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Behavioral responses of 129/Sv, C57BL/6J and DBA/2J mice to a non-predator aversive olfactory stimulus

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Abstract. We examined the behavior of three inbred mouse strains (129/SvPasIco, C57BL/6J, and DBA/2J) exposed to an object soaked with the chemical component of the aversive scent (toluquinone odor) emitted by a myriapod species (*Ommatoiulus sabulosus*) in the presence of a predator. Subjects were exposed to the odor for three consecutive days. Behavioral responses to the toluquinone odor were characterized both by an approach phase of risk assessment and by a repeated series of approach-avoid episodes. Results indicate that toluquinone exposure reduced completely, and in a strain independent fashion, selected behaviors such as crouching, catching and eating object. Other responses were strain-dependent: the DBA (DBA/2J) strain displayed defensive burying at high levels, C57 (C57BL/6J) mice performed high levels of withdrawal while the 129/Sv (129/SvPasIco) strain showed also high levels of stretch attend posture. Compared to other tasks, this test is ethological, simple, cheap and is not affected by strain differences in appetitive-sensory responses, as shown by some strain-independent responses. These features make this task as a good complement to any exploration-anxiety test battery.

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Key words: aversive behavior, olfaction, genetic background, inbred strains, 2-methyl-1,4 benzoquinone, behavioral phenotyping, mice

INTRODUCTION

Olfaction is the main sensory modality for rodents. Olfactory cues are important for basic activities such as finding a mate, food, identifying young and avoiding danger (Blanchard et al. 2001a,b, 2003a,b, Dielenberg and McGregor 2001, Kavaliers and Choleris 2001). The natural defensive behaviors of rats and mice have been studied in both seminatural and highly structured situations, such as the mouse-defense test battery (Blanchard et al. 2001a) and characterized in terms of the relationship between specific behaviors and the stimuli that elicit them (Alleva et al. 1995, Blanchard et al. 2001a,b, 2003b). A number of test situations developed to investigate aversive responses are based on forced confrontations of rodents with a predator or unconditioned predator stimuli, such as odors (i.e., faeces, urine and scent products of cats, red fox and snake) (Berton et al. 1998, Dell'Omo and Alleva 1993, Dell'Omo et al. 1994, Lester and Fanselow 1985, Vernet-Murray et al. 1968). Depending upon the features of the environment, threat stimuli can elicit flight, hiding, freezing or a systematic species-typical risk assessment pattern of orientation approach and investigation of the stimulus (Blanchard and Blanchard 1989, Blanchard et al. 1991, 2001b, 2003b).

Responses to sources of aversive stimulation have been studied in both rats and mice (Blanchard et al. 2001a, De Boer and Koolhaas 2003). Although the defense patterns of the two most investigated species of laboratory rodents are generally similar, mice show risk-assessment on initial exposure to highly threatening stimuli, while rats do not (Blanchard et al. 2001a). In mice, relatively few data are available on the behavioral patterns of avoidance when the key stimulus is a novel, noxious non-predator odor (Crawley 2000, Dielenberg and McGregor 2001, Mihalick et al. 2000, Schellinck et al. 2001). Recently, a novel method set up for screening the behavioral responses to a repellent olfactory stimulus in mice (Capone et al. 2002, Olivieri et al. 2001) demonstrated the ability of both sexes of the outbred CD-1 strain to detect and remember the aversive odor secreted by the striped millipede *Ommatoiulus sabulosus* (L.). A large panel of avoidance, including risk-assessment, and non-avoidance behaviors are displayed by mice in the presence of the noxious olfactory stimulus (Capone et al. 2002, Olivieri et al. 2001).

Many different outbred and inbred genetic lines of rodents have been generated so far to be exploited in a variety of experimental investigations. Most of these lines show differences in behavior which, when seeking functional explanations for specific neural mechanisms, can make data interpretation difficult. Thus, it is important to characterize the different behavioral phenotype specific for each mouse strain and to develop easy tests to assess their behavioral competences (Crawley 2000, Holmes et al. 2002, Wahlsten et al. 2003).

The aim of this study was to use the test previously developed to characterize the behavioral patterns, including risk assessment and avoidance strategies, in 129/SvPasIco, C57BL/6J, and DBA/2J mice, three of the most commonly used strains in studies dealing with genetically modified mice. Compared to the most commonly used tests, this appears rather appealing since it does not require a complex set-up and relies upon exposing the animals to a naturalistic stimulus. The latter approach should make functional analysis more effective (Blanchard and Blanchard 2003).

