Effects of under- and overcrowding on exploratory behavior in the elevated plus-maze

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Abstract

The present work investigated whether the number of rats housed in a cage affects exploration of an elevated plus-maze. Male Wistar-derived rats were kept 1, 2, 3, 4, 6, 8, 12, 16, or 24 to same size cages either for 1 or 14 days and tested in the elevated plus-maze. Rats kept 6 to a cage were arbitrarily considered controls because this is the housing condition adopted in many laboratories, ours included. In comparison to controls, 1-day housed rats kept 1, 2, 16, and 24 to a cage decreased the percentage of entries into the open arms. Similar decreases were also found in the time spent in the open arms, the only exception being the group with rats kept 16 to a cage which failed to show significant differences from the control group. Fourteen-day housed rats kept 1, 2, 16, or 24 to a cage decreased the percentage of entries and time spent in the open arms. We found plus-maze exploration to be similar in groups in which rats were kept from 4 to 12 to a cage. The present data indicate that anxiogenic effects resulting from under- and overcrowding should be taken into consideration in behavioral studies.

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1. Introduction

The elevated plus-maze, firstly reported by Handley and Mithani (1984), is a modification of a procedure proposed 5 decades ago (Montgomery, 1955), which has broadly been used to investigate anxiolytic and anxiogenic compounds (Handley and Mithani, 1984) and to study anxiety neurobiology (Knox and Berntson, 2006; Weitemier and Ryabinin, 2005; Patiasul et al., 2005). The method has behavioral, physiological, and pharmacological validation (Pellow et al., 1985). The test consists of placing an animal in a plus-shaped maze elevated above the floor level, with closed arms (i.e., walled) and open arms (i.e., with no walls). A rat explores both the closed and the open arms but typically will enter more frequently and stay longer in the closed arms. Open arm measures (i.e., entries and time spent) are taken as anxiety indexes, with more intense anxiety being correlated with lower exploration of the open arms (Handley and Mithani, 1984; Pellow et al., 1985).

In spite of the apparent simplicity of this test situation, the aversion to the open arms is influenced by many factors, such as treatments applied to the subject or its intrinsic characteristics such as gender, species, etc. (Johnston and File, 1991; Wigger and Neumann, 1999; Manaf et al., 2003). Aversion is also influenced by the test situation itself (Fernandes and File, 1996; File and Zangrossi, 1993; Griebel et al., 1993; Hogg, 1996; Morato and Castrechini, 1989; Treit et al., 1993). Pretest conditions, such as the way rats are transported to the test room (Morato and Brandão, 1996) or the place and how long they are kept there before testing (Morato and Brandão, 1997) can also alter open arm aversion.

Individual housing is known to increase the aversiveness of the open arms (Frussa-Filho et al., 1991; Maisonnette et al., 1993; Motta et al., 1992; Ruis et al., 1999; Serra et al., 2000; Weiss et al., 2004). There are also reports of increased fearful-
ness due to crowding (Armario et al., 1984; Gamallo et al., 1986; Haller et al., 2004; Thiebot et al., 1977). Housing conditions can be appropriately evaluated if information concerning the number of animals living in a delimited space is available. Curiously, scientific reports including little or no such information are abundant. Also, there are reports that do provide information about the number of rats grouped in a cage but do not specify its dimensions. Furthermore, it is worth noticing that the number of rats grouped in a cage varies from only one (e.g., Crombag et al., 2000; Harro et al., 1999; Krebs-Thomson et al., 2001; Lawson et al., 2000; Ness et al., 1995), two to six (Lawson et al., 2000; Harro et al., 1999; Krebs-Thomson et al., 2001; Ness et al., 1995; Harro et al., 1999; Gallate et al., 2003), or even 10 (e.g., Satanovskaya and Bardina, 1998).

The present work was aimed at comparing the effects on rat exploratory behavior caused by housing the animals in different numbers in same size cages. We also investigated the possibility that the period of housing might interfere in such effects. For these purposes, anxiety was assessed with the use of an elevated plus-maze.

2. Material and methods

2.1. Animals

Male Wistar rats weighing 200–230 g were housed 6 to a cage (41 cm × 34 cm × 17 cm) for an habituation period of at least 3 days before being submitted to the experimental conditions. Room temperature was maintained at 24–26 °C in both the vivarium and the test room, which was adjacent to it. Throughout the experiment, animals had rat chow (Nuvital, Brazil) and tap water ad libitum. The vivarium was maintained in a 12-h light/12-h dark photoperiod (lights on at 7:00 a.m.). Cage cleaning procedures were performed three times a week and wood shavings were used as bedding. All testing was performed between 7:30 and 10:30 a.m.

