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# Selection, evolution of behavior and animal models in behavioral neuroscience

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## Abstract

We investigated whether genetic differences in various forms of intraspecific aggression and anxiety in four different genetic lines of mice (i.e. wild, outbred Swiss-CD1, inbred DBA/2 and inbred C57/BL6N) may reflect modifications in behavioral strategy. Experiments 1 and 2 used ethologically based paradigms to analyze aggressive and anxiety responses both in social (i.e. aggression) and non-social (i.e. novel environment exploration) contexts. In Experiment 3, an anxiolytic drug (chlordiazepoxide (CDP)) was used to examine possible differences in proximal mechanisms underlying anxiety-related behaviors. The data show that intrasexual aggression, infanticide and maternal aggressions are related and covarying. Genetic lines with the highest levels of intermale attack (i.e. Wild and Swiss-CD1) also have highest levels of infanticide, interfemale attack and maternal aggression but, interestingly, the lowest levels of anxiety. In fact, exploratory behavior is lower and risk assessment behavior markedly higher in DBA/2 and C57/BL6N mice (i.e. the less aggressive strains) compared to Swiss and Wild genetic lines. Although reproductive status influences anxiety levels in female mice, our findings show that (contrary to previous studies) lactating mice are more anxious than virgin females in terms of risk assessment activities. These data demonstrate the importance of studying behavior in a more ecologically-relevant context which emphasizes the function of behavior in a specific situation. Moreover, differential strain sensitivity to the behavioral effects of CDP suggests that genetic lines of mice may differ in the underlying mechanisms mediating behavior. It is therefore possible that artificial selection of different genotypes has resulted in differences in proximate mechanisms modulating the levels of aggression and anxiety, thereby leading to modification of social behavior. Overall, the results presented here suggest that subtle genetic alterations in specific underlying neural mechanisms are likely to cause profound effects on behavioral responses and their adaptive significance. Implications for behavioral neuroscience research that seeks to understand both the proximal and ultimate mechanisms of behavior are discussed. © 1999 Published by Elsevier Science Ltd. All rights reserved.

**Keywords:** Genes; Strain; Selection; Chlordiazepoxide; Aggression; Anxiety; Infanticide; Exploration; Mice

Slight changes in structure almost cause vast changes in behavior

Stuart Kaufmann in *The origin of order, self organization and selection in evolution* (1993)

## 1. Introduction

The implications of the theory of natural selection for the evolution of behavior were scientifically addressed by Darwin [1] in his book 'The expression of emotions in man and animals'. In this century, a powerful contribution

to the study of behavior based on this Darwinian background has been the development of ethology. Ethology has borrowed concepts from comparative anatomy to understand the evolution of behavior. This is accomplished by the comparative analysis of behavior in related species that share homologous anatomo-physiological structures [2–5]. The vertebrate neuroendocrine system is a clear example of the homology principle in that its development and organization is substantially similar across the various classes [6]. Similarly, the vertebrate serotonergic system, formed by a small number of neurons located in the raphe or close to the wall of the fourth ventricle, has been preserved in so far as basic structure (at molecular and morphological levels) and associated functions (e.g. the modulation of socio-sexual behaviors [7]) are concerned. These examples reflect the 'conservative' nature (both in terms of proximal and ultimate mechanisms) of structures and related behaviors which

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are critical for survival and reproductive success (i.e. competitive and protective aggression, sexual and territorial behavior).

Based on this evolutionary evidence, animal models can be used in behavioral neuroscience to better understand the general principles whereby neurochemical mechanisms control behavior. However, in this field of research, the majority of data are essentially collected from two species of rodent: rats and mice. Furthermore, it is known that many different outbred and inbred genetic lines of rodents have been generated for use in such experimental investigations. Most of these lines show high behavioral variability which, when seeking functional explanations for specific neural mechanisms, makes data very difficult to interpret and extrapolate. Such differences suggest that the choice of a given genotype in a particular study will affect the behavioral expression of any experimental manipulation. As such, choice of genotype and the dependent behavioral variable/s is becoming one of the major problems in behavioral neuroscience. In this context, advances in the neurobehavioral genetics of aggression and anxiety have been particularly rapid in the last few years and has resulted in a significant contribution to our understanding of the interactions between the central nervous system, neurosecretions and the evolution of behavior by means of natural and artificial selection.

## 2. Genes, aggression and anxiety

It is widely acknowledged that individual differences in mouse aggressive behavior are related to genetic inheritance. Most of these genetic studies have involved the use of inbred and outbred strains, recombinant inbred lines or selected lines in order to map or identify genes for aggressive behavior, and to determine the mechanisms for the effect of genes on aggressive behavior [8,9].

The use of genetic lines selected for aggression was initiated by Lagerspetz [10]. Since then, other investigators have worked on genetic lines of mice selected for more or less aggressive behavior in both males [11–13] and females [14]. As all of these studies succeeded in selecting, within few generations, animals with different levels of aggressive behavior, a significant genetic regulation of aggression in mice was suggested. Similar conclusions were reached in studies that compared different strains of mice [8]. Despite these very promising results, only a few genes with effects on aggression have been systematically investigated. Thanks to the recent advances in the field of molecular biology and neurobehavioral sciences, it has been possible to identify some of these genes and to understand their effects on aggression. Recently, Cases et al. [15] have found increased aggression in transgenic mice in which the gene encoding for the monoamine oxidase A (MAOA) had been inactivated. Other investigations revealed that 5HT<sub>1B</sub> knockout mice exhibited increased aggression [16],

a result that has been interpreted in terms of low serotonergic activity due to decreased activation of the 5HT<sub>1B</sub> receptor. Using a different approach, it has been possible to identify genes that affect aggression in a specific region of the chromosome, e.g. by positional cloning [17] or positional candidates [18]. For example, it has been shown that the non-pseudoautosomal region (NPAR) of the Y chromosome influences aggression. By comparing reciprocal F1's, it has been shown that variants of one or more genes on the Y chromosome (NPAR) differ in their levels of aggression [19].

Artificial selection for genetic lines that differ in their levels of aggression has in the last few years provided important contributions to our understanding of the possible relationship between aggressive behavior and neurochemical parameters. Recently, it has been shown that male mice selected for short (SAL) or long (LAL) attack latency differ in many physiological and neurochemical parameters. In LAL mice, aromatase activity is higher in the pre-optic area than the SAL mice [20]; significantly, aromatase activity in the pre-optic area is inversely correlated with aggression. Furthermore, it has been shown that SAL male mice possess smaller hippocampal intra- and infra- pyramidal mossy fiber terminal fields compared to LAL mice [21], a finding that appears to be related to differences in spatial ability.

Strain comparisons and the selective breeding of mice offer an opportunity to study covariation of behavioral/physiological factors related to aggression. For example, it has been shown that SAL and LAL mice not only differ in their levels of aggression, but also in their behavioral response to challenging situations. SAL males flee or escape from a physically stronger resident male [22], perform better on a two-way active avoidance task [23], and do not suppress its activity when exposed to an inescapable shock [24]. In contrast, LAL males respond with immobility when confronted with a stronger male or inescapable shock. Other aggressive and non-aggressive genetic lines of mice, selected for isolation-induced intermale aggression, have been found to differ in olfactory communication, marking behavior, maternal and predatory aggression, locomotor activity, and learning [25]. It has been proposed that these differences in behavioral response in several experimental situations between genetic lines selected for more or less aggressive behavior reflect heritable, fundamentally different, yet equally valuable, alternative coping strategies [26]. These studies also emphasize that there may be a significant relationship between levels of aggression and the responses of male mice to potentially dangerous environments.