To this purpose, we exposed, for three consecutive days, male mice of the three strains to an aversive odor consisting of the chemical component (toluquinone, methyl-p-benzoquinone) of the repelling exudate naturally emitted by the millipede *Ommatoiulus sabulosus*.

MATERIALS AND METHODS

Subjects

Twenty-four adult male mice for each of the three inbred strains 129/SvPasIco (129/Sv), C57BL/6J (C57), and DBA/2J (DBA) were purchased from a commercial breeder (Charles River, Calco, Italy). Upon arrival at the laboratory, the animals were housed in an air conditioned room (temperature $21 \pm 1°C$, relative humidity $60 \pm 10\%$) under a reversed 12/12 h light/dark cycle with lights off from 08:00 A.M. to 08:00 P.M. and kept in these housing conditions for at least 2 weeks prior to behavioral testing. Adult virgin mice were individually housed in $33 \times 13 \times 14$ cm Plexiglas boxes, in order to avoid intermale aggression. Food consumption (enriched standard diet purchased from Mucedola, Italy) and tap water were freely available.

All animal handling and experimental procedures were performed in accordance with the EC guidelines (EC Council Directive 86/609, 1987) and with the Italian legislation (Decreto L.vo 116/92) on animal experimentation.

Procedure

Mice were around 3-months-old when the experiment started. All experimental manipulations were conducted between 10:00 A.M. and 03:00 P.M. in a quiet experimental room with the same temperature, humidity and light conditions as the colony room. On the day of testing each subject was individually transferred in the experimental room and individually exposed in its home cage to the stimulus object (SO), consisting of a sponge resembling a millipede in shape and dimension (0.8-cm diameter, length of 4-cm) previously soaked in: (i) a solution containing 5 g of 2-methyl-1,4 benzoquinone (Methyl-p-benzoquinone; p-toluquinone, Fluka Catalogue 2003-2004, p. 1025. Product number: 89590) dissolved in 100 ml of distilled water – toluquinone group; (ii) the vehicle of the solution – control group. The SO was introduced in the right corner of the animal cage at the opposite end from the pellet food (for more details see Capone et al. 2002).

Each mouse was randomly assigned either to the toluquinone or to the control group and was exposed for 12 min to the SO for three consecutive days (day 1, day 2 and day 3). The control group was always tested before the toluquinone one.

Behavioral observation

Behavioral responses of each experimental subject were video-recorded using a Panasonic AG-6200 apparatus equipped with a CH-1400CE video-camera for red lights. Frequency and duration of each behavioral response induced by SO exposure were registered and scored using the software Observer 3.0 (Noldus 1991). Scoring was by trained observers blind to the condition of the animals.

We used an ethogram previously described by us (Capone et al. 2002, Olivieri et al. 2001) as well as by others (Birke and Sadler 1986, Blanchard et al. 2003b, Carere et al. 1999, De Boer and Koolhaas 2003) including a large repertoire of avoidance as well as of non-avoidance behaviors.

The following items were scored: risk assessment, avoidance behaviors – defensive burying (the animal digs the sawdust in the direction of the SO, with the forelimbs often kicking it away with the hindlimbs), stretch attend posture (the animal approaches and sniffs the SO with flat back and stretch neck), withdrawal (approach-avoid) (the animal approaches the SO without contacting it, immediately withdrawing from it – this behavior is performed numerous times during the test); non-avoidance behaviors – locomotion (time spent moving), inactivity (the animal spends time sleeping or without moving), crouching over object (the animal investigates and crouches over the SO – this behavior usually precedes catching and involves contact with the SO), catching (the animal takes the SO in its mouth), eating object (the animal eats the SO), food consumption (the animal eats the pellet food freely available in its cage), grooming (the animal licks its own fur, sometimes using its forepaws, passing them over the nose with a series of brief horizontal movements), bar holding (the animal grasps the metal top of the cage holding himself above the level of the ground), rearing (the animal stands on its hindlimbs), wall rearing (the animal stands on its hindlimbs and touches the wall of the cage with its forelimbs), digging (the animal digs the sawdust with the forelimbs often kicking it away with the hindlimbs).

Statistical analysis

Frequency and duration of each behavioral item were analyzed by parametric mixed models of ANOVA considering exposure condition (control *versus* toluquinone exposure) and strain as between-subject factors and day as repeated measure (within-subject factor). Multiple comparisons were carried out using the Tukey HSD test. Table II and III summarize ANOVA's results.