2.2. Apparatus

Rats were tested in an open field (60 cm × 60 cm × 30 cm with the floor divided into 15-cm squares) and in an elevated plus-maze described elsewhere (Setem et al., 1999). Briefly, the maze was elevated 50 cm from the floor and consisted of two open arms (with 1-cm Plexiglas edges) and two closed arms (with 40-cm high wooden walls and no roof) arranged in such a way that like arms were opposite to each other.

The open-field and the maze were located in a room lit by a 15-W light bulb 1.75 m above the central part of the maze. All data were recorded using standard Grason-Stadler (Concord, Massachusetts, USA) electromechanical equipment in the test room.

2.3. Procedure

Immediately after the habituation period, the rats were randomly divided into groups of different sizes and kept for 1 or 14 days before testing. Some of the rats were kept for 1 day in numbers of one rat per cage (N = 12), two rats per cage (N = 12), three rats per cage (N = 12), four rats per cage (N = 12), six rats per cage (N = 18), eight rats per cage (N = 16), 12 rats per cage (N = 12), 16 rats per cage (N = 16), or 24 rats per cage (N = 24). Similarly, another set of rats was kept for 14 days in numbers of one per cage (N = 12), two per cage (N = 12), three per cage (N = 12), four per cage (N = 12), six per cage (N = 12), eight per cage (N = 16), 12 per cage (N = 12), 16 per cage (N = 16), or 24 per cage (N = 24). The animals came from different cages and had no contact with each other except for the groups with 12 and 24 rats, which were composed by the rats coming, respectively, from two or four cages. All groups started the experimental period in a fresh cage, not their own. All cages used were the same size (41 cm × 34 cm × 17 cm).

Each rat was placed individually in the open-field for 5 min (where the number of squares crossed was recorded) and then transferred to the elevated plus-maze where it was gently placed with the nose facing one of the closed arms. The number of entries and time spent in each type of arm was recorded for 5 min by an experimenter standing as still as possible and away from the maze. The person recording the sessions was unaware of the group to which each rat belonged; data were analyzed by a different person. An entry was recorded when all four paws were placed inside one arm. The number of squares crossed in the open-field was used to assess locomotor deficits, if any. Percent of entries into the open arms were calculated in relation to total entries in both types of arms.

2.4. Data analysis

Data were analyzed by means of a two-way analysis of variance (Anova) with the factors housing period (two levels: 1 and 14 days) and number of rats per cage (nine levels: 1, 2, 3, 4, 6, 8, 12, 16, and 24). Rats grouped six to a cage for 1 or 14 days were considered controls because this is a number of rats per cage often used in many behavioral studies and also the usual number of rats kept in our laboratory. Whenever appropriate, group means were compared using all pairwise Duncan’s multiple range tests. When a group differed from its respective control in the number of entries in both types of arms, analysis of covariance (within groups housed for the same period) was performed to determine whether the increase or reduction in open-arm entries was independent of any effect on closed-arm entries. Analysis was carried out with entries into the open arms as the dependent variable and entries into the closed arms as the covariant. A significant effect indicated that the alterations in open-arm entries were independent of effects on closed-arm entries.

3. Results

3.1. Open-field

Fig. 1 shows the number of crossings in the open-field for 1- and 14-day housed rats and Table 1 presents two-way Anova results. There were significant effects of the housing period and of the number of rats per cage, but no significant interaction. Post
hoc comparisons showed that, within groups housed for 1 day, rats kept 24 to a cage crossed significantly less squares than the ones kept 6 to a cage (Duncan, $P < 0.05$). In general, there were no differences between groups with the same number of rats in a cage but housed for different periods, the exception being the group kept 12 to a cage for 14 days, which crossed fewer squares than the ones kept 12 to a cage for 1 day (Duncan, $P < 0.05$).

3.2. Elevated plus-maze

Anova showed a significant effect of the number of rats per cage on the percentage of entries into the open arms but no effect of the housing period or a significant interaction between the two factors (see Table 1). Post hoc comparisons showed that within the groups housed for 1 day, those kept 1, 2, 16, and 24 to a cage entered less the open arms than their respective controls. Similarly, rats housed for 14 days 1, 2, 16, and 24 to a cage entered less the open arms than their respective controls.