In this context, several authors have pointed to a possible relationship between aggression and anxiety in animals [27–29]. Consistent with this view, it has been reported that male mice rated as highly aggressive during dyadic encounters display higher levels of anxiety compared to non-aggressive counterparts [28]. Genetic studies with 10 different inbred mouse strains have shown large differences

in the levels of intermale aggression and in the level of anxiety measured in the light/dark preference paradigm [30]. More specifically, males of the more attacking strains have higher levels of anxiety, as compared to low aggressive strains, but do not differ in their level of activity. Behavioral research based on comparison of strains which are known to differ in the levels of aggression have reported large differences in the levels of plus-maze anxiety both for rats [31–33] and mice [34]. A comprehensive study on 16 inbred mouse strains has confirmed major strain differences in plus-maze anxiety measures, at least 70% of which could be attributed to genetic factors [35]. It has been shown that mice of different inbred and outbred genetic lines display pronounced differences in defense reactions in a recently-developed fear/defense test battery [36–38]. This strain-comparison approach has been proposed as a very useful tool in the study of the biological mechanisms involved in stress-related behaviors [39]. Moreover, the behavioral characterization of different strains on a number of stress-related measures may reveal correlations between behavioral measures of anxiety and aggression as well as the possible neurochemical substrates that these two phenomena may share.

A considerable number of studies have suggested that genetic differences in anxiety relate to neurochemical differences. For example, the GABA/benzodiazepine receptor complex plays a key role in anxiety and, in this context, genetic lines of mice that exhibit high levels of anxiety have lower specific benzodiazepine binding in brain compared to ‘non-emotional’ strains [40]. Other investigations have revealed that animals displaying ‘more anxious’ behavior in the elevated plus-maze have a significantly lower number of cortical benzodiazepine and GABA receptors [41]. Furthermore, research on rats selected for rapid (RHA) and non-rapid (RLA) acquisition of two-way active avoidance has demonstrated that these two lines differed significantly in the level of cortical GABA-stimulated  $\text{Cl}^-$  uptake [42].

Pharmacological studies with benzodiazepine anxiolytics provide further evidence of a link between anxiety and aggression. Benzodiazepines have been used for over 30 years in the treatment of human anxiety disorders and are known to reduce anxiety-related behavior in many animal models [43,44]. However, from the very outset, it has been known that these drugs also exhibit ‘anti-aggressive’ properties in humans and animals. In fact, benzodiazepines are renowned for their profound effects in reducing different forms of aggressive behavior, and particularly those that are defensively motivated [45–49]. This property seems to reflect a relatively greater sensitivity of defensive behavior to benzodiazepines rather than a non-specific inhibitory action on attack [50]. A wealth of literature supports the view that benzodiazepines inhibit defensive responses at doses much lower than those required to inhibit offensive behavior [49] and, for this reason, it is frequently assumed that their anti-aggressive properties are directly related to their anxiolytic efficacy.

In the present paper, we report a series of original experiments involving a comparative analysis of intraspecific aggression and anxiety in a wild stock population and three genetic lines of laboratory house mouse (*Mus musculus domesticus*). The first objective of the present study was to investigate the relationship between levels of aggression (in different social contexts) and emotional responsivity in different genotypes. The second objective was to examine whether genetic differences in aggression and anxiety reflect a modification in behavioral strategies. For these reasons, we assessed the behavioral responses of mice in both social and non-social contexts, and used the anxiolytic drug, chlor-diazepoxide (CDP), to explore possible differences in the proximal mechanisms underlying behavior.

## 2.1. General methods

### 2.1.1. Animals and husbandry

Four genetic lines of mice were used throughout these studies: (1) wild mice (fifth generation laboratory reared originally captured on the river Po Valley, Poggio, Reggio-Emilia); (2) Swiss albino outbred mice CD-1 born and reared in our laboratory (originally purchased from Charles River, Italy); (3) DBA/2ncr1br inbred mice born and reared in our laboratory (originally purchased from Charles River Italia); (4) C57/BL6N inbred mice born and reared in our laboratory (originally purchased from Charles River, Italy). After weaning (around 25–28 days of age), same strain mice were housed with same sex peers in-groups of 8–10 (transparent Plexiglas cages measuring  $55 \times 33 \times 20 \text{ cm}^3$ ). They were maintained under controlled temperature (22–24°C) and a 12L:12D reversed light cycle (lights on 0700 h). Water and food were available ad libitum.

### 2.1.2. Ethical considerations

The procedures used in the present work followed the rules of the European Community and of the ASAB Ethical Community [51]. Throughout this study, care was taken to minimize the stress imposed on animals, both adults (during aggression tests) and infants (during the infanticide tests). The number of adults and pups used in each experimental group were kept to a viable minimum. In particular males and females were confronted with a single pup, except during maternal aggression tests in which the presence of the whole litter was necessary to elicit maternal attack [52]. However, to prevent unnecessary animal suffering [53] the tests were immediately terminated in the following cases: (a) as soon as fighting escalated to severe biting, and (b) as soon as a pup received a bite. The injured pups that did not die as a result of attack were anaesthetised and killed.

## 3. Comparing different forms of aggression

Intraspecific aggression is an important factor in shaping social structure and population dynamics in the house mouse. Depending on specific socio-ecological conditions, house

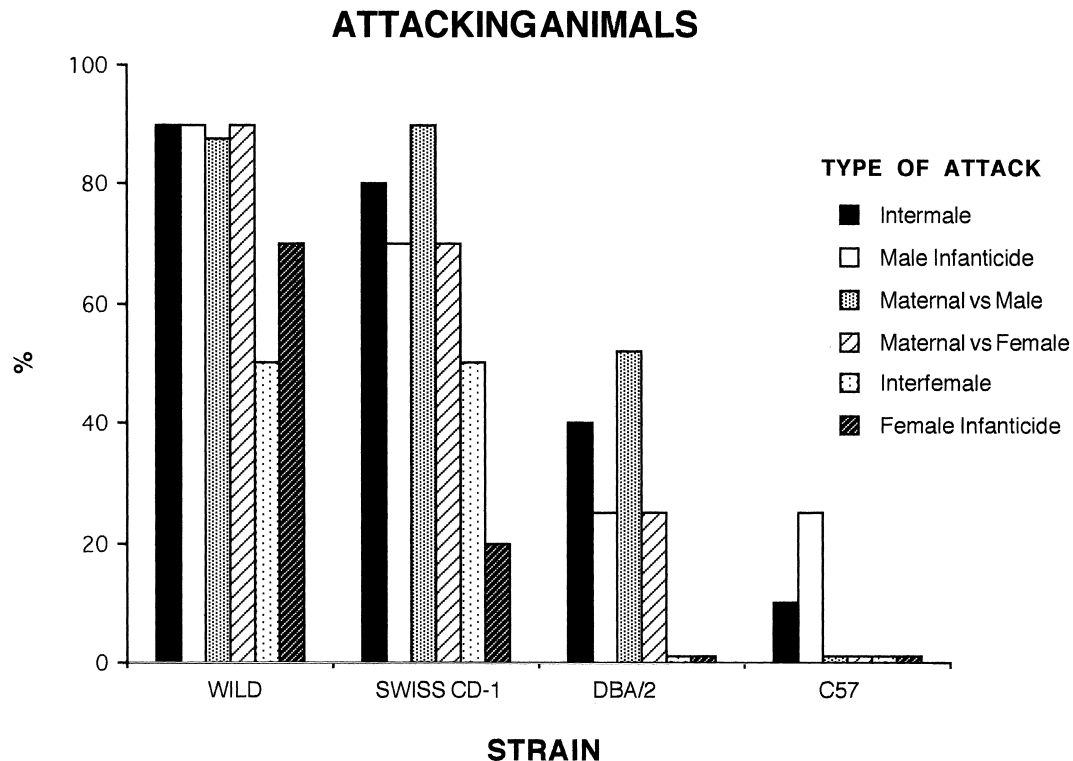


Fig. 1. Proportion of animals attacking conspecific intruders in different genetic lines of mice.