RESULTS

Risk Assessment-Avoidance Behaviors

Exposure to toluquinone strongly affected the risk assessment-avoidance behavioral responses of the three strains considered (see Fig. 1 and Table I). In particular:

Defensive burying (DB): exposure to the toluquinone odor induced a specific increase in DB in all subjects ($F_{1,66}$=30.1, P=0.0000 and $F_{1,66}$=23.17, P=0.0000, respectively for frequency and duration). This effect was strain dependent ($F_{2,66}$=4.22, P=0.0188 and $F_{2,66}$=3.79, P=0.0277, respectively for frequency and duration). A significant interaction between strain × exposure condition was found both for frequency and duration ($F_{2,66}$=4.26, P=0.0182 and $F_{2,66}$=3.82, P=0.0269, respectively). *Post-hoc* comparisons carried out for dura-

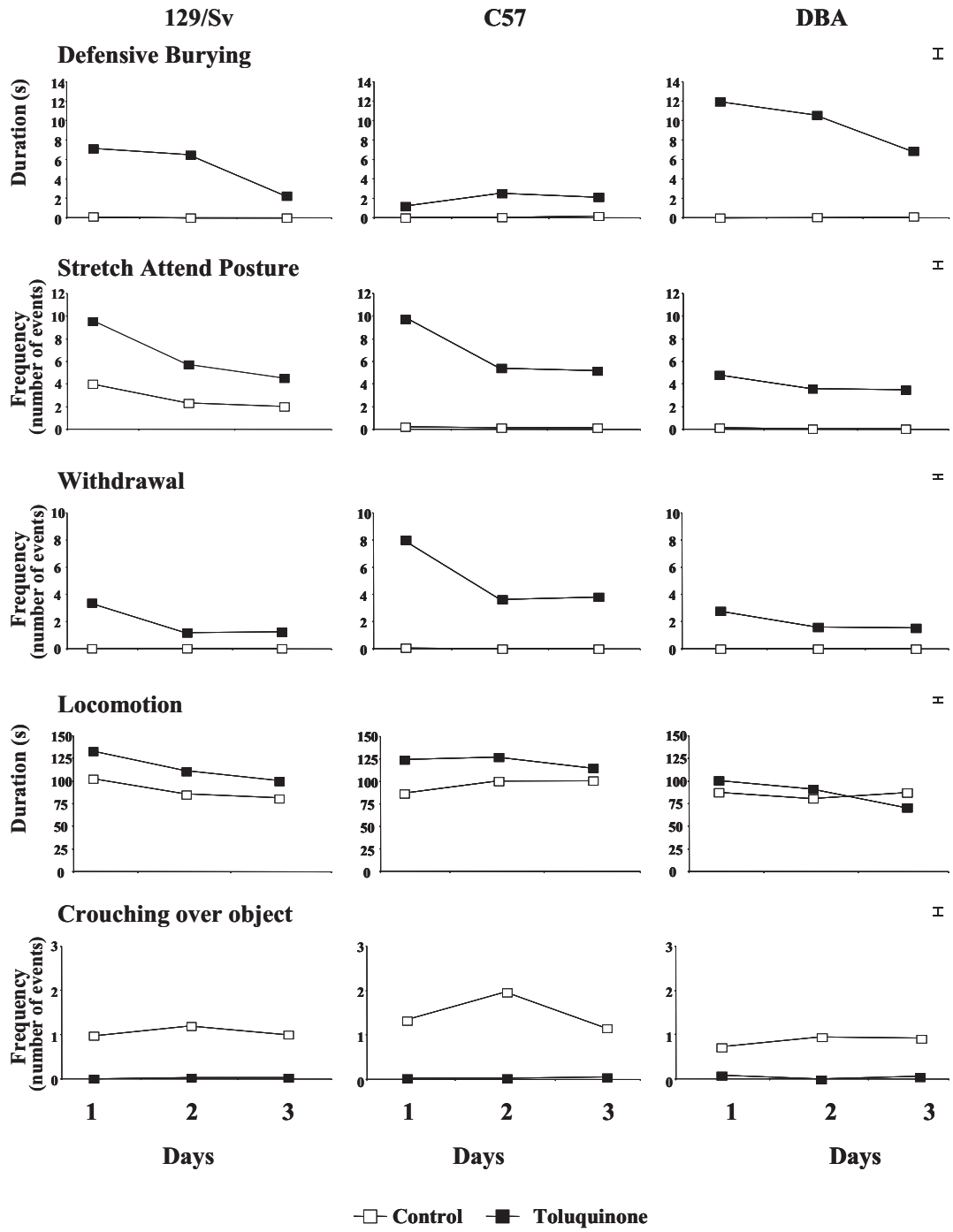

Fig. 1. Risk assessment and non-avoidance behaviors in three inbred strains of mice. Frequency or duration of defensive burying, stretch attend posture, withdrawal, locomotion and crouching over object performed by 129/Sv, C57 and DBA mice exposed for 12 min, over three consecutive days, to a stimulus object, consisting of a sponge resembling a millipede soaked in distilled water (C) or in toluquinone solution (T). Data are means. For each strain and in each C and T group n=12 subjects. The vertical bars on the right hand side indicate the pooled SEM. The Pooled SEM is the standard error of mean pooled across all subgroups under comparison. It is the error term for the assessment of the difference between subgroups in multiple comparisons tests (such as the Tukey's HSD test). It is computed dividing the mean square of the error term appropriate for the assessment of the effect under evaluation in the ANOVA by the (common) number of observations in each subgroup, and extracting the square root of this ratio (namely, pooled SEM = sqrt (MSerror/n). See the results section for a description of the main effects of strain, experimental condition and day.

Table I