Time spent in the open arms is shown in Fig. 2. According to the two-way Anova, there were significant effects of both the number of rats per cage and an interaction between this factor and the housing period. Duncan’s multiple range test showed that the rats housed for 1 day 1, 2, and 24 to a cage spent less time in the open arms than those from their respective control group. Among animals housed for 14 days, those grouped 1, 2, 16, and 24 to a cage spent less time in the open arms than their respective control group.

As can be seen in Fig. 3, decreases in total entries by rats housed in small or large numbers were partly due to decreases in the open arm entries. According to Anova, there was a significant effect of the number of rats per cage on the frequency of entries into the open arms; there was also a significant inter-

Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$F$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Housing (d.f. = 1,244)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percent of entries into the open arms</td>
<td>0.08</td>
<td>0.774</td>
</tr>
<tr>
<td>Entries into the open arms</td>
<td>2.64</td>
<td>0.105</td>
</tr>
<tr>
<td>Entries into the closed arms</td>
<td>18.74</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time spent in the open arms (s)</td>
<td>0.61</td>
<td>0.435</td>
</tr>
<tr>
<td>Squares crossed in the open-field</td>
<td>43.70</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Rats per cage (d.f. = 8,244)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percent of entries into the open arms</td>
<td>84.59</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Entries into the open arms</td>
<td>90.15</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Entries into the closed arms</td>
<td>17.77</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time spent in the open arms (s)</td>
<td>66.02</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Squares crossed in the open-field</td>
<td>10.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Interaction housing vs. rats per cage</td>
<td>(d.f. = 8,244)</td>
<td></td>
</tr>
<tr>
<td>Percent of entries into the open arms</td>
<td>0.72</td>
<td>0.673</td>
</tr>
<tr>
<td>Entries into the open arms</td>
<td>3.65</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Entries into the closed arms</td>
<td>1.71</td>
<td>0.097</td>
</tr>
<tr>
<td>Time spent in the open arms (s)</td>
<td>2.52</td>
<td>0.012</td>
</tr>
<tr>
<td>Squares crossed in the open-field</td>
<td>1.40</td>
<td>0.196</td>
</tr>
</tbody>
</table>

$F$, $F$-values; $P$, probability; d.f., degrees of freedom.
action between the frequency of entries into the open arms and the housing period. Post hoc comparison pointed that within 1-day housed rats those kept 1, 2, 3, 16, and 24 to a cage entered less the open arms than their respective controls. Among the 14-day housed rats, only those kept 1, 2, 16, and 24 to a cage showed decreases in open arm entries as compared to their respective control group. Closed arm entries, according to the Anova, showed significant effects of the period of housing and of the number of rats per cage but there was no significant interaction between the factors. Comparison between groups (Duncan, $P < 0.05$) only found that 14-day animals housed 1 or 24 to a cage entered less the closed arms than their respective control group. Analysis of covariance showed that the decreases in open-arm entries were independent of those occurring in the closed-arm entries, for both 1- and 24 to a cage entered less the closed arms than their respective control group. Among the 14-day housed rats, only those kept 1, 2, 3, 16, and 24 to a cage entered less the open arms than their respective controls. Among the 14-day housed rats those kept 1, 2, 3, 16, and 24 to a cage entered less the open arms than their respective controls.

### 4. Discussion

The present work found that grouping rats in extremely low or large number in comparison to the often used six-rats-per-cage, increases anxiety as evaluated in the elevated plus-maze. Housing the animals in large groups for 14 days seemed to intensify anxiogenic effects. The decreases in exploratory activity were probably not related to deficits in locomotor activity, since there were, in general, no significant differences in the number of squares crossed in the open-field.

Housing rats in small numbers may result in increased anxiety. In the present experiment, groups in which rats were housed 1 or 2 per cage showed most consistently such an effect. Individual housing is known to result in anxiogenic effects in the plus-maze (Ferrari et al., 1998; Frussa-Filho et al., 1991; Maisonnette et al., 1993; Morato and Brandão, 1996; Motta et al., 1992; Ruis et al., 1999; Vasar et al., 1993; Weiss et al., 2004; Wright et al., 1991). See Hilakiv et al. (1989), however, for a report in which individually-housed mice actually increased the percentage of time exploring the open arms. Such an effect could be due to a difference in species or to the fact that the animals were previously exposed to the holeboard test before test in the plus-maze and Pellow et al. (1985) had already shown that rats submitted to this sequence of protocols may present changes in exploratory activity. The present results also showed that housing two rats per cage may not be enough to avoid the anxiogenic effects usually seen in individually-housed rats. Such a result is useful as a warning for future experiments in which, similarly to housing individually, even pairs of rats housed together may be in a somewhat disturbing condition.