mice can assume a variety of social organizations, ranging from exclusive male territoriality to hierarchical groups [54]. Males and females of this species show differences in the display and timing of aggression toward conspecifics of differing age and reproductive state [50,55,56]. Therefore, in order to assess possible relationships between genetic background and different forms of aggression, we examined genetically different mice for aggressive behavior in different social contexts.

**Methods:** At 60 days of age, male and female mice of each genetic line were randomly assigned to infanticide and intrasexual aggression tests. Females were also tested for maternal aggression towards conspecific intruders of differing sex (see below the description for each experiment).

**Experimental apparatus:** Each apparatus consisted of two Plexiglas chambers (40 × 20 × 20 cm), linked by a 40 cm Plexiglas tube, and which could be closed by removable barriers at both ends. Food, water and nest materials (cotton wool) were provided and the cage bottom was covered by sawdust. The walls facing the observer were made of transparent Plexiglas to facilitate observation and behavioral data recording by means of digital electronic counters (see for details in Ref. [57]).

**Statistical analysis:** Because of the ethical considerations outlined above (i.e. tests were terminated before animals received severe injuries), all the duration data reported in each behavioral tests represent the time spent in a given behavior as a function of total encounter duration (i.e. percentage measures). The data were analyzed by non-parametric tests appropriate for the scale of measurement

and type of comparison: Chi-square and Fisher's exact probability tests (e.g. proportion of animals attacking or exhibiting infanticide).

### 3.1. Intermale aggression

#### 3.1.1. Method

Males ( $n = 20$  for each genetic line) were individually housed in the apparatus for 24 h. This procedure was employed because this time has been proven to be sufficient to stimulate defense of an area from same-sex conspecifics in Swiss and Wild mice [57,58]. Five minutes prior to testing, each animal was confined to the chamber in which the nest was located by closing the end of the tunnels with Plexiglas barriers. An unfamiliar and unrelated intruder male, of similar size, same age and same genetic line as the resident male was introduced into the opposite chamber. After 5 min, the barriers were removed. Behavioral recordings began as soon as the animals contacted each other and the proportion of fighting pairs were recorded.

#### 3.1.2. Results

As shown in Fig. 1, levels of intermale aggression induced by 24 h individual housing varied considerably among the genetic lines. A significant strain effect ( $X^2 = 32.27$ ,  $p < 0.0001$ ) was found for proportion of attacking males. The majority of Swiss and Wild resident males attacked intruders, whereas only 40% of DBA/2 ( $X^2 = 6.66$ ,  $p < 0.01$  vs. Swiss;  $X^2 = 10.36$ ,  $p < 0.01$  vs. Wild)

and 10% of C57/BL6N ( $X^2 = 19.79$ ,  $p < 0.0001$  vs. Swiss;  $X^2 = 24.63$ ,  $p < 0.0001$  vs. Wild) showed overt attack. However, DBA/2 males showed a higher incidence of fighting than did C57/BL6N males ( $X^2 = 4.8$ ,  $p < 0.05$ ), confirming that the level of competitive aggression measured as defense of an area after individual housing is strongly affected by genotype. Thus, Swiss and Wild genetic lines showed similar profiles of intermale attack and were seen to be more aggressive than the DBA/2 and C57/BL6N genetic lines.

### 3.2. Interfemale aggression

#### 3.2.1. Method

Virgin females ( $n = 15$  for each genetic line) were exposed to the odor of an unfamiliar male by placing them individually for 24 h into the experimental apparatus which had been inhabited for the previous 24 h by a male of the same genetic line. This procedure is known to stimulate intrasexual female attack both in Swiss and wild stocks of mice [58,59]. Five minutes prior to the intruder tests, females were confined to the chamber where they built the nest, by closing the entrance of the tunnel with Plexiglas barriers. A female intruder of similar size, same age and genetic line was then introduced into the chamber opposite to the nest. Five minutes later, the barriers were removed and animals allowed to interact for 10 min, following the first contact between the two animals. The proportion of fighting pairs was recorded.

#### 3.2.2. Results

Levels of interfemale aggression varied considerably among genetic lines (see Fig. 1). A significant strain effect was found for the proportion of attacking animals ( $X^2 = 26.44$ ,  $p < 0.0001$ ). 50% of Swiss and Wild resident females attacked the intruder, whereas none of DBA/2 or C57/BL6N females showed biting attack toward intruders ( $X^2 = 13.33$ ,  $p < 0.0005$  vs. Swiss;  $X^2 = 13.01$ ,  $p < 0.0005$  vs. Wild). As for the intermale aggression study, females of the Swiss and Wild genetic lines were more aggressive towards female conspecifics than were females of either the DBA/2 or C57/BL6N genetic lines.

### 3.3. Male and female behavior towards alien pups

#### 3.3.1. Method

Sexually naive males ( $n = 12$ ) and females ( $n = 12$ ) of each genetic line were individually housed in the experimental apparatus for 24 h. Five minutes prior to testing each animal was confined in the chamber where its nest was located by closing the entrances to the tunnel with Plexiglas barriers. A single pup (2–3 days of age) of the Swiss genetic line was placed into the opposite chamber. After 5 min, the barriers were removed and the behavior of the test animals observed for 30 min. Animals that bit the pup once were scored as infanticidal and the test was immediately interrupted.

#### 3.3.2. Results

*Males:* Statistical analysis revealed a significant strain effect for the proportion of animals attacking an unfamiliar pup ( $X^2 = 14.94$ ,  $p < 0.002$ , see Fig. 1). Swiss and Wild mice showed higher incidence of infanticide relative to DBA/2 ( $X^2 = 6.66$ ,  $p < 0.01$ ;  $X^2 = 10.98$ ,  $p < 0.001$  respectively) and to C57/BL6N ( $X^2 = 7.61$ ,  $p < 0.01$  vs. Wild and  $X^2 = 3.95$ ,  $p < 0.05$ , vs. Swiss) lines.

*Females:* Statistical analysis revealed a significant strain effect for the percentage of animals attacking an unfamiliar pup ( $X^2 = 35.52$ ,  $p < 0.0001$ , see Fig. 1). Wild females showed the highest incidence of infanticide relative to the other strains ( $X^2 = 10.41$ ,  $p < 0.005$  vs. Swiss;  $X^2 = 19.25$ ,  $p < 0.0001$  vs. DBA/2 and C57). While Wild and Swiss males showed similar high levels of infanticide, Wild females differed significantly from all other strains, including Swiss.

