodents. Gross analysis revealed concentrated almond, raspberry and orange odors to be  
32 as salient as diluted odors (Figure 6A; Mann-Whitney tests:  $U=174$ ;  $p=0.27$ ,  $U= 109.5$ ;  $p=0.63$   
33 and  $U= 151$ ;  $p=0.39$  respectively) while concentrated banana, toffee and chicory was  
34 significantly more salient compared to diluted odors (Mann-Whitney test:  $U=53.5$ ;  $p=0.023$   
35 for banana and  $U= 8$ ;  $p<0,0001$  for toffee and  $U= 58$ ;  $p=0.028$  for chicory). Detailed analysis  
36 confirmed these results by showing that first presentations of banana (Mann-Whitney test:

1 U= 53.5; p=0.004), toffee (Mann-Whitney test: U= 12; p<0.0001) and chicory (Mann-Whitney  
 2 test: U= 81.5 ; p=0.014) were significantly attractive for mice whereas almond tended to be  
 3 aversive as time spent sniffing the first presentation was decreased (Mann-Whitney test: U=  
 4 15; p<0.0001) (Figure 6B). No significant differences were observed for raspberry and orange  
 5 (Mann-Whitney test: U=109.5; p=0.49 and U= 151; p=0.39 respectively).  
 6



7  
 8 **Figure 6: Difference between non-social odors attractiveness. (A)** Cumulative time spent  
 9 investigating various non-social odors namely almond, banana, raspberry, orange, toffee and  
 10 chicory presented either at high concentration (pure odor) or diluted. Dilutions were  
 11 prepared as follow in respect of respective odor intensity: almond 1/25, banana 1/1000,  
 12 raspberry, 1/100, orange 1/10, toffee 1/600 and chicory 1/100. **(B)** Time spent investigating  
 13 each presentation of all non-social odors. n=16 in each group. \* p<0.05; \*\* p<0.01 and \*\*\*  
 14 p<0.001 compared to the diluted group.

15  
 16 Taken together, this set of data indicates that the dependent variable is not sensitive to  
 17 urinary mixtures constitution and breeding conditions, meaning that variation of  
 18 independent variables of interest are not affected by these parameters. However, the choice  
 19 and the concentration of non-social odors has to be strictly controlled between experiments  
 20 as dependent variable may be strongly affected. In addition, our results suggest that urinary  
 21 mixtures must be tightly controlled in their composition as social odor 1 may impact  
 22 behavioral response to social odor 2.



### 3.2) Construct validity

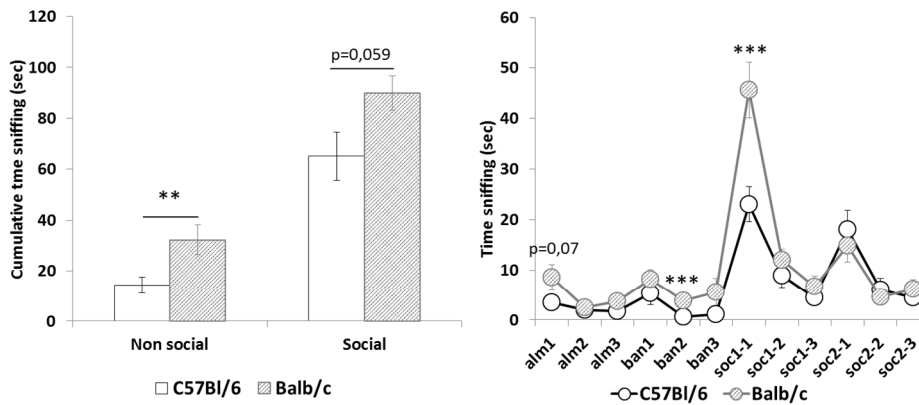
Construct validity is more and more considered as one of the most important validation criteria of behavioural tasks for rodents (Keeler and Robbins, 2011; Nestler and Hyman, 2010b; Stewart and Kalueff, 2015). Here, construct validity of our setup was assessed by using genetic, physiological and pharmacological manipulations known to produce well characterized behavioral alterations in mice.

#### 3.2.1) Experiment 6 (Trait and state anxiety)

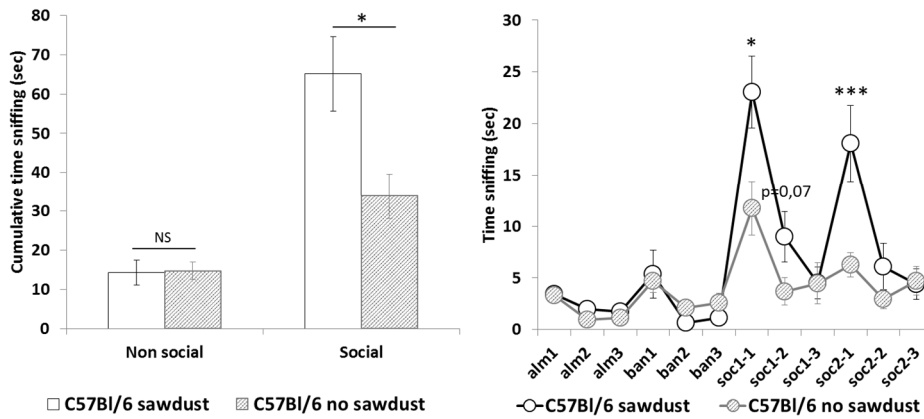
We first examined the effect of trait anxiety by comparing behavioral response of male Balb/cJRj mice vs. C57BL/6JRj mice in our olfactory test. Balb/cJRj mice are recognized as a validated model of trait anxiety, displaying recurrent reluctance to explore the environment and higher level of defensive postures in a novel area compared to other inbred mice (C57BL/6JRj , C3H, CBA/JRj, DBA/2, NZB, SJL) (Griebel et al., 1993; Griebel et al., 2000). Interestingly, we observed that Balb/cJRj mice spent more time investigating both the non-social (Mann-Whitney test:  $U= 16$ ;  $p=0.0076$ ) and social odors (Mann-Whitney test:  $U= 26$ ;  $p=0.059$ ) (Figure 7A; left graph). Difference between the 2 strains was especially evident during the first presentation of the first social odor: Balb/cJRj mice spent significantly more time sniffing almond 1 (Mann-Whitney test:  $U= 27$ ;  $p=0.07$ ), banana 2 (Mann-Whitney test:  $U=27$ ;  $p=0.0001$ ) and social odor 1 (Mann-Whitney test:  $U=12$ ;  $p=0.0025$ ) (Figure 7A, right graph). Then, we wanted to assess whether exposure to an acute environmental stressor (no sawdust within test cage) would result in changes in time spent investigating the odors. We addressed this question by using C57BL/6JRj and Balb/cJRj mice known to differ in stress reactivity (Ducottet et al., 2004). Interestingly, we found the 2 groups of mice to be differently affected by the stressor: the former did not display any alterations regarding non-social odors (Mann-Whitney test:  $U= 82$ ;  $p=0.49$ ) but were significantly less interested by social odors (Mann-Whitney test:  $U= 151.5$ ;  $p=0.013$ ) compared to their unstressed counterparts (Figure 7B). Balb/cJRj mice significantly spent less time investigating both the non-social (Mann-Whitney test:  $U= 179$ ;  $p=0.0049$ ) and social odors (Mann-Whitney test:  $U= 203$   $p=0.00019$ ) (Figure 7C).