Mean frequency and/or duration of selected behavioral responses recorded during each of the three exposures to a stimulus object soaked with vehicle (C) or toluquinone (T)

			129/Sv		C57		DBA			
		Day	C	T	C	T	C	T	MS1	MS2
Inactivity	Dur	1	9.19	9.92	0.17	0.17	0.06	1.06	2 003.79	525.54
		2	30.36§§	39.22§§	0.33	0.72	0.06	0.06		
		3	51.08§§	47.80§§	0.14	13.69	0.11	0.00		
Bar holding	Fr	1	0.11	0.03	3.86	2.92	3.22	3.08	13.35	1.65
		2	0.14	0.11	3.36	3.36	3.67##	5.33##		
		3	0.03	0.08	3.58	2.78	4.75##	4.30##		
	Dur	1	0.31	0.03	29.78	34.64	30.28	36.72	1 514.17	254.47
		2	0.31	0.28	25.50	33.75	39.72##	63.44##		
		3	0.03	0.78	33.39	34.83	44.67##	54.08##		
Catching	Fr	1	1.11	0.00	1.83	0.00	2.11	0.00	4.84	2.00
		2	1.22	0.00	3.25	0.00	2.75	0.00		
		3	1.44	0.00	3.17	0.00	2.61	0.00		
Eating object	Fr	1	3.06	0.03	4.39	0.00	3.69	0.00	5.53	1.39
		2	3.17	0.00	6.05£	0.00	3.86	0.00		
		3	3.08	0.00	4.94	0.00	4.00	0.00		
	Dur	1	60.88$	0.08	67.33$	0.00	52.03$	0.00	946.20	330.33
		2	49.22$	0.00	47.97$	0.00	51.89$	0.00		
		3	28.19$	0.00	39.77$	0.00	42.64$	0.00		
Digging	Fr	1	0.28	0.31	0.92	2.14	3.00	1.56	3.75	1.17
		2	0.14	0.31	1.56	1.97	2.17	1.14		
		3	0.22	0.36	1.47	1.00	2.25	0.64		
	Dur	1	0.56	0.72	1.94	4.81	8.39	2.94	42.46	21.40
		2	1.39	0.81	4.86	4.25	9.94	4.53 &		
		3	0.36	0.56	2.81	1.56	4.14	1.28		
Food consumption	Fr	1	0.69	1.36	0.75	1.11	1.58	2.72	4.76	2.14
		2	1.86	2.25	0.53	2.06	1.86	2.56		
		3	1.47	2.50	0.83	2.89	1.69	2.67		
	Dur	1	3.11	14.22	2.67	4.28	9.22	33.00	1 901.62	409.14
		2	17.92	16.03	2.56	13.11	11.72	25.06		
		3	17.75	31.53**	7.69	26.17**	14.58	69.44**		
Wall rearing	Dur	1	14.83	25.58$	27.17	34.58$	27.31	26.80$	400.11	73.84
		2	14.78	24.94$	25.28	32.50$	20.61	23.28$		
		3	12.44	19.00$	25.31	19.17$	19.64	17.03$		
Rearing	Fr	1	2.31	3.22	8.08	5.89	4.97	5.11	13.66	4.17
		2	3.42	2.92	7.53	5.75	4.83	6.44		
		3	3.06	2.31	7.44	4.97	6.22	5.69		
Grooming	Fr	1	1.03	1.69	1.39	1.28	1.08	1.33	1.20	0.69
		2	1.56	1.42	1.25	1.28	1.33	1.22		
		3	1.67	1.36	1.47	1.69	1.50	0.89		
	Dur	1	19.14	13.92	5.08	6.75	7.78	8.58	268.76	171.16
		2	18.50	17.53	6.61	6.25	8.31	9.25		
		3	28.58	21.14	8.31	10.11	7.97	5.50		