### 3.4. Maternal aggression

#### 3.4.1. Experimental procedures

At the age of 65–70 days, females of each genetic line were paired with males of the same age and stock (Swiss:  $n = 40$ ; Wild, DBA/2, C57/BL6N:  $n = 30$  for each strain). Approximately one week before parturition, the females were separated from their mates. After the delivery of the pups (3–5 days postpartum), and 24 h prior to testing, females with their litters were individually introduced into the experimental apparatus. Lactating females were randomly allocated to two experimental groups confronting either a sexually naive male or virgin female intruder of the same genetic line. Intruders, approximately 80 days old, had previously been housed in same-sex groups (6–8 per cage) since weaning (Plexiglas cages:  $45 \times 28 \times 13$  cm<sup>3</sup>). Male and female intruders were used once only and marked with dye on the tail before testing for individual recognition. Testing commenced by closing the ends of the tunnel thereby confining the resident dam to the nest chamber. An intruder was then placed into the opposite chamber and, 5 min later, tunnel barriers were removed, allowing free interactions between animals. Intruder tests lasted for 10 min beginning from the first contact between the mother and the intruder. The proportion of intruders attacked was recorded. In order to assess whether male and female intruders would differ in their behavior, the following elements of intruders' behavior were also recorded: the proportion of intruders attacking the lactating female and the proportion of intruders attacking pups (i.e. infanticide).

#### 3.4.2. Results

For clarity, results are presented separately for maternal aggression towards intruders of differing sex. Fig. 1 shows data on the proportion of lactating females attacking male and female intruders.

*Male intruder:* Statistical analysis revealed a significant strain effect for the percentage of dams attacking male

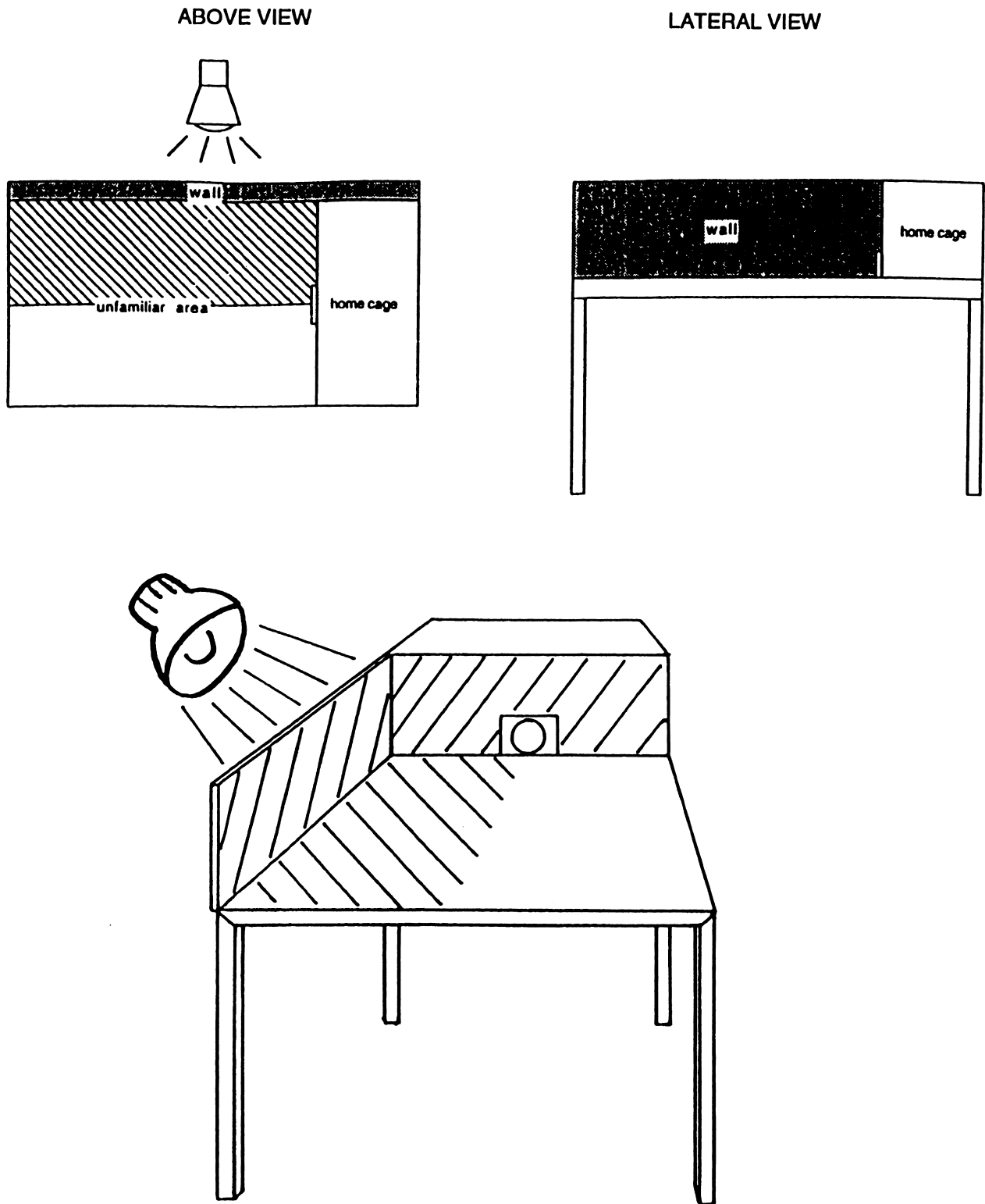


Fig. 2. Modified free-exploratory apparatus. Top left figure shows apparatus as seen from above. Top right figure shows apparatus as seen from one side.

intruder ( $X^2 = 19.44, p < 0.0005$ ). Swiss and Wild mice showed a higher incidence of maternal attack towards male intruders when compared to DBA/2 ( $X^2 = 6.03, p < 0.05$ ;  $X^2 = 3.96, p < 0.05$ , respectively) and C57/BL6N

( $X^2 = 14.72, p < 0.0001$  and  $X^2 = 10.99, p < 0.001$ , respectively) lines.

*Female intruder:*  $X^2$  analysis revealed a significant strain effect for the percentage of dams attacking female intruders

( $X^2 = 28.99$ ,  $p < 0.0001$ ). Swiss and Wild dams showed higher levels of maternal attack towards female intruders than did dams of the DBA/2 ( $X^2 = 6.44$ ,  $p < 0.05$  and  $X^2 = 13.88$ ,  $p < 0.0005$ , respectively) and C57/BL6N ( $X^2 = 14.03$ ,  $p < 0.0005$  and  $X^2 = 22.53$ ,  $p < 0.0001$ , respectively) strains. Swiss and Wild mice showed the highest frequency and intensity of attack towards female intruders. However, while there was no difference in the frequency of maternal attack among Swiss and Wild mice when confronting female intruders, attack intensity reached the highest level in Wild females. Overall, these findings parallel to those recorded for intermale aggression in showing that Swiss and Wild mice were the most aggressive genetic lines when assessed for maternal attack.

It is interesting to note that only in Swiss and Wild mice some male intruders counterattacked and subdued the female and attempted to attack the pups.