Taken together, these results indicate that both trait and state anxiety can be assessed in our setup and that mice strains differing in emotional reactivity can be segregated.

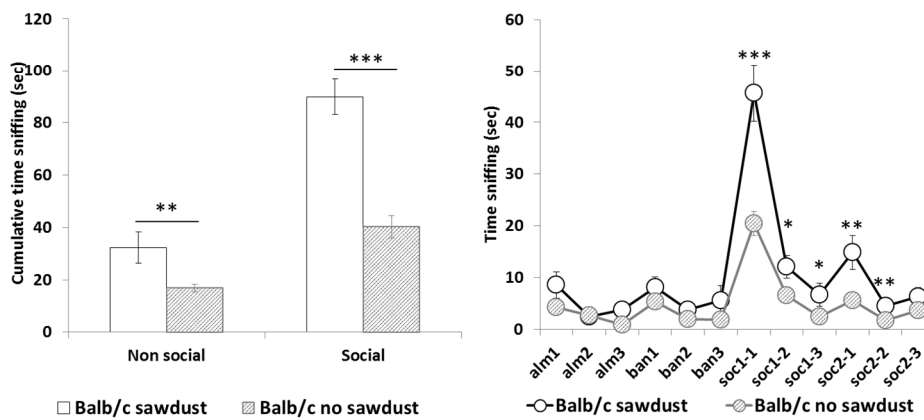
### A. Effects of trait anxiety: Balb/c vs. C57Bl/6 mice



### B. Effects of state anxiety: environmental stress on C57Bl/6 mice



### C. Effects of state anxiety: environmental stress on Balb/c mice



1

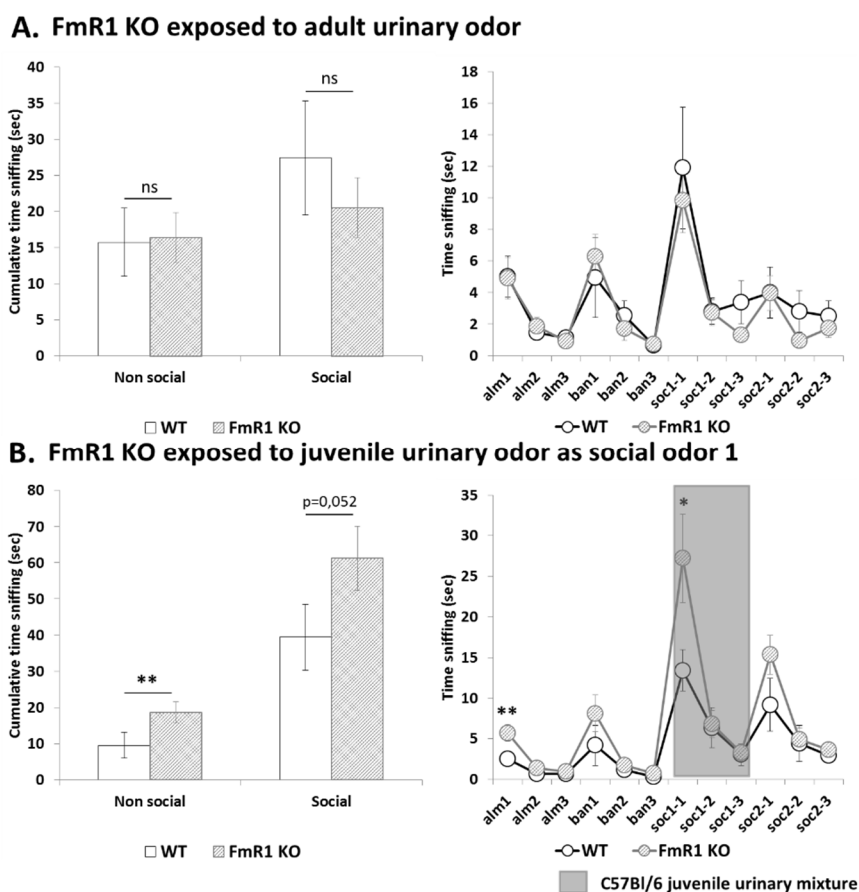
2 **Figure 7: Effect of state and trait anxiety models. (A)** Time spent investigating non-social  
 3 and social odors by two cohorts of mice differing in their level of trait anxiety: C57BL/6JRj  
 4 (n=13) and Balb/cJRj mice (n=11). \*\* p<0.01 and \*\*\* p<0.001 compared to C57BL/6JRj mice.  
 5 **(B)** Time spent investigating non-social and social odors by C57BL/6JRj mice exposed to  
 6 control conditions (sawdust in the testing box, n=10) or stressful conditions (no sawdust in  
 7 the testing box, n=9). \* p<0.05 and \*\*\* p<0.001 compared to C57BL/6JRj sawdust. **(C)** Time  
 8 spent investigating non-social and social odors by Balb/cJRj mice exposed to control  
 9 conditions (sawdust in the testing box; n=10) or stressful conditions (no sawdust in the  
 10 testing box; n=11). For all these experiments, non-social odors were almond 1/25 and banana  
 11 1/1000 and social odors were urinary mixtures collected from adult C57BL/6JRj male mice. \*  
 12 p<0.05; \*\* p<0.01 and \*\*\* p<0.001 compared to Balb/cJRj sawdust. Left panel: cumulative  
 13 time spent exploring and right panel: time spent exploring each presentation of odors.

14

15

### 3.2.2) Experiment 7 (Mental retardation model: FmR1 KO mice)

1  
2 FmR1 KO mice did not display any abnormalities regarding neither non-social nor social  
3 odors (Figure 8A; Mann-Whitney test:  $U=69$ ;  $p=0.45$  for non-social odors and  $U= 82$ ;  $p=0.94$   
4 for social odors). However, investigation time of social odors were especially low compared  
5 to that observed in all other experiments we performed with C57BL/6JRj male mice. FmR1  
6 KO mice have already been shown to express abnormal social behavior in response to  
7 juvenile conspecifics (Pietropaolo et al., 2011). In respect of this social impairment, we  
8 exposed FmR1 KO mice and their wild type controls to urinary mixture collected from  
9 juvenile C57BL/6JRj male mice as social odor 1 to verify whether maladaptive response could  
10 be observed. In this case, FmR1 knockout mice spent more time exploring both the non-  
11 social (Mann-Whitney test:  $U= 160$ ;  $p=0.0031$ ) and social odors (Mann-Whitney test:  $U= 55$ ;  
12  $p=0.052$ ) (Figure 8B, left graph). Detailed analysis revealed FmR1 knockout mice to be more  
13 attracted by almond odor (first presentation) (Mann-Whitney test:  $U= 46$ ;  $p=0.009$ ) and  
14 juvenile odor (first presentation) (Mann-Whitney test:  $U= 55$ ;  $p=0.023$ ) (Figure 8B, right  
15 graph).  
16 Overall, these data indicate that deficits in several dimensions of social behavior of mice  
17 models of mental retardation can be addressed in the setup.  
18