Abbreviations: (Dur) duration (s); (Fr) frequency (events/12 min) (n=12); (MS1) error mean square for the assessment of the between-subject factor; (MS2) error mean square for the assessment of the within-subject factor; (§§) P<0.01 between-strains difference independent from exposure condition; (##) P<0.01 between-day difference within the same strain; ($) P<0.05 between-day difference within the same exposure condition; (£) P<0.05 vs. 129/Sv independently from day; (&) P<0.05 C vs. T within the same strain; (**) P<0.01 day3 vs. day2 and day1 only in T group. For main effects see Table I and Table II.

tion and frequency evidenced that toluquinone exposure markedly increased the time spent in DB, with DBA mice performing more DB than C57 and 129/Sv (frequency) or performing it for a longer time than C57 (duration) ($P<0.01$). A significant interaction between exposure condition and day was also found for frequency of DB ($F_{2,132}=3.11$, $P=0.0048$). Indeed more DB was observed in toluquinone-exposed mice on day 1 and day 2 when compared to day 3 ($P<0.05$) (see Fig. 1).

Stretch attend posture (SAP): exposure to toluquinone odor increased frequency and duration of SAP in all subjects ($F_{1,66}=94.69$, $P=0.0000$ and $F_{1,66}= 16.11$, $P=0.0002$, respectively for frequency and duration). A main effect of strain was detected ($F_{2,66}=9.96$, $P=0.0002$ and $F_{2,66}=17.47$, $P=0.0000$, respectively for frequency and duration). Toluquinone differentially affected the frequency of SAP in the three strains (strain × exposure condition: $F_{2,66}=3.59$, $P=0.0332$) inducing a greater increase in the number of SAP episodes, particularly in C57 and 129/Sv mice in comparison with DBA ($P<0.05$). It is interesting to notice that, differently from the other strains, 129/Sv mice performed this behavior also in the absence of the odor ($P<0.05$). Moreover a significant interaction between exposure condition and day was found both for frequency and duration of SAP ($F_{2,132}=13.90$, $P=0.0000$ and $F_{2,132}=6.42$, $P=0.0022$, respectively for frequency and duration), indicating that this behavior decreased, only in toluquinone exposed mice, throughout the three days considered, confirming the capability of the experimental subjects (independently from strain) to habituate to the SO (day 1 *versus* day 2 and 3: $P<0.01$, for toluquinone mice only).

Withdrawal (W): exposure to toluquinone odor increased specifically frequency and duration of W in all subjects ($F_{1,66}=273.57$, $P=0.0000$ and $F_{1,66}=151.25$, $P=0.0000$, respectively for frequency and duration). A main effect of strain was detected ($F_{2,66}=34.49$, $P=0.0000$ and $F_{2,66}=17.8$, $P=0.0000$, respectively for frequency and duration), with C57 mice performing more W than 129/Sv and DBA ($P<0.01$). A three-way significant interaction strain × exposure condition × day was found for frequency of W ($F_{4,132}=3.83$, $P=0.0056$). Toluquinone-exposed C57 and 129/Sv mice performed W more frequently on day 1 *versus* day 3 ($P<0.01$ after *post-hoc* comparisons). This difference was not found in DBA mice, indicating a lack of habituation in this strain (see Fig. 1).

Toluquinone exposure showed to clearly affect in different manners the non-avoidance responses performed by 129/Sv, C57 and DBA mice. In particular:

Locomotion (L): in general, exposure to the toluquinone-embedded SO increased locomotion (duration) in all subjects (exposure condition main effect:

Table II

ANOVA results for avoidance behaviors

		Strain	*P*	Exposure condition	*P*	Exposure condition × strain	*P*	Day	*P*	Strain × day	*P*	Exposure condition × day	*P*	Exposure condition × day × strain	*P*
		$F_{2,66}$		$F_{1,66}$		$F_{2,66}$		$F_{2,132}$		$F_{4,132}$		$F_{2,132}$		$F_{4,132}$	
Defensive	F	4.22	0.02	30.1	0.000	4.26	0.018	2.92	ns	0.71	ns	3.11	0.048	0.66	ns
burying	D	3.79	0.027	23.17	0.000	3.82	0.027	2.51	ns	0.97	ns	2.69	ns	0.87	ns
Stretch	F	9.96	0.0002	94.69	0.000	3.59	0.0332	30.2	0.000	3.94	0.0047	13.9	0.000	1.8	ns
attend	D	17.47	0.000	16.11	0.0002	0.62	ns	30.42	0.000	8.97	0.000	6.42	0.002	0.92	ns
posture															
With-	F	34.49	0.000	273.57	0.000	34.3	0.000	32.28	0.000	4.15	0.0034	31.36	0.000	0.006	0.000
drawal	D	17.8	0.000	151.25	0.000	17.95	0.000	25.19	0.000	1.68	ns	25.43	0.000	1.68	ns
Crouching	F	2.52	ns	89.09	0.000	2.57	ns	1.19	ns	0.48	ns	1.54	ns	0.48	ns
over object	D	1.28	ns	49.72	0.000	1.3	ns	0.49	ns	0.23	ns	0.76	ns	0.18	ns
Catching	F	2.36	ns	52.43	0.000	2.36	ns	1.56	ns	0.38	ns	1.56	ns	0.38	ns
	D	3.06	ns	32.99	0.000	3.06	ns	0.98	ns	0.91	ns	0.98	ns	0.91	ns
Eating	F	3.39	0.039	158.11	0.000	3.45	0.038	1.32	ns	0.94	ns	1.4	ns	0.91	ns
object	D	0.15	ns	136.32	0.000	0.15	ns	7.38	0.001	0.89	ns	7.35	0.001	89.000	ns

(D) duration (s); (F) frequency (events/12 min) (n=12)

Table III

ANOVA results for non-avoidance behaviors

		Strain	*P*	Exposure condition	*P*	Exposure condition × strain	*P*	Day	*P*	Strain × day	*P*	Exposure condition × day	*P*	Exposure condition × day × strain	*P*
		$F_{2,66}$		$F_{1,66}$		$F_{2,66}$		$F_{2,132}$		$F_{4,132}$		$F_{2,132}$		$F_{4,132}$	
Locomotion	F	15.76	0.000	2.77	ns	0.13	ns	13.21	0	0.26	ns	9.82	0.0001	1.86	ns
	D	4.55	0.0141	8.16	0.0057	1.5	ns	6.59	0.0019	3.44	0.0104	4.46	0.0133	0.46	ns
Inactivity	F	35.64	0.000	24.64	0.000	0.16	ns	4.01	0.0205	2.51	0.0451	0.48	ns	1.71	ns
	D	10.74	0.0001	0.15	ns	0.4	ns	8.12	0.0005	5.57	0.0004	0.08	ns	0.52	ns
Bar holding	F	24.08	0.000	0.03	ns	0.3	ns	2.64	0.0749	3.28	0.0134	3.21	0.0433	1.24	ns
	D	24.98	0.000	1.31	ns	0.52	ns	3.01	0.0527	3.32	0.0125	1.12	ns	0.59	ns
Food	F	2.57	ns	10.93	0.0015	0.37	ns	3.73	0.0266	1.23	ns	0.91	ns	0.81	ns
consumption	D	3.02	ns	7.43	0.0082	1.5	ns	13.89	0.000	1.21	ns	5.71	0.0042	1.6	ns
Wall	F	25.97	0.000	0.17	ns	0.55	ns	16.98	0.000	2.08	ns	4.03	0.02	1.54	ns
rearing	D	3.71	0.0298	1.87	ns	0.87	ns	12.31	0.000	1.08	ns	3.78	0.0253	0.99	ns
Rearing	F	19.58	0.000	1.51	ns	2.41	ns	0.25	ns	1.13	ns	1.32	ns	0.87	ns
	D	10.17	0.0001	0.77	ns	1.95	ns	1.57	ns	1.15	ns	0.4	ns	0.48	ns
Digging	F	12.59	0.000	1.198	ns	4.24	0.018	2.18	ns	1.56	ns	1.53	ns	1.32	ns
	D	8.57	0.000	2.63	ns	3.14	0.049	5.35	0.005	1.14	ns	0.42	ns	0.81	ns
Grooming	F	0.83	ns	0.01	ns	0.24	ns	0.46	ns	0.41	ns	1.7	ns	1.25	ns
	D	13.45	0.00	0.31	ns	0.57	ns	1.31	ns	1.03	ns	0.18	ns	0.19	ns

(D) duration (s); (F) frequency (events/12 min) (n=12)