#### 4. Exploration and anxiety

In the past few decades, a number of animal tests have been developed in order to study drugs with anti-anxiety properties [43,44]. Those tests based on spontaneous behavior have been suggested to have a high degree of ecological validity in that they rely upon unconditional reactions to potentially threatening novel situations [60]. These animal tests of anxiety are based on the ‘two-factor theory’ of the dynamic relationship between exploratory behavior and anxiety typically displayed in unfamiliar environments [61,62]. Furthermore, they have been shown to have high sensitivity to anti-anxiety agents [43,60]. However, in most of these tests, animals are ‘forced’ into a novel environment and several authors have questioned whether the so-called fear reactions might be elicited by such a ‘forced’ situations rather than by novelty per se [63]. Recently, a paradigm has been developed which may offer a more ecologically relevant situation in which to study the defensive reactions elicited by unfamiliar environments: the free-exploratory paradigm [63]. In this test, animals have the opportunity to choose between a novel and a familiar compartment.

In the present experiment, we used a modified free-exploratory paradigm that allowed to measure specific defensive reactions of mice (such as risk assessment) which have been shown to be a reliable index of anxiety in other fear/defence paradigms [60,64,65]. The risk assessment activities include scanning of the potential dangerous open area and approaches that can be characterized by postures and behaviors oriented to the open area. These behaviors have also been shown to be sensitive to anxiolytic compounds [65]. In accord with the comparative approach adopted throughout this series, we used this test to examine exploration and anxiety levels in different genetic lines previously characterized for their levels of intraspecific aggression in order to study possible relationships between aggression and anxiety.

#### 4.1. Methods

Animals of each genetic line were randomly assigned to three experimental groups ( $n = 12$ – $18$  per group): (1) group-housed sexually naive males; (2) group-housed virgin females; and (3) lactating females. Lactating females were obtained by mating sexually naive females aged 70–80 days with same strain sexually-naive males of the same age in Plexiglas cages ( $22 \times 17 \times 13$  cm<sup>3</sup>) placed in a breeding room. Approximately one week before parturition, the females were separated from their mates. Two days after the delivery of the pups, and 24 h prior to testing, females with their litters were individually introduced into the experimental apparatus.

#### 4.2. Experimental apparatus

The apparatus (see Fig. 2) consisted of two sections. The home cage ( $40 \times 20 \times 20$  cm<sup>3</sup>) was a burrow of black Plexiglas, in which animals were singly housed for 24 h before testing. The floor of this cage was covered by sawdust and food and water were freely available. The frontal wall of the home cage was made of transparent Plexiglas to allow observation and videotape recording. The unfamiliar area (an open-field) comprised a rectangular white Plexiglas platform, that was raised 60 cm above the floor and which was bordered along one length by a 20 cm high white Plexiglas wall. A bright light (60 W) was positioned behind and above the wall so as to cast a shadow along the length of the open-field. The home-cage burrow could be connected to the open-field by means of a small opening (5 cm diameter) which was closed with a removable barrier until testing as shown in the Fig. 2.

#### 4.3. Procedure

Before 24 h of testing, subjects (lactating females were introduced together with the litter) were singly housed in the home cage burrow. Lactating females were tested for anxiety on day 2 of lactation (day 0 was considered as the day of pup delivery). Tests were conducted between 10:00 and 16:00 h in a darkened room illuminated with the white light which was positioned behind and above the wall of the open-field. During observation, the experimenter sat 2.5 m distant from the apparatus. 5 min before testing, the home cage was placed at one end of the rectangular open-field. Once the barrier was removed, behaviors (risk assessment behaviors) were recorded from the first approach to the entrance, and a cut-off of 12 min was used for animals that did not emerge onto the surface of the open-field. These animals were included in the statistical analysis with a latency to enter the open field of 12 min. The test was considered to have started after the first entry into the unfamiliar open-field (with all four paws) and lasted 5 min. To reduce any lingering olfactory cues, the apparatus was wiped with a clean damp cloth between successive tests. Animals were used only once. Sessions were recorded

## EXPLORATION IN THE NOVEL AREA

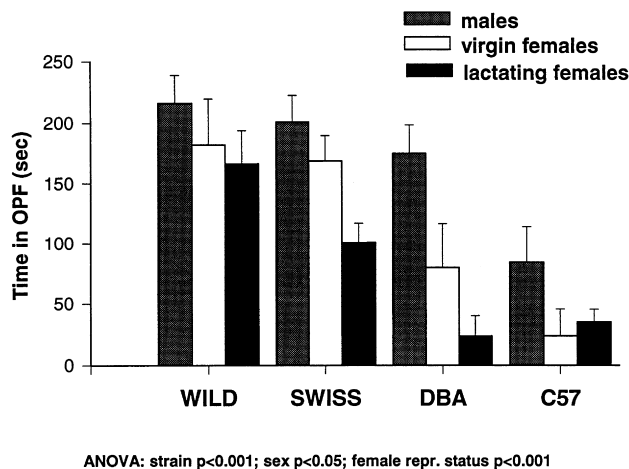


Fig. 3. Exploratory behavior in the open area of the modified free-exploratory paradigm in different genetic lines of mice. For each genetic line data are shown as means  $\pm$  SEM. The behavior referred to sexually naive males, virgin females and lactating females of each genetic line. For more details on statistical analysis see text.

on videotape by a VHS videocamera and VCR situated 3 m distant from and 0.5 m above the apparatus.

**Behavioral analysis:** Behaviors were scored off videotape by a trained observer who remained blind to treatment conditions until data analysis was complete. Data were logged by a series of electronic counters and timers. Behavioral measures were scored as durations (s): time spent in the open-field (TO) and risk assessment behavior (RA) defined as forward elongation of the head and shoulders with scanning the unfamiliar open-field (occurring from the home cage). This posture was recorded as total time spent before the first exit into the open-field.

**Statistical analysis:** All duration (percent time) data were initially arcsin-transformed to give normal distributions. Behaviors of sexually naive males and virgin females were analyzed by two-way analysis of variance ( $2 \times 4$  ANOVA; independent factors of sex and strain). Behaviors of virgin and lactating females were compared by two-way analysis of variance ( $2 \times 4$  ANOVA; independent factors of reproductive status and strain;  $2 \times 4$  ANOVA). Unplanned comparisons were used for binary contrasts (Newman–Keuls analyses).

### 4.4. Results

#### 4.4.1. Sexually-naive males vs. virgin females (see Fig. 3).

**Time spent in the open-field:** ANOVA revealed a significant main effect for strain ( $F = 12.41, p < 0.0001$ ) and sex ( $F = 4.66, p < 0.04$ ). DBA/2 was the only strain to show sex differences in TO, in that males showed more TO than females ( $p < 0.02$ ). Wild, Swiss and DBA/2 males showed comparable levels of TO. However, these strains showed higher levels of TO when compared to C57/BL6N

( $p < 0.004, p < 0.003$  and  $p < 0.03$ , respectively). While Wild and Swiss females had similar levels of TO, they showed much higher levels of TO when compared to DBA/2 ( $p < 0.002$  and  $p < 0.02$ , respectively) and C57/BL6N ( $p < 0.0002$ ) females.

**Risk assessment:** ANOVA revealed a significant main effect of strain ( $F = 10.76, p < 0.0001$ ) and sex ( $F = 12.82, p < 0.0006$ ). Post-hoc comparisons showed sex differences in Swiss and DBA/2 mice, with males showing higher levels of RA compared to females ( $p < 0.03$  and  $p < 0.01$ , respectively). In males, DBA/2 and Swiss mice showed higher levels of RA compared to C57/BL6N ( $p < 0.05$  and  $p < 0.06$ , respectively) and Wild ( $p < 0.05$  and  $p < 0.0003$ , respectively). In females, DBA/2 mice had higher levels of RA than Wild and C57/BL6N ( $p < 0.05$ ).