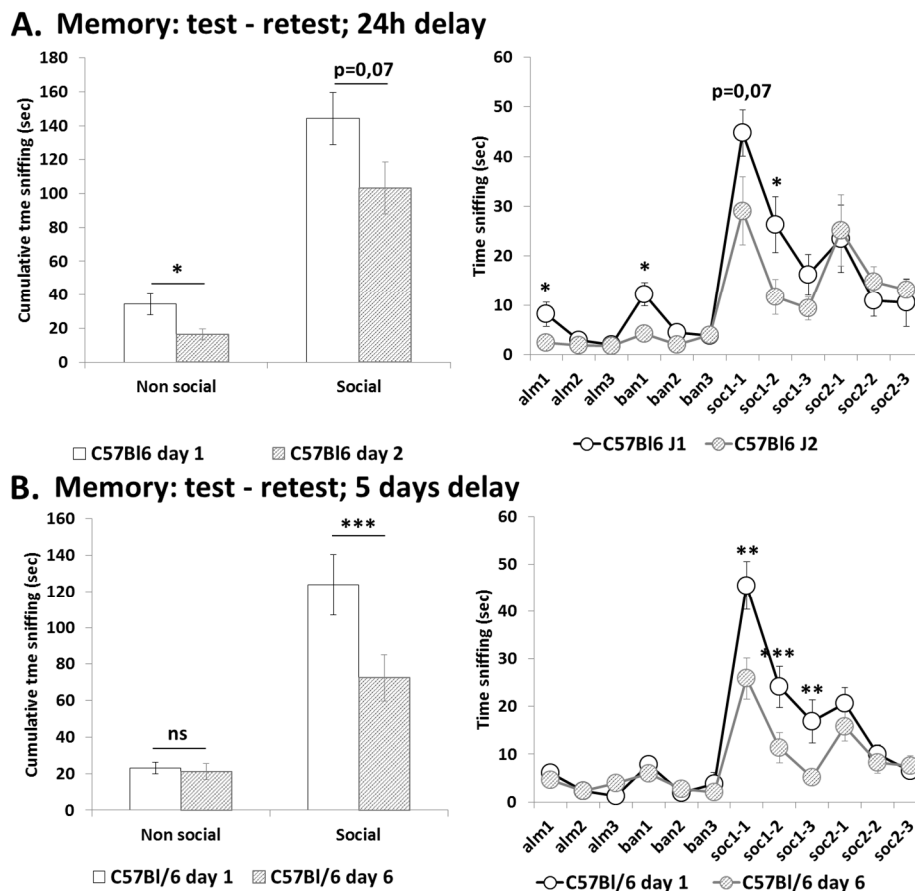
19  
20 **Figure 8: Mental retardation model: FmR1 knockout mice. (A)** Time spent investigating non-  
21 non-social and social odors by two cohorts of FmR1 knockout ( $n=14$ ) mice and their wild type  
22 controls ( $n=12$ ). Non-social odors were almond 1/25 and banana 1/1000 and social odors  
23 were two urinary mixtures collected from adult C57BL/6JRj male mice differing in their  
24 composition. **(B)** Time spent investigating non-social and social odors by two other cohorts of  
25 FmR1 knockout ( $n=15$ ) mice and their wild type controls ( $n=13$ ). Non-social odors were the  
26 same as previously mentioned and social odors were one urinary mixture collected from  
27 juvenile C57BL/6JRj male mice as social odor 1 and one urinary mixture collected from adult  
28 C57BL/6JRj male mice as social odor 2. \*  $p<0.05$ ; and \*\*  $p<0.01$  \*\*\* compared to wild type

1 controls. Left panel: cumulative time spent exploring and right panel: time spent exploring  
 2 each presentation of odors.

3  
 4

### 3.2.3 Experiment 8 (Memory)

5 We sought to further support the construct validity of our setup by addressing the question  
 6 of olfactory learning and memory. We were first interested in assessing the duration of both  
 7 non-social and social odors' memory by changing the delay between 2 exposures to the  
 8 same non-social and social odors. In this way, exposing mice to the same odors with a 24h  
 9 inter trial delay resulted in a decrease in investigation time regarding both non-social odors  
 10 (Wilcoxon test:  $U = 27$ ;  $p = 0.031$ ) and social odors (Wilcoxon test:  $U = 27$ ;  $p = 0.07$ ) (Figure 9A;  
 11 left graph). In support of this, mice were less attracted by almond and banana during their  
 12 first presentation (Wilcoxon test:  $U = 26$ ;  $p = 0.015$  and  $U = 26$ ;  $p = 0.047$  respectively) (Figure  
 13 9A; right graph). This was also true for the 2 first presentations of social odor 1 (Wilcoxon  
 14 test:  $U = 24$ ;  $p = 0.07$  and  $U = 24$ ;  $p = 0.048$  respectively) (Figure 9A; right graph).  
 15 In a second experiment, we increased the inter trial delay between test and retest sessions  
 16 and observed that mice investigated non-social odors as importantly as during the first  
 17 session (Wilcoxon test:  $U = 85$ ;  $p = 0.63$ ) whereas they were significantly less interested in  
 18 social odors (Wilcoxon test:  $U = 130$ ;  $p = 0.0004$ ) (Figure 9B; left graph). Interestingly, this  
 19 latter effect was evident especially during the 3 presentations of social odor 1 (Wilcoxon  
 20 test:  $U = 123$ ;  $p = 0.0027$ ,  $U = 129$ ;  $p = 0.0006$  and  $U = 127$ ;  $p = 0.001$  respectively) (Figure 9B; right  
 21 graph).  
 22



23

24 **Figure 9: Investigation of memory processes. (A)** Time spent investigating non-social and  
 25 social odors by one cohort of C57BL/6J mice ( $n = 8$ ) during two sessions, with a 24h inter trial  
 26 delay. \*  $p < 0.05$  compared to day 1. **(B)** Time spent investigating non-social and social odors  
 27 by another cohort of C57BL/6J mice ( $n = 16$ ) during two sessions, with a 5-day inter trial  
 28 delay. \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  compared to day 1. For the two experiments, non-social

1 odors were almond 1/25 and banana 1/1000 and social odors were two urinary mixtures  
2 collected from adult C57BL/6JRj male mice differing in their composition. Left panel:  
3 cumulative time spent exploring and right panel: time spent exploring each presentation of  
4 odors.