$F_{1,66}$=8.16, P=0.0057). A significant interaction between exposure condition and day was observed for both frequency and duration ($F_{2,132}$=9.82, P=0.0001; $F_{2,132}$=4.46, P=0.0133, respectively for frequency and duration). L was high, following toluquinone exposure, on the first day, decreasing thereafter. *Post-hoc* comparisons carried out for duration indicated a significant difference between control and toluquinone mice on day 1 and day 2, with toluquinone mice performing more L in comparison with controls (P<0.01). Furthermore, toluquinone mice performed more L on day 1 in comparison with day 3 (P<0.01) and on day 2 compared to day 3 (P<0.05). No differences were found in control animals as a function of day of testing.

The C57 strain spent overall more time moving than the DBA strain (P<0.05), the 129/Sv strain being intermediate between the two. However, the 129/Sv strain showed a lower frequency of locomotion, which suggests a less "fragmented" pattern of behavior compared to the other two strain (P<0.01) (strain main effect: $F_{2,66}$=15.76, P=0.0000; $F_{2,66}$=4.55, P=0.0141 for frequency and duration, respectively). Significant differences between strains were also observed in the three days. Indeed, a strain × day interaction was found for L duration ($F_{4,132}$=3.44, P=0.01). Mice of the 129/Sv strain decreased their locomotor activity over the three days of testing (P<0.01 after *post-hoc* comparisons) while in C57 and DBA mice a failure to habituate was found (see Fig. 1).

Crouching over object: exposure to toluquinone odor reduced frequency and duration of this behavior in all subjects ($F_{1,66}$=89.09, P=0.0000 and $F_{1,66}$=49.72, P=0.0000, respectively for frequency and duration) (see Fig. 1).

Catching: toluquinone-exposed C57 and DBA mice, never caught the sponge (exposure condition main effect: $F_{1,66}$=52.43, P=0.0000 and $F_{1,66}$=32.99, P=0.0000, respectively for frequency and duration).

Eating object: a main effect of exposure condition was detected also for eating object ($F_{1,66}$=158.11, P=0.0000 and $F_{1,66}$=136.32, P=0.0000, respectively for frequency and duration). A main effect of strain was detected for eating object ($F_{2,66}$=3.39, P=0.0396, for frequency only). A significant interaction between strain and exposure condition was observed for frequency only ($F_{2,66}$=3.45, P=0.0376), C57 mice showing more eating than the 129/Sv strain (P<0.05) (see Table I).

DISCUSSION

In this study, presentation of toluquinone odor elicited a strong suppression of consummatory behaviors in all strains tested. Furthermore, approach-avoidance behaviors were also elicited that appeared to show some strain-dependent features.

Most of previous studies focusing on behavioral responses to an olfactory stimulus in mice used a predatory cue (Blanchard et al. 2003a). Conversely, in this experiment mice were confronted with a non-predator noxious olfactory stimulus. As shown in rats (Blanchard et al. 2001b, Dielenberg and McGregor 2001), we found that aversive responses to a non-predator stimulus overlap with selected, but not all, behavioral responses elicited by a predator threat. For example, in the presence of the toluquinone-embedded object all subjects showed an increase in locomotion. Conversely, in rats exposed to non-predatory odors, such as amyl acetate and butyric acid (McGregor et al. 2002) no change in locomotor activity occurs, while decreases in locomotion are seen in the case of cat odor-exposed rats.

In this study, behavioral responses to the toluquinone odor were characterized both by an approach phase of risk assessment, allowing investigation of the threat source, and/or by a repeated series of approach-avoid episodes with no contact with the object, possibly associated with gathering of information about the potential danger. These data agree with previous findings showing that, while manifest and tangible threats tend to elicit flight, hiding or freezing, depending upon the specific features of the environment, situations in which a threat is suggested by partial stimuli (odors) or learned or unlearned cues (e.g., the odor of a predator) maximally elicit risk assessment, as shown for mice in the mouse-defense test battery (Blanchard and Blanchard 1988, Blanchard et al. 2001a).

As far as strain differences are concerned, 129/Sv mice performed the stretch attend posture even in the absence of the toluquinone odor, suggesting that, for this behavior, the subjects responded to the novelty represented by the SO, rather than responding to the odor *per se*. These data, suggestive of high levels of anxiety in this strain, are in line with the performance of 129/Sv mice in the elevated plus maze and the light–dark exploration test (Holmes et al. 2002, Podhorna and Brown 2002, Voikar et al. 2001).