#### 4.4.2. Virgin females vs. lactating females (see Fig. 3)

**Time spent in the open-field:** ANOVA revealed a significant main effect of reproductive status ( $F = 17.78, p < 0.0001$ ) and strain ( $F = 14.61, p < 0.0001$ ). Swiss and DBA/2 lactating females spent less time in the unfamiliar open-field compared to their virgin counterparts (Swiss:  $p < 0.02$ ; DBA/2:  $p < 0.006$ ). In Wild and Swiss mice, virgin females spent more TO than C57/BL6N ( $p < 0.0005$  and  $p < 0.05$  respectively); similarly, virgin Wild mice spent more TO than did virgin DBA/2 mice ( $p < 0.007$ ). In lactating females, Wild and Swiss mice showed higher levels of TO compared to C57/BL6N ( $p < 0.0001$  and  $p < 0.01$ , respectively) and DBA/2 ( $p < 0.0001$  and  $p < 0.02$ , respectively). Lactating Wild females also spent more TO than Swiss ( $p < 0.0005$ ).

**Risk assessment behavior:** The analysis of variance revealed a significant strain effect ( $F = 4.15, p < 0.009$ ) for RA. In virgin females, C57/BL6N mice showed higher levels of RA than the other strains (Wild:  $p < 0.008$ ; Swiss:  $p < 0.02$ ; DBA/2:  $p < 0.06$ ). In lactating females, Wild mice showed lower levels of RA than Swiss ( $p < 0.003$ ) and C57 ( $p < 0.04$ ). In Swiss mice, lactating females showed higher levels of RA than virgin females ( $p < 0.003$ ).

## 5. Ethopharmacology of anxiety in different genetic lines of mice

In this final phase, the modified free exploratory paradigm was used to assess the sensitivity of the different genetic lines to the behavioral effects of the benzodiazepine anxiolytic, chlordiazepoxide. In view of the sample sizes required for pharmacological investigations, wild mice were not used in this study.

### 5.1. Methods

**Apparatus and procedures:** The apparatus and procedures are described in the previous experiment. However, in

## TIME SPENT IN THE NOVEL AREA

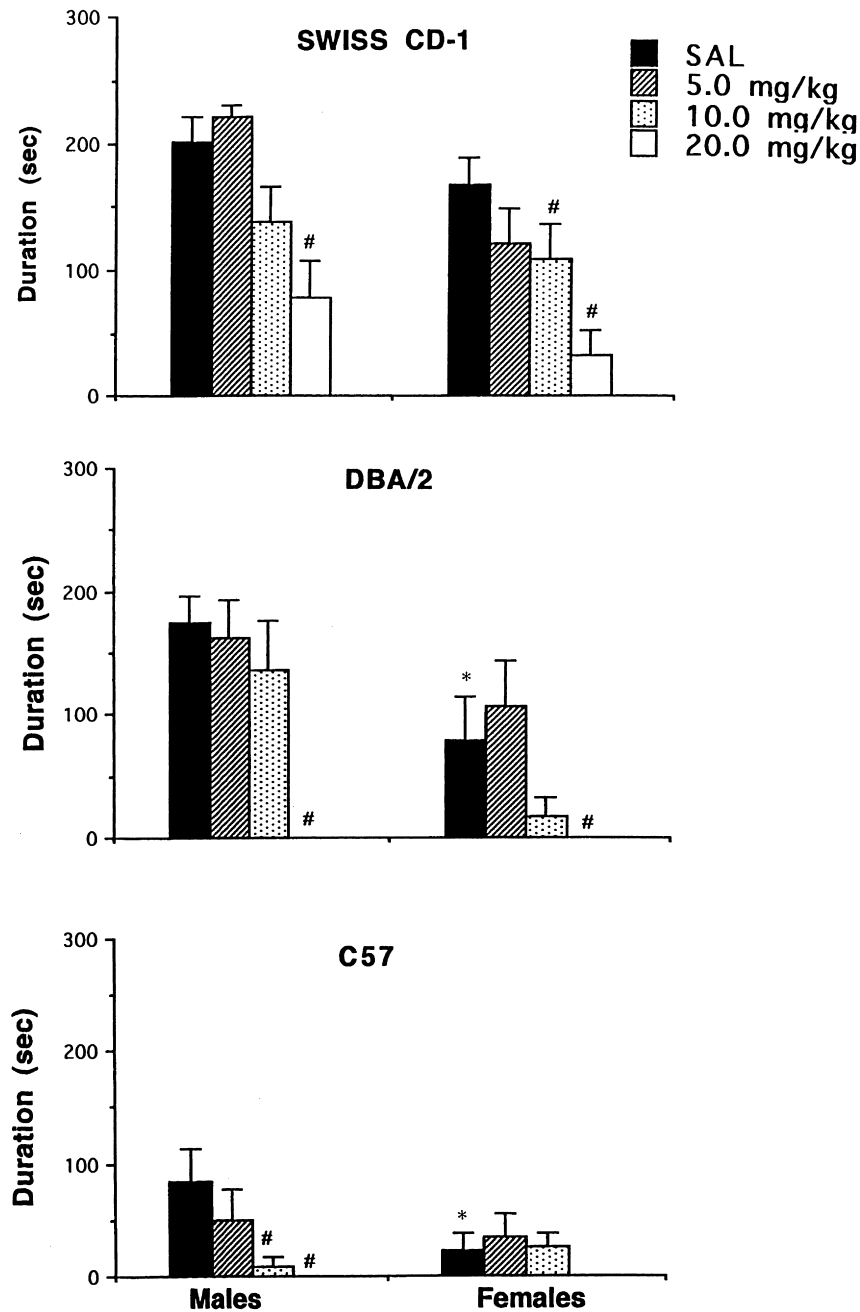


Fig. 4. Effects of chlordiazepoxide on exploratory behavior in the modified free-exploratory paradigm in three genetic lines of mice. Data are shown as means  $\pm$  SEM. For more details on statistical analysis see text.

addition to the measures of time open and risk assessment, this study included a measure of immobility to evaluate possible sedative effects of the drug. On test days, animals were randomly assigned to one of the following treatment conditions: saline control ( $n = 10\text{--}12/\text{strain}$ ), 5.0 mg/kg ( $n = 9\text{--}12/\text{strain}$ ), 10.0 mg/kg ( $n = 9\text{--}12/\text{strain}$ ) or 20.0 mg/kg ( $n = 8\text{--}12/\text{strain}$ ) CDP. Drugs were administered in a volume of 10 ml/kg i.p. 30 min after injection, and

5 min before testing, the home cage was placed at one end of the rectangular open-field.

*Statistical analysis:* All duration (percent time) data were initially arcsin-transformed to give normal distributions. These data were analysed by three-way analysis of variance (independent factors of strain, sex and drug treatment;  $4 \times 2 \times 4$  ANOVA). Unplanned comparisons were used for binary contrasts.