5  
6

### 3.3) Predictive validity

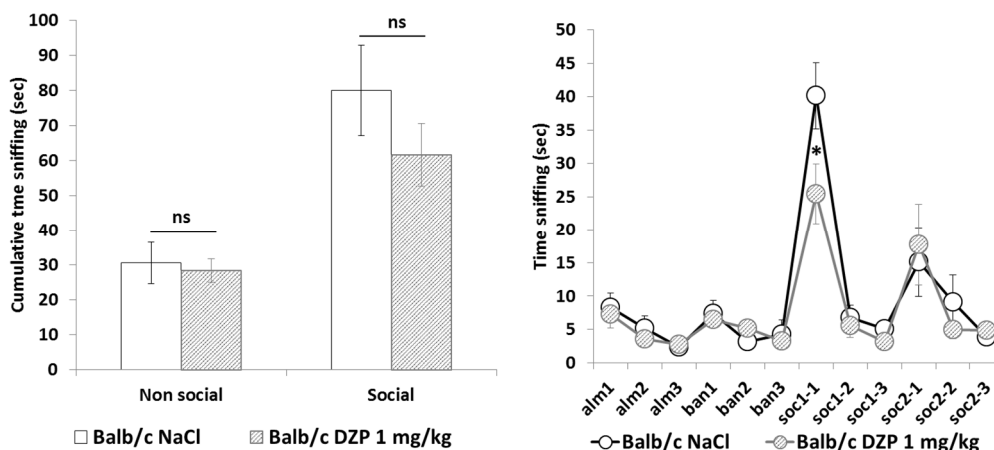
7

#### 3.3.1) Experiment 9 (Pharmacological relief of anxiety)

8 To further implement our system, we wanted to verify whether anxiety symptoms could be  
9 relieved pharmacologically through the use of a molecule known to decrease emotional  
10 reactivity. With this aim, the benzodiazepine diazepam was administered 30 minutes before  
11 testing and then Balb/cJRj mice were run within the setup. This strain was chosen as it is  
12 recognized to express persistent high levels of anxiety (Griebel et al., 2000). Results showed  
13 no effect of diazepam on time spent investigating both the non-social (Mann-Whitney test:  
14  $U = 28$ ;  $p = 0.95$ ) and social (Mann-Whitney test:  $U = 38$ ;  $p = 0.28$ ) odors (Figure 10; left graph).  
15 However, by considering only the first presentation of social odor 1, data showed that  
16 diazepam significantly reduced mice interest for social cues (Mann-Whitney test:  $U = 46$ ;  
17  $p = 0.028$ ) (Figure 10; right graph). This effect could not be attributed to diazepam-induced  
18 hypolocomotion as locomotor activity was not affected by the treatment (see  
19 supplementary data; Figure S1).

20 These data not only show that the exaggerated olfactory response of Balb/cJRj mice to social  
21 odors can be attenuated by anti-anxiety treatments, but more importantly that the setup  
22 may be used to address questions about emotional reactivity deficits and its treatment.

23



24

25 **Figure 10: Effect of the anti-anxiety compound diazepam. (A)** Time spent investigating non-  
26 social and social odors by two cohorts of Balb/cJRj mice ( $n = 8$  for each group) with one  
27 receiving diazepam intraperitoneally 30 minutes before testing. The other group of mice  
28 received injection of vehicle. Non-social odors were almond 1/25 and banana 1/1000 and  
29 social odors were two urinary mixtures collected from adult C57BL/6JRj male mice differing in  
30 their composition. Left panel: cumulative time spent exploring and right panel: time spent  
31 exploring each presentation of odors. \*  $p < 0.05$  compared to vehicle-treated mice.

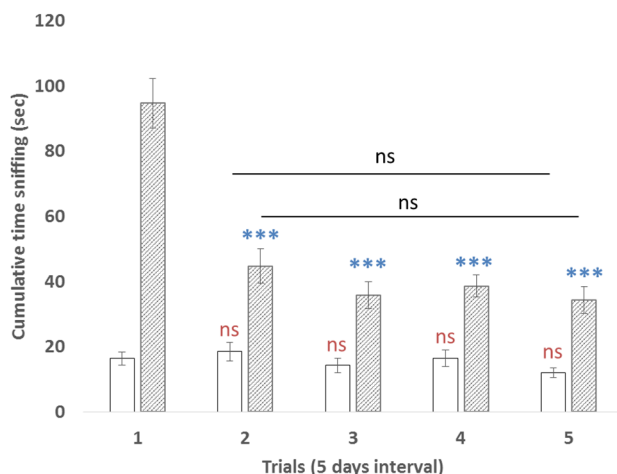
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33

#### 3.4) Experiment 10 (Test – Retest strategy: repeated testing)

34 In order to verify that mice could be repeatedly tested in the apparatus, we exposed one  
35 cohort of mice to both non-social and social odors on several sessions. We made the  
36 assumption that changing all the odors from one session to another, we could avoid

1 habituation process which is the main issue in longitudinal behavioral studies. As expected,  
 2 mice spent as much time investigating non-social odors during the first session as during all  
 3 the other ones (Figure 11; **Friedman test, ANOVA:  $Q=6.81$ ;  $p=0.15$** ). Regarding social odors,  
 4 we were surprised to observe a reduced investigation time during the second session  
 5 compared to the first one (**Friedman test, ANOVA:  $Q=30.9$ ;  $p<0.0001$ ; Pairwise difference  
 6 between trail 1 and trial 2: **Dunn's test;  $p<0.0001$** ), suggesting that something was recalled  
 7 during the second trial despite the fact that social mixtures were different from one trial to  
 8 another. For all other sessions, time spent investigating social odors was not changed  
 9 compared to the second session (Figure 11).  
 10 Such results showed that attractiveness of both non-social and social odors is reliable even  
 11 when multiple exposures (with each trial with different odors) to the setup are performed.  
 12**



13  
 14 **Figure 11: Effect of repeated testing. (A)** Time spent investigating non-social and social  
 15 odors by one cohort of C57BL/6JRj mice ( $n=16$ ) during repeated sessions with a 5-day inter  
 16 trial interval. Non-social odors were as follow: first session: almond 1/25 and banana 1/1000;  
 17 second session: orange 1/10 and toffee (1/600); third session: cotton candy (1/400) and  
 18 exotic fruits (1/200); fourth session: pineapple (1/100) and peach (1/100); fifth session red  
 19 fruits (1/100) and vanilla (1/100) and sixth session: chicory (1/100) and lemon (1/100). All  
 20 social odors used in this series of experiment were collected from unknown adult C57BL/6JRj  
 21 male mice and were different in their composition from one session to another (10 urinary  
 22 mixtures were subsequently used). For non-social odor, respective dilution factors were  
 23 determined according to their intensity rated by Human experimenters. \*\*\*  $p<0.0001$   
 24 compared to vehicle-treated mice.

25  
 26

## 4) Discussion

For some years now, there is accumulating evidence strengthening the need for behavioral testing to evolve by developing new experimental bioassays or by improving already existing tests (Hanell and Marklund, 2014; Homberg, 2013; Nestler and Hyman, 2010a). Such advances have to be made in respect of the 3Rs principles which both protect animal welfare and ensure valid and reliable data acquisition for scientists (Russell, 1959). This declaration states that researchers have to Replace, Reduce and Refine animal use in their experiments. In agreement with this, we described here how a well-known olfactory-based task can be optimized with the main objective to explore many dimensions of mice behavior in only one experimental paradigm.

### 4.1) Few changes for important refinement

As stated by many authors, new or improved behavioral bioassays should be thinking or rethinking such as to be simple, economical, reliable and reproducible (Hanell and Marklund, 2014; Nestler and Hyman, 2010a). In line with this, an increasing number of researchers have, during the last decade, performed home-made modifications of already existing apparatus to assess various dimensions of mice behavior in a more prolific manner (Deacon, 2013; York et al., 2013). We consider this movement to be of particular interest for the field of behavioral testing, and it is in that path we modified the current version of the habituation/dishabituation olfactory task. The proposed adaptations presented here fulfilled most of the criteria stated above. First, experimental materials were not expensive and were obtained from hypermarkets for plastic boxes, and from providers of lab equipment for Eppendorf plastic tubes. This demonstrates that each lab, interested in using the setup, can easily install it at low cost. Non-social odors were purchased at very cheap prices from a private company specializing in food flavouring development. Social odors are completely free of charge as they were collected from mice reared in our facilities. For data acquisition, we used webcams also obtained in hypermarkets. The only significant investment to be done is related to the acquisition of a Video tracking software. However, this is crucial for the Refinement process to be efficient. Indeed, automation of data acquisition and data analysis through the use of such a system provides significant benefits such as objectivity of the scoring procedure, reduced work effort and reduced time spent performing the experiment, avoid animal experimenter interaction during testing and allow simultaneous tracking of individuals. All these criteria are in line with two of the three “R” of the Russell and Burch principle. The Refinement process, as they contribute to reduce mice distress during testing (by avoiding interactions with human investigators) and significantly increase data accuracy through increased objectivity of measurements. The “Reduce” process, as they decrease the total number of animals used in an experiment because each mouse can be used much more efficiently. More importantly, our experiments on the retest strategy strongly suggest that each animal can be used as its own control: this might be of particular value in drug discovery processes when dose-effect responses are to be assessed. As evidence of the efficacy of such a procedure, we acquired preliminary data showing that dose-effect response to acute fluoxetine might be studied (Supplementary data, Figure S2).