High levels of withdrawal characterized C57 mice upon exposure to the aversive odor, a response which was reduced upon re-exposure, while DBA performed considerable higher levels of defensive burying than the other two strains, in agreement with a previous study (Dell'Omo et al. 1994). Thus, compared to the DBA strain, which tended to bury the aversive stimulus, C57 subjects were characterized by a cautious investigation of the SO. The differences in behavior shown by DBA and C57 could be interpreted as reflecting different levels of anxiety experimented by the two strains in the test situation. Previous studies on anxiety-related behaviors in these mouse strains have shown contradictory results, possibly due to the adoption of different test paradigms and test conditions (Rogers et al. 1999, Trullas and Skolnick 1993). Our own results are suggestive of high levels of anxiety in the DBA strain, as manifested through marked expression of defensive burying, a behavior which has been extensively used as an index of fear/anxiety since it is specifically suppressed by anxiolytic drugs (De Boer and Koolhaas 2003).

About non-avoidance behaviors, the most striking effect of exposure to the toluquinone odor was the absence of crouching, catching and eating object, three behaviors consistently observed in control subjects, characterized by making contact, manipulating and chewing the sponge used as SO. Among controls, C57 mice ate the SO more frequently than the other strains (as previously shown; see Holmes et al. 2002). When control subjects contacted and chewed the sponge, further approaches to the SO were prevented because subjects spent most of the session in direct contact with the object. The behavioral approach in the presence of the noxious stimulus was radically different, since subjects went close to the toluquinone-smelling SO but withdrew immediately after, a behavior never involving contact and suggesting a conflict between curiosity and avoidance as if avoidance responses dominated over an innate tendency for novelty exploration.

While defensive burying was specifically increased following exposure to toluquinone, digging was decreased, but only in DBA mice, which indicates a "switch" between these two behaviors in this strain. Exposure to the toluquinone smell also increased the duration of wall rearing, but only on the second day of exposure. Other behaviors, such as rearing and bar holding were not affected by exposure to the aversive odor, but were differentially expressed by mice of the different strains. Indeed, compared to the other two strains, 129/Sv mice performed very low levels of bar holding

and wall rearing, while spending a considerable amount of time grooming or being inactive.

It is interesting to note that, in the presence of the noxious odor, all subjects increased the amount of food consumption. This could be interpreted as an indirect index of emotionality, since consummatory behaviors are known to reduce arousal (Levine et al. 1979). This increase possibly prevented any increase in grooming in the subjects exposed to the odor. Indeed an interesting strain-difference was found for grooming duration, 129/Sv mice performing this behavior to a greater extent than the other strains, a result which, to our knowledge, not described previously.

Even if a habituation was observed in all mice, the levels of risk assessment and avoidance items in toluquinone-exposed subjects were above those observed in controls, even on the third day of exposure. More importantly, non-avoidance responses such as crouching, catching and eating object did not habituate over the three days of exposure. It has been reported that, while responses to predator odors habituate, noxious olfactory stimuli representing only partial predator cues do not habituate as well (Dielenberg and McGregor 2001, McGregor et al. 2002).

Other tests to measure olfaction abilities have been developed and validated in transgenic mice, such as the olfactory-guided foraging task (Ferguson et al. 2000). This test, differently from ours, measures animal's ability to locate an attractive odor cue. However, strain differences have been reported in behavioral taste responses to sweeteners which might bias the results (Bachmanov et al. 2001). In addition, the procedure used in this test is a lengthy one, involving a few days of habituation. Compared to this, the toluquinone assay described in this paper appears particularly appealing. First of all, it is an easy and fast procedure. Secondly, meaningful results are obtained on the first exposure. In fact, compared to the olfactory-guided foraging task, the animals do not have to undergo long familiarization procedures. In addition, the extent of the response is the same in all the three mouse strains employed. Since previous work has indicated differences in olfactory sensitivity in inbred strains of mice, this test might be especially useful since it appears to be devoid of such bias (Lee et al. 2003).

CONCLUSIONS

Taken together, these results confirm that the toluquinone exposure assay, previously defined (Capone et al. 2002, Olivieri et al. 2001) and here validated in different strains of mice, is an ethological, easily testable, quick (it could be limited to day 1) and low-cost method to measure exploration and the emotional profile in mice. Since changes in behaviors only involve approach-avoidance items and are not influenced by confounding sensory or appetitive variables as in other exploration-anxiety tasks (e.g., vision in the elevated plus maze), this test could be a great complement to any exploratory-anxiety battery (Kalueff and Tuohimaa 2004).

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