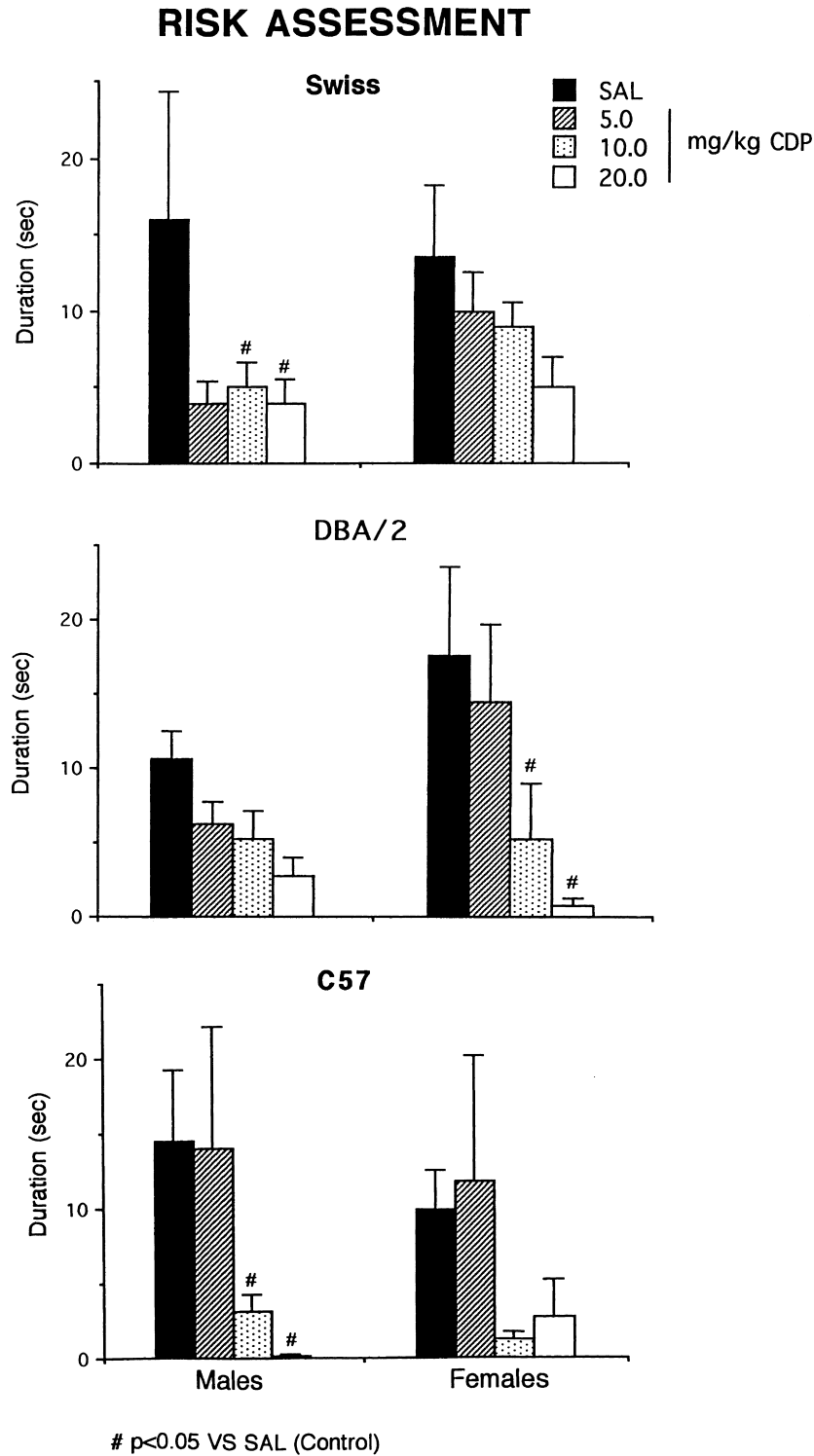


Fig. 5. Effects of chlordiazepoxide on risk assessment behavior in the modified free-exploratory paradigm in three genetic lines of mice. Data are shown as means  $\pm$  SEM. For more details on statistical analysis see text.

5.2. Results

The results are summarized in Figs. 4 and 5.  
*Time spent in the open-field:* ANOVA revealed a significant main effect of sex ( $F = 17.05, p < 0.0001$ ), strain

( $F = 39.48, p < 0.0001$ ) and drug treatment ( $F = 23.34, p < 0.0001$ ) on the time spent exploring the open-field. In the control condition, Swiss and DBA/2 males spent more time in the open field compared to C57/BL6N males ( $p < 0.005$  and  $p < 0.05$ , respectively). In females, only

Swiss showed higher levels of TO than DBA/2 ( $p < 0.0001$ ) and C57/BL6N ( $p < 0.005$ ). DBA/2 and C57 lines also showed sex differences in TO, with males spending more time exploring the open field than females ( $p < 0.005$  and  $p < 0.06$ , respectively). In males, 10 mg/kg CDP decreased TO only in C57/BL6N ( $p < 0.02$ ) whereas, at 20.0 mg/kg, decreases in TO were observed in all strains: Swiss ( $p < 0.0005$ ), DBA/2 ( $p < 0.0001$ ) and C57 ( $p < 0.01$ ). CDP did not affect TO in female C57/BL6N mice at any dose. However, decreases in TO were observed in Swiss females at 10.0 mg/kg ( $p < 0.05$ ) and 20.0 mg/kg ( $p < 0.0001$ ), and in DBA/2 females at 20.0 mg/kg ( $p < 0.05$ ).

**Risk assessment:** ANOVA revealed a significant main effect of drug treatment ( $F = 8.53$ ,  $p < 0.0001$ ). Swiss and C57/BL6N males were more sensitive to drug treatment than females: 10.0 and 20.0 mg/kg CDP reduced RA in male Swiss and C57 mice (10.0 mg/kg:  $p < 0.06$  for both strains; 20.0 mg/kg:  $p < 0.05$  for both strains). In C57 and Swiss females, CDP did not significantly affect RA at any dose tested. However, in DBA/2 females, CDP decreased RA at both 10.0 mg/kg ( $p < 0.05$ ) and 20.0 mg/kg ( $p < 0.01$ ).

**Immobility:** ANOVA revealed a significant interaction between the genetic line and sex ( $F = 3.93$ ,  $p < 0.05$ ). In saline controls, only DBA/2 showed sex differences in immobility, with females spending more time immobile than males ( $p < 0.05$ ). Swiss females showed lower levels of Immobility compared to C57/BL6N and DBA/2 females ( $p < 0.005$  and  $0.05$ , respectively). 10.0 mg/kg CDP increased Immobility in Swiss and DBA/2 females ( $p < 0.05$  for both strains), and in C57 males ( $p < 0.001$ ). The highest dose used (20.0 mg/kg) increased Immobility in males and females for all 3 strains: Swiss ( $M$ :  $p < 0.0005$ ;  $F$ :  $p < 0.0001$ ), C57 ( $M$ :  $p < 0.0001$ ;  $F$ :  $p < 0.01$ ) and DBA/2 ( $M$  and  $F$ :  $p < 0.0001$ ).