### 4.2) Internal validity and comprehensive analysis of behavior during the task

However, our goal was not to improve testing procedure at the expense of high quality research. This is the reason why we undertook a series of simple experiments which not only aimed at characterizing the fundamentals of the setup but also at describing relevant variables potentially acting as confounding factors. The first relevant point to be noticed is

1 that results we obtained, in terms of time spent investigating odors, were quite similar to  
2 those reported in other studies using the classical apparatus (Briellmaier et al., 2012;  
3 Silverman et al., 2011; Silverman et al., 2010; Yang et al., 2012; Yang and Crawley, 2009b).  
4 Indeed, odor habituation (decreased time spent investigating odors over successive  
5 presentations) and dishabituation (increase of time spent investigating when a novel odor is  
6 presented) were observed in each experiment. It was also the case for preference for social  
7 olfactory cues over non-social ones. We could show that these intrinsic characteristics were  
8 present each time the test was performed, not only within the same laboratory but also in  
9 different laboratories, demonstrating good reliability and replicability of the test. Such  
10 results are of importance as many behavioral assays are known to provide different data  
11 when performed in different labs even though experimental conditions are strictly  
12 controlled . It is classically argued that overall differences between laboratories may not  
13 realistically be preventable, as subtle differences in background noise and its daily pattern in  
14 modern concrete buildings, diversity of breeding devices and maintenance procedures,  
15 handling of animals by breeding staff as well as other variables, such as olfactory cues,  
16 cannot reasonably be controlled for, but are most probably of significant impact on some  
17 mice behavioral outputs (Crabbe et al., 1999; Wahlsten et al., 2003). In order to better  
18 address the accuracy of the measure, we studied the effect of relevant factors known to  
19 strongly affect mice behavior during testing. Differences in breeding conditions are  
20 recognized to be causatively related to inter lab discrepancies (Olsson and Dahlborn, 2002)  
21 but no changes were observed when we tested this hypothesis. Such a between lab  
22 consistency indicates that this behavior is not sensitive to varying breeding conditions.  
23 Another possibility for this stability is related to the significant decrease of Human – animal  
24 interaction during testing. Indeed, it has already been shown that adopting such a strategy,  
25 through an increased automation of the apparatus, strongly contributed to the enhanced  
26 consistency of behavioral results obtained across laboratories (Krackow et al., 2010).  
27 However, only one condition was replicated within two laboratories (presentation of same  
28 non-social and social odors). Ideally, other conditions should be tested such as the effect of  
29 unrelated strain urinary mixtures.

#### 30 31 **4.2.1) Test refinement: on the use of non-social odors**

32  
33 With the objective of further exploring internal validity of the setup, we performed  
34 experiments directed to the field of non-social odors' interest. Our findings indicate that our  
35 setup may be used to study non-social odor hedonics. Indeed, in almost all experiments we  
36 performed, we could see that time spent investigation banana odor was higher than that of  
37 almond odor. Such a result was already observed in previous experiments carried-out not  
38 only in our lab (Laugeray et al., 2014) but also in others (Rattazzi et al., 2015; Silverman et  
39 al., 2011), while some studies reported no such preference (Hitti and Siegelbaum, 2014;  
40 Lugo et al., 2014; Silverman et al., 2010; Yang et al., 2012). Such inconsistencies could be  
41 explained by odor concentration used in these works. However, data exposed in the present  
42 study strongly strengthen the former assumption as increasing almond concentration  
43 resulted in less interest while increasing banana concentration resulted in more attraction.  
44 Moreover, such a result is in line with the fact that benzaldehyde odor (the smell of almond)  
45 is well-known to be aversive for mice (Saunders et al., 2013). In agreement with this, the  
46 findings obtained when mice were exposed to caramel-like odors (toffee and chicory)  
47 strikingly demonstrated that the apparatus can be used to address the field of non-social  
48 odor hedonics. Indeed, we found these two odors to be highly salient when their  
49 concentration was raised. The effect was so high that the amount of time spent investigating  
50 these odors (at high concentration) was as important as that of social odors. Such an  
51 assumption was verified through an additional experiment (Supplementary data; Figure S3).



1 This result is of crucial value as it demonstrates that the motivational drive needed to spend  
2 a high amount of time investigating a non-social odor can be dissociated from the  
3 motivational drive needed to explore social cues. Actually, it adds another dimension to be  
4 assessed in the apparatus which is in agreement with the 3Rs principle. Additionally, this  
5 finding may not only be useful to complete traditional interpretations made in the literature  
6 (on the preference of mice for social olfactory cues over non-social ones) but also to provide  
7 a new behavioral tool to assess anhedonia-like symptoms in animal models of depression for  
8 example.

#### 10 **4.2.2) Test refinement: on the use of social odors**

11  
12 In the classical practice of the test, social odors are described to be more attractive than  
13 non-social odors (Silverman et al., 2010; Yang et al., 2012; Yang and Crawley, 2009b).  
14 However, such an interpretation need to be performed very carefully in regard of what we  
15 discussed above concerning some non-social olfactory cues provoking high level of  
16 investigation. Indeed, without including a salient non-social odor in their olfactory tasks,  
17 researchers may falsely conclude on the social specificity of an effect whereas it may reflect  
18 an overall decrease of mice motivational drive, not necessarily detected when only “neutral”  
19 non-social odors are used. Such a strategy will significantly contribute to refine the test as it  
20 will be more informative and more useful for researchers in order to better dissociate brain  
21 disorders in which social deficits are observed. In this way, autism-like deficits in which only  
22 the social field should be disturbed, will be dissociated from depression-like alterations, in  
23 which the motivational drive for both non-social and social olfactory should be affected.

24 Another key characteristic deserves to be addressed as it would allow to explore another  
25 dimension of mice behavioral repertoire. In all the experiment we performed, the second  
26 social odor induced less interest than the first one. This effect is in agreement with most  
27 studies reported in the literature (Silverman et al., 2010; Yang et al., 2012; Yang and Crawley,  
28 2009b). This is quite surprising considering that urinary mixtures were of different  
29 composition which should theoretically induce similar interest. Such a finding suggests not  
30 only that some memory processes are at work during social odor 2 presentation but also  
31 that this trial may provide additional information on mice cognitive abilities. This  
32 phenomenon can be explained by the type of information conveyed by urinary mixtures.  
33 Indeed, the urine of mice contains a high concentration of Major Urinary Proteins (MUP)  
34 which informs conspecifics about numerous information such as strain, sex, reproductive  
35 and social status, the time since the mark was deposited, and the identity and relatedness of  
36 the donor (Hurst; Hurst et al., 2001). As in most of our experiments, the two social odors  
37 were collected from male mice of the same strain, it is very likely that these odors contained  
38 a common set of MUP (conveying the same information, i.e. strain, sex, species) which  
39 rendered the second social odor less interesting as it was just encountered shortly before.  
40 Such findings implies that changes in interest for social odor 2 may provide information of  
41 different behavioral relevance (social cognition) and then must be more extensively studied.  
42 In line with this, we also showed that the ethological meaning of the first social odor induced  
43 behavioral changes in response to social odor 2 when the latter is of different significance.  
44 Indeed, the fact for adult male mice to be exposed to juvenile odor as social odor 1  
45 significantly increased their interest for adult male mice odor when presented as social odor  
46 2. This finding suggests that social odor 1 “quality” may be used to manipulate the  
47 ethological context of the test, i.e. place the test animal in a specific internal/biological state,  
48 and then explore behavioral responses emitted when various olfactory stimuli are  
49 presented. For example, interest for female odors in a context of social dominance should be  
50 evaluated by presenting urinary mixtures collected from dominant mice as social odor 1 (to  
51 place test animals in the context of social dominance) and mixtures obtained from female

1 mice as social odor 2. Of course, these assumptions should be experimentally verified, but if  
2 so, such a feature would constitute a very promising tool to perform multidimensional  
3 analysis of mice behavior in only one task.