Housing rats in large numbers can also result in increased anxiety, as measured by the decrease in the exploration of the open arms. Such a result was most consistently found in the rats housed 16 or 24 to a cage. There are reports using the open-field, the Y-maze and the holeboard (Armario et al., 1984; Thiebot et al., 1977) describing similar decreases in exploratory behavior and locomotion by rats in overcrowded housing conditions. But, there are also descriptions of increases in these behaviors caused by the same overcrowding (Morrison and Thatcher, 1969; Thiessen, 1964). It is difficult, however, to compare studies with overcrowding because they vary broadly in the number of animals kept per cage, the time the animals are kept in the condition, and the kind of tests and species used.

In spite of previous reports on anxiogenic effects of crowding, the present study is, to our knowledge, the first report of such an effect in the elevated plus-maze. Unexpectedly, the effect was only found in rats housed in the largest group sizes tested. Even housing 12 rats per cage (a number twice as large as the one of the group assumed as control) resulted in no effect on anxiety. Therefore, in experiments in which crowding is studied it seems that care should be taken in grouping rats in numbers that could result in no effects, or in undesirable effects. On the other hand, as no effect on open arm exploration was found when rats were grouped 4–12 per cage, the present work indicates that there are several possible numbers of rats per cage that do not increase anxiety. In the long run, behavioral studies in general should take precautions in order not to influence their results with the number of rats kept to a cage.

It may be argued that the results could be influenced by the fact that rats kept 12 or 24 per cage came from 6-rat cages and were familiar with each other. However, in these cases there were also unfamiliar subjects composing the groups and the results obtained with rats grouped 12 to a cage were dissimilar to the ones obtained with rats grouped 24 to a cage. Therefore, we believe the behavior patterns to be the result of the size of the group, instead of familiarity. In fact, this is a discussion – the composition of which rats compose a group and are kept in one cage – which is rarely, if ever, seen in the literature.

Increasing the period of housing seemed to intensify anxiogenic crowding effects. Time spent in the open arms by rats housed 16 to a cage was significantly decreased only in the group maintained in this condition for 14 days, but not in those maintained for 1 day. The increased housing period, however, did not intensify the effects of housing rats in small numbers.

In addition, the fact that more extreme conditions in our experiment also decreased closed arm entries is interesting. This parameter is usually considered an index of locomotor activity (File and Zangrossi, 1993; Cruz et al., 1994) based on studies involving factorial analysis. A factorial study (Lister, 1987), however, reported that total entries in the plus-maze loaded only partly on a factor related to anxiety. Also, later studies (Morato and Brandão, 1996, 1997) showed that more aversive conditions drastically reduced the number of entries into the closed arms. An explanation can be that, in experimental situations which do not provoke much anxiety the exploration of closed arms may be more related to locomotion while in a more aversive context it may reflect anxiety.

Obviously, what can be considered a large number or an optimal number of rats per cage depends on the size of the cage. The same frequently used number of six rats per cage may represent overcrowding in a small sized cage. Similar concerns should be present in respect to the size of the animals under study. For instance, infant rats may need less space than
adult ones. While there are recommendations about the area which should be accounted for each animal (National Research Council, 1996), there are however no established values of area per rat which reliably are less anxiogenic. Thus, it is necessary to have experimental data on the influence of different sized areas on rat anxiety.

In conclusion, the present work indicates that there is a group size range that seems to evoke less anxiety in rats. When grouped in numbers out of this range, rats exhibit increased anxiety. The present results should be taken into consideration in behavioral experiments grouping rats in numbers extremely small or large. Further studies, however, may be necessary to understand what characteristic of the experimental situation the rats are responding, since there are many variables present in the situation which were not addressed. Thus, the animals might be sensitive to density, as measured in rats per cage area or volume. Current research from our laboratory is assessing the effect of density on behavior of rats in the elevated plus-maze and preliminary results suggest a complex relation between these factors.

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