## 6. Conclusions and perspectives

The present findings show that intrasexual aggression, infanticide and maternal aggression are usually related and covarying. More specifically, compared to the other strains tested, genetic lines that showed the higher levels of intermale attack also show higher levels of interfemale attack, infanticide and maternal aggression. In this context, it is worth noting that, in terms of social behavior, the Swiss albino CD-1 strain is rather similar to the wild line, suggesting that the process of domestication has left the behavior of certain laboratory mice relatively unchanged. These findings also suggest that phenotypically-different forms of aggression such as intrasexual attack and infanticide, which share a similar function (i.e. competition for mates and resources, [66]), also share genetic inheritance in both sexes. In fact, in those genetic lines where the level of intermale attack is high, interfemale attack and infanticide are also high. These data are consistent with recent

ethopharmacological studies which show that the motivational and neural substrates underlying different forms of aggression (such as intrasexual attack and infanticide) in wild and Swiss CD-1 mice, are similarly regulated at the serotonergic level [67]. This finding suggests that certain genetic lines, which are not strongly modified from the wild behavioral phenotype, can be used to investigate both proximal and ultimate mechanisms of behavior. For example, the aggressive behaviors of Swiss males are quantitatively and qualitatively similar to those of wild male mice (see also Ref. [67]). In contrast, compared to wild females, Swiss females showed profound behavioral differences in competitive aggression (i.e. lower levels of infanticide). This difference implies that, whereas Swiss males can be useful models not only to investigate proximal mechanisms but also adaptive significance of aggressive behavior, Swiss CD-1 females may be not fully suitable for understanding competitive strategies in mice. Our data further suggest that intrasexual attack and infanticide in female Swiss mice may have been selected against by laboratory breeding practices [68].

The present findings also show that, compared to the other strains tested, those genetic lines with higher levels of aggression have lower levels of anxiety as measured in the modified free-exploratory paradigm. In fact, the exploratory behavior of Swiss and Wild strains is comparatively higher than C57/BL6N and DBA/2 strains. In addition, risk assessment behavior is markedly higher in C57/BL6N and DBA/2 strains compared to both Wild and Swiss. This finding apparently contrasts with other studies showing that more aggressive strains have higher levels of anxiety as assessed in the light-dark exploration test [30]. However, this discrepancy may be related to the use of different exploratory paradigms which may reflect/measure different facets of anxiety [69]. In fact, Griebel et al. [63] has suggested that the free-exploratory test may be devoid of those constraining and stressful components of the experimental situation typical of the classical animal models of anxiety, such as the elevated plus-maze and the light/dark procedures. Despite this controversy, what appears clear from these data is that animals with a particular genotype adopt a specific coping strategy with respect to environmental challenge. In this context. It is already known that wild mice bidirectionally selected for attack latency, show major differences in a wide range of behavioral, physiological, and neuroendocrinological traits, suggesting important genetic differences in coping style [24].

The second experiment showed that female reproductive state influences anxiety levels as measured in the free-exploratory paradigm. Contrary to previous research [27,70–72], lactating mice were observed to be more anxious when compared to virgin females. However, in those previous studies, anxiety was measured by keeping the mother away from the litter or in a unfamiliar context [27,70–72]. Such procedures could stimulate specific behavioral responses in dams, such as seeking for the pups or

searching for a safer nesting area, both of which could be wrongly interpreted as reduced anxiety. When given the opportunity to explore an unfamiliar area, our experimental paradigm showed that dams did not leave the pups and nest area for long periods and displayed activities consistent with detection of possible threats to the mother and the litter (i.e. risk assessment). Thus, the free-exploratory paradigm clearly outlined the importance of studying dams' exploratory behavior in a more ecologically-relevant context which may emphasize the function of behavior in a specific situation. Overall, these results from the modified free-exploratory paradigm indicate that genotype can strongly affect the response of mice to environmental challenges. In contrast, the influence of gender on anxiety levels measured in the modified free-exploratory paradigm is more equivocal, in that only comparatively minor sex differences were detected (mainly in the DBA/2 strain), as also reported for other experimental tests [60].

The free exploratory paradigm has also been proposed as a useful animal model for investigating anti-anxiety drugs [63], particularly agents that act at the GABA-benzodiazepine receptor. Many behaviors related to emotionality and aggression have been linked to the GABAergic functioning. In this context, strain-specific behaviors characteristic of anxiety may provide useful tools for determining brain mechanisms mediating anxiety and thus predicting drug response to anxiolytics [73]. Consistent with this view, present data clearly show that genotype and gender can strongly influence the behavioral effects of CDP in the free exploratory paradigm, and that such differences cannot solely be attributed to different behavioral baselines. In the interest of brevity, and given the overall lack of significant effects of the lowest dose tested, comments will be limited to the pattern of change seen at the two higher doses (i.e. 10 and 20 mg/kg). Firstly, the induction of significant immobility in males and females of all three strains indicates that effects observed at 20 mg/kg should be considered behaviorally non-selective. Secondly, it is evident that female Swiss and DBA/2 mice are more sensitive to the behavioral suppressant effects of CDP than their male counterparts. Thus, in both strains, immobility was significantly increased in females at 10 and 20 mg/kg whereas, in males, this effect was seen only at 20 mg/kg. Interestingly, however, the reverse was true for C57 mice where males were more sensitive than females to this action of the drug. Finally, 10 mg/kg CDP significantly reduced risk assessment in male Swiss mice without influencing other behavioral measures, whereas no such behaviorally-selective effect was observed in females of this line or, indeed, males or females of the other two strains. Other reports using different paradigms have also shown that genotype may profoundly affect the action of benzodiazepines on aggressive behavior [74] and anxiety [73]. This differential strain sensitivity to the behavioral effects of CDP suggests the distinct possibility that mouse strains may differ in the underlying mechanisms mediating

behavior (e.g. receptor binding, receptor number and/or receptor distribution in the brain). Further evidence in favor of this hypothesis comes from investigations showing large strain differences in the GABA/benzodiazepine receptor complex [40].

Our findings have shown that artificial selection of different genetic lines of mice has resulted in differences in proximate mechanisms of aggression and anxiety, in terms of reactivity to social and non-social stimuli. From an evolutionary point of view, it is possible that different levels of aggression (i.e. intrasexual, infanticide) or anxiety (i.e. risk assessment behaviors) displayed by different genetic lines of mice may be predictive of different social structures. In fact, genetic lines characterized by high levels of aggression (i.e. Wild and Swiss) showed, when introduced in large territorial enclosures, social structures based on exclusive male territories accompanied by intense reproductive competition (i.e. high rate of intrasexual attack and infanticide) between females. Conversely, lines with low levels of aggression (e.g. DBA/2) form highly tolerant hierarchically-organized groups with a virtual absence of competition between females [58,68,75]. Thus, variation in proximal mechanisms may preadapt animals to different socio-ecological conditions. Indeed, artificial selection operated by man on animals through the process of domestication was the foundation of Darwin's [76] reasoning that led to the theory of evolution by means of natural selection. Consequently, because 'slight changes in structure almost always cause vast changes in behavior' [77], the use of a specific genetic line implies the knowledge that the alteration of proximal mechanisms is accompanied by the modification of behavior which may affect its adaptive significance. This evolutionary perspective, which takes into account the adaptive significance of behavior and the selective pressures acting on behavior, has recently been applied to behavioral pharmacology [78]. Together, these findings and considerations have profound implications for studies in behavioral neuroscience. Behavior is the end-point of a complex interaction of different integrated systems. Subtle alterations in any of the component systems are likely to be reflected at the behavioral level. It follows that investigations at any specific level of biological organization (from neuron to behavior) must take into consideration the relationships with other levels of organization. Hence, the study of neurochemical mechanisms of a specific behavior demands consideration of the relative context and function which implies the knowledge of its ontogenetic and evolutionary processes. Similarly, when studying the adaptive functions of behavior, it is of paramount importance to understand the mechanisms upon which selective pressures have acted to shape that specific behavior. This concept is clearly expressed in the emergent properties principle which claims that any structure (e.g. genes, hormones, receptors and neurotransmitters) and its function are correlated at all levels of biological organization including behavior.

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