#### 4 5 **4.2.3) One task for a multi-parametric use**

##### 6 7 **- Emotional reactivity impairments**

8  
9 To better validate our system and then understand on which behavioral dimensions it can  
10 provide informative data, we subjected mice to several conditions known to result in  
11 behavioral changes. The comprehensive assessment of mouse models of CNS disorders is  
12 based on genetic, pharmacological and environmental manipulations. So, we decided to use  
13 these manipulations to further address the validity of our system. First we chose to  
14 investigate the realm of emotional reactivity. Emotional reactivity is defined as the tendency  
15 to react intensively to emotion-generating stimuli, and is expressed in terms of high  
16 emotional sensitivity and low emotional endurance (Dragan and Dragan, 2013). To address  
17 that point, we used one of the most popular model of trait anxiety, the highly anxious  
18 Balb/cJRj mice (Belzung and Griebel, 2001). According to our data, high level of emotional  
19 reactivity is associated with a rise in interest for odors, especially for social odors. However,  
20 it seems to be true only when the concerning odor has the status of “novel odor” (i.e. first  
21 presentation). Such a result suggests some kind of increased novelty seeking behavior  
22 (increased interest for novel stimulus). However, Balb/cJRj mice are known to display low  
23 level of novelty seeking behaviors (Belzung and Berton, 1997; Griebel et al., 1993). In  
24 accordance with data obtained from other studies performed in our lab, one can assume  
25 that this result may be related to high aggressiveness inherent to Balb/cJRj mice (Van Loo et  
26 al., 2003). Indeed, when we tested another strain known to display high motivation to  
27 aggressive behaviors (CBA/JRj male mice), we also observed an increased interest for social  
28 odor 1 (first presentation) compared to male C57BL/6JRj mice (see supplementary data,  
29 Figure S4). Interestingly, this mice model also displayed high level of anxiety-like behaviors.  
30 However, such an assumption has to be addressed in detail. The link between this olfactory  
31 behavioral trait of Balb/cJRj and anxiety was considerably highlighted by the fact that  
32 injection of the well-known anxiolytic diazepam completely reversed these alterations, not  
33 only demonstrating that anxious features can be assessed in our system but also that  
34 efficacy of putative anti-anxiety treatments can be studied.

35 Our data also show that emotional reactivity can be assessed in this system by rendering  
36 environmental conditions during testing more challenging for mice. Indeed, sawdust  
37 removal is known to be stressful for rodents, inducing both behavioral and physiological  
38 changes (Laviola et al., 2002). In this aversive context, mice interest for odors was reduced  
39 depending on strain and odor types. Indeed, in C57BL/6JRj mice, only social odor  
40 investigation was affected whereas in Balb/cJRj mice, both non-social and social odors  
41 became less attractive. This is in agreement with other studies showing that exposing mice  
42 to a stressful event during testing modifies some of their olfactory-based behaviors  
43 (Arakawa et al., 2008). This result shows that the respective behavioral response of these  
44 two strains, which are not equally affected by environmental stress, can be dissociated by  
45 the system, highlighting the accuracy of the provided measurements. Total or partial  
46 reversion of these olfactory changes by anti-anxiety compounds would be of interest to  
47 further validate that point.

##### 48 49 **- Social impairments**

1 The second realm we were interested to explore was the social dimension of mice  
2 behavioral repertoire. Social disturbances are pathological features recurrently observed in  
3 mice models of CNS disorders such as depressive-like disorders, schizophrenia and autism-  
4 spectrum disorders (ASD). We chose to expose FmR1 knockout mice in our setup for two  
5 reasons: to further implement our system with a recognized model of ASD-like symptoms  
6 and then because FmR1 knockout mice had never been tested in an olfactory-based task so  
7 far. FmR1 knockout mice constitute a mouse model of the Fragile X Syndrome (FXS), a  
8 genetic disease associated with ASD-like symptoms including deficits in social adaptation  
9 (Bernardet and Crusio, 2006). When we exposed FmR1 knockout mice to the setup, no  
10 alterations were observed in response to non-social and social odors when these were  
11 composed of adult male mice odors. However, when these mice were confronted to juvenile  
12 odors as social odor 1, they were highly attracted compared to their wildtype controls. Such  
13 findings are in accordance with studies reporting anomalous social behaviors in response to  
14 juvenile encounter (Gkogkas et al., 2014; Pietropaolo et al., 2011) but are not consistent  
15 with other works showing reduced social interaction in response to adult male mice  
16 (Bernardet and Crusio, 2006; Hébert et al., 2014). However, this latter deficit is not  
17 consistently reported in the literature with some studies showing decreased or equal while  
18 others report increased social skills during social interaction tests (Bernardet and Crusio,  
19 2006). It is also of importance to notice how reduced was the time spent investigating adult  
20 social odors compared to all other experiment involving C57BL/6JRj male mice. This is  
21 especially true considering that this incongruity was observed in the two experiments we  
22 performed. Such an irregularity would deserve to be explored more thoroughly. Beyond  
23 fundamental questions raised by our results, data collecting from our experiments  
24 demonstrate the ability of the setup to detect abnormal social behaviors in a well-known  
25 mice model of ASD.

### 26 - Cognitive impairments

29 Our data also indicate that learning and memory processes are likely to be evaluated in the  
30 system. Indeed, we were able to show that mice exposed to non-social and social odors  
31 during two consecutive sessions with a 24h inter trial interval can recognize all the odors  
32 presented before, irrespective of their ethological meaning. Such findings are in agreement  
33 with other studies showing that C57BL/6JRj mice could remember social olfactory cues 24h  
34 after a previous exposure (Arakawa et al., 2008). Importantly, it shows that short-term  
35 memory impairments can be assessed in the apparatus. In order to evaluate olfactory  
36 memory duration, the interval between the two sessions was increased to 5 days. In this  
37 case, data indicated that mice were able to remember only the social odors indicating that  
38 duration of long-term memory depends on the significance of the stimuli. This finding is  
39 consistent with the view that emotional tone / arousal level associated to ethologically  
40 relevant stimuli such as social olfactory cues modulates consolidation of lasting memories  
41 (Conversi et al., 2014; Tandler and Wagner, 2015). This outcome is of particular importance  
42 in respect of the refinement strategy to which we are committed as it highlights the  
43 multidimensionality of data provided by the task, offering researchers the opportunity to  
44 explore both social and non-social memory processes in only one test. As far as social  
45 memory is concerned, the work by Arakawa *et al.* showed that mice could not remember  
46 social olfactory cues anymore after a 7 day interval between the two sessions (Arakawa et  
47 al., 2008), indicating that social memory lasted no more than 7 days in their setup. Their  
48 findings were in agreement with studies reporting that rodent social memory has a relatively  
49 short term duration (Sekiguchi et al., 1991; Winslow and Camacho, 1995). However, one  
50 report showed long-lasting (at least 7 days) social memory in a similar paradigm (Kogan et  
51 al., 2000) . Even if our data did not allow to conclude about the duration of long-term

1 memory, our results were in agreement with the study by Kogan *et al.*, as it seems to last at  
2 least 5 days in the present setup.

3 In addition, the two experiments we performed to explore the field of olfactory memory also  
4 emphasized the cognitive particularity inherent to social odor 2. Indeed, we were surprised  
5 to not observe odor habituation (compared to session 1) during the second session as should  
6 have been the case if the two odors were learned equally. We cannot explain this  
7 phenomenon but it strengthens the fact that what happens during exposure to social odor 2  
8 is of specific ethological meaning and deserves be addressed more thoroughly.

## 9 10 **5) Limitations as concluding remarks**

11  
12 The proposed adaptations of this well-known olfactory-based task allow the flexibility to  
13 measure a wide range of emotional and cognitive processes by small adjustments, as  
14 described above. Indeed, changing various aspects inherent to either mice biological and  
15 psychological state, environmental conditions before and / or during testing, or behavioral  
16 meaning conveyed by olfactory stimuli, may provide crucial information on the behavioral  
17 adjustment mice perform in specific ethological contexts and then contribute to increase  
18 interpretations refinement. Such a testing procedure has the advantage to require neither  
19 behavioral training or conditioning, nor behavior-altering procedures as it is based on innate  
20 mouse behaviors. Even though the results exposed here are non-exhaustive, our approach is  
21 in accordance with the strategy behavioral researchers working on bioassay  
22 development/improvement should adopt in order to pave the way for other non-behavioral  
23 researchers who wish to perform behavioral analysis of their model.

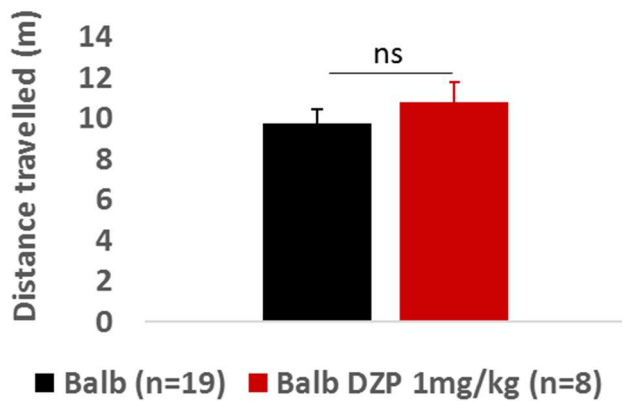
24 Despite all the benefits provided by this optimized behavioral task, there are some caveats  
25 to be addressed. First of all, it is to be noticed that some of our results, even though they  
26 strengthen internal validity of the task, are not consistent with what is reported in some  
27 studies. Indeed, according to our results, using urinary mixtures differing in their  
28 composition (especially when urine of other strains were used) did not induce any significant  
29 changes in terms of time spent investigating social odors. This is somewhat surprising in  
30 regard to the literature highlighting the ability for mice to distinguish individuals belonging  
31 to its own strain from those belonging to unrelated strains (Isles *et al.*, 2001; Zomer *et al.*,  
32 2009). However, this could be explained by confounding factors inherent to testing  
33 conditions which is often associated with result inconsistencies in the field of behavioral  
34 testing. Additional data suggest that urinary MUPs profile can be recognized very precisely  
35 as they convey specific information about individual identity (Robertson *et al.*, 1997). In  
36 support of this statement, we should have observed variable levels of attractiveness  
37 depending on the identity of mice included in the mixtures. For instance, a urinary mixture  
38 containing high amount of dominant MUP should have been more investigated than one  
39 containing more neutral MUP. However, it was not the case. An explanation for this  
40 phenomenon could be related to the high number of individuals we used to constitute  
41 urinary mixtures. Another explanation has support in the literature showing that individuals  
42 from highly inbred strains are unable to discriminate between each other's urinary odors as  
43 they have the same MUP patterns (Arakawa *et al.*, 2008; Nevison *et al.*, 2000). In this way,  
44 making mixtures varying in their composition should have limited consequences on  
45 investigation time.

46 This setup offers new perspectives not only in the fundamental field of mice behavioral  
47 exploration but also for drug discovery as pointed out by our results on the repeated testing  
48 strategy.

Experiment	Name of experiment	Associated figure	Experimental group	Individuals / group
1	Within laboratory replicability	2A	2	16-14
	Inter laboratory replicability	2B	2	16-20
2	C57Bl/6 mice exposed to different C57Bl/6 urinary mixtures	3A	2	15-16
	C57Bl/6 mice exposed to C57Bl/6 vs CBA/J urinary mixtures	3B	2	13-15
	C57Bl/6 mice exposed to C57Bl/6 vs CD1 urinary mixtures	3C	2	18-18
3	Influence of ethological meaning of social odor 1 over social odor 2 interest	4A, 4B, 4C	2	13-14
4	Effects of breeding conditions	5	3	10-11-14
5	Non-social odor aversion vs. salience	6	2	16-16
6	Effect of trait anxiety: Balb/c vs. C57Bl/6 mice	7A	2	11-13
	Effect of state anxiety: environmental stress on C57Bl/6 mice	7B	2	9-10
	Effect of state anxiety: environmental stress on Balb/c mice	7C	2	10-11
7	FmR1 KO exposed to adult urinary odor	8A	2	12-14
	FmR1 KO exposed to juvenile urinary odor as social odor 1	8B	2	13-15
8	Mid-term memory: 24h inter trial with the same odors	9A	1	8
	Long-term memory: 5 days inter trial with the same odors	9B	1	16
9	Pharmacological relief of anxiety	10	2	8-8
10	Test-Retest strategy: repeated testing	11	1	16

**Table S1:** Details on experimental design. All experiments used animals that were naïve for the setup. No animals have been used in multiple experiments. Only animals involved in experiment 8 and 10 have been exposed several times to the setup.

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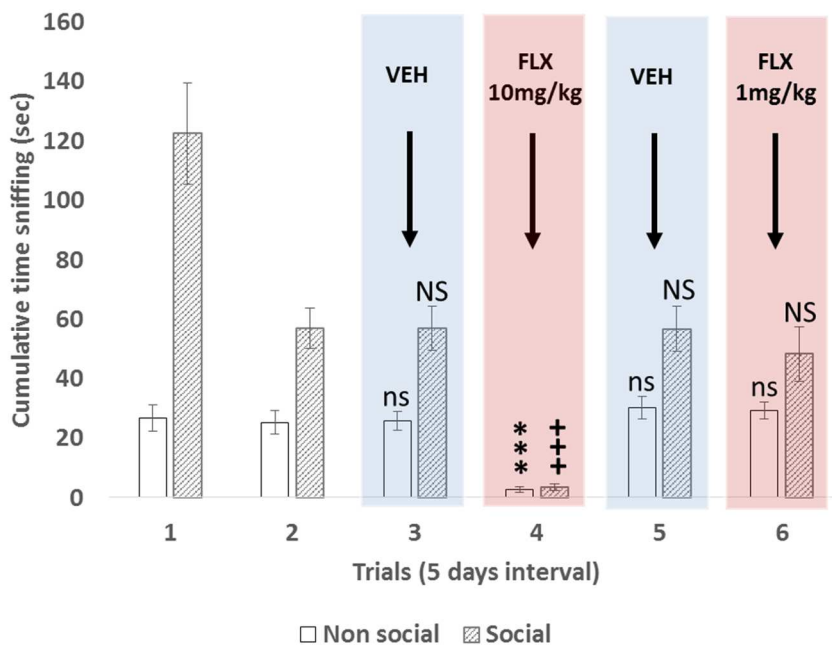
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16 **Figure S1: Diazepam does not change locomotor activity in Balb/cJRj mice. (A)** Total  
 17 distance travelled (m) during the habituation session, 30 minutes before olfactory testing.  
 18 Adult Balb/cJRj male mice (n=8) were injected with diazepam 1mg/kg i.p and left to explore  
 19 the apparatus during 30 minutes.

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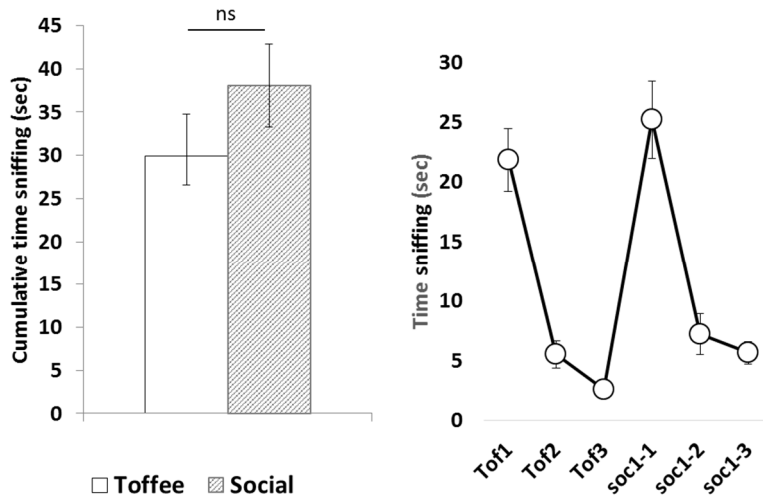
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23 **Figure S2: Dose - effect of the anxiogenic compound fluoxetine (FLX) during repeated**  
 24 **testing.** Cumulative time spent investigating non-social and social odors by one cohort of  
 25 adult C57BL/6JRj male mice (n=15) repeatedly exposed to the setup. Non-social odors were  
 26 as follow: first session: almond 1/25 & banana 1/1000; second session: orange 1/10 and  
 27 toffee (1/600); third session: cotton candy (1/400) and exotic fruits (1/200); fourth session:  
 28 pineapple (1/100) and peach (1/100); fifth session red fruits (1/100) and vanilla (1/100) and  
 29 sixth session: chicory (1/100) and lemon (1/100). All social odors used in this series of  
 30 experiment were collected from unknown adult C57BL/6JRj male mice and were different in  
 31 their composition from one session to another (12 urinary mixtures were used). For non-  
 32 social odor, respective dilution factors were determined according to their intensity rated by  
 33 Human experimenters. ns: compared to non-social odors, session 2. NS: compared to social  
 34 odor, session 2. \*\*\* p<0.001 compared to non-social odor, vehicle-treated mice, session 3.  
 35 +++ compared to social odor, vehicle-treated mice, session 3.

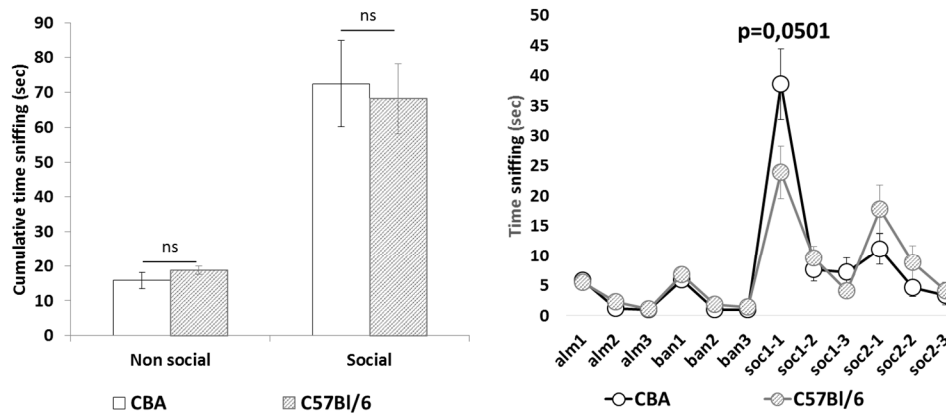
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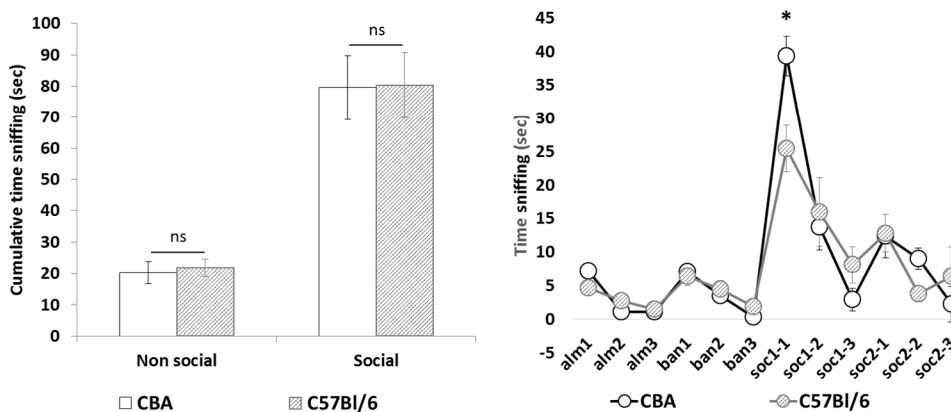
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**Figure S3: Some non-social odors are as attractive as social cues.** Time spent investigating the non-social odor toffee (not diluted) and a urinary mixture collected from adult C57BL/6JRj male mice. Tested mice were adult C57BL/6JRj male mice (n=20).

**A. CBA & C57Bl/6 mice exposed to CBA urinary mixtures**



**B. CBA & C57Bl/6 mice exposed to C57Bl/6 urinary mixtures**



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**Figure S4: CBA/JRj mice display higher interest for social odor 1 than C57BL/6JRj mice.** (A) CBA/JRj (n=15) and C57BL/6JRj (n=19) mice exposed to non-social odors (almond 1/25 and banana 1/1000) and two urinary mixtures collected from CBA/JRj individuals. Left panel: cumulative time spent investigating; Right panel: detailed analysis of time spent investigating each odor. (B) CBA/JRj (n=13) and C57BL/6JRj (n=13) mice exposed to non-social odors (almond 1/25 and banana 1/1000) and two urinary mixtures collected from C57BL/6JRj individuals. Left panel: cumulative time spent investigating; Right panel: detailed

1 *analysis of time spent investigating each odor. \* p<0.05 compared to time spent*  
2 *investigating social odor by C57BL/6JRj mice.*

3

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5

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7 University of Orléans (France).

8

#### 9 **Declarations of interest:**

10

11 None.

12

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