Voorwoord

Dit is het eindverslag van mijn afstudeerproject voor de studie Life Sciences, dierkunde aan de Hogeschool Utrecht. Deze opdracht heb ik gedurende een periode van 6 maanden op de afdeling Proefdierkunde van het departement Dier, Wetenschap en Maatschappij (Faculteit Diergeneeskunde) aan de Universiteit van Utrecht mogen uitvoeren.

Het doel van dit project was om inzicht te krijgen in het cognitieve functioneren van twee inteelt muisstammen. Uit voorgaand onderzoek is gebleken dat een van deze stammen moeilijk tot niet habitueerd. Een defect in het cognitieve vermogen zou daar de oorzaak van kunnen zijn. Dit project was een klein onderdeel van het nog lopende promotie onderzoek van drs. Amber R. Salomons. Zij gaat binnen haar onderzoek op zoek naar een "pathologisch angstig" diermodel, wat in de toekomst gebruikt kan worden in diverse onderzoeken naar angst. Daarnaast zal dit onderzoek ook meer inzicht geven in de eigenschappen van deze muisstammen waardoor gebruik van deze dieren verder geoptimaliseerd kan worden.

Amber wil ik dan ook als eerste bedanken voor deze leuke opdracht en fijne samenwerking. Voor alle obstakels en problemen vonden we altijd wel een goede oplossing. Door haar feedback en van haar directe begeleidsters Saskia Arndt en Frauke Ohl zijn we tot een mooi eindresultaat gekomen. Ondanks jullie drukke werkzaamheden gaven jullie mij altijd het gevoel dat ik met mijn vragen mocht aankloppen, bedankt daarvoor. Tot slot wil ik Amber, Elise, Janneke, Jelle, José, Marijke, Niels, Saskia en Susanne bedanken voor het iedere dag gezellig samen lunchen! En natuurlijk ook alle andere collega's en medestudenten van Dier Wetenschap en Maatschappij (DWM) voor hen getoonde interesse en de heel gezellige tijd!

December 2007, Judith van Luijk

Summary

Anxiety instigates a series of behavioural, neuronal and physiological responses that prepare an animal to new environmental challenges. When anxiety in animals appear to lack adaptive value and severely interferes with normal interaction of the sufferer with its physical and social environment, it might be defined as pathological. In previous research it was found that the BALB/c mouse strain habituates to an initially stressful novel environment while the 129P3/J mouse strain did not show habituation. To investigate if this lack of habituation in the 129P3/J strain is caused by cognitive impairment, an object recognition task was performed. According to the results of the previous research we hypothesized that the lack of habituation in the 129P3/J strain is not caused by cognitive impairment and therefore expected to see no difference between habituated and non habituated groups with respect to object memory. We also hypothesized to see higher anxiety related behaviour in the BALB/c strain compared to the 129P3/J strain. In addition we expected that the BALB/c mice will be able to habituate to the test arena whilst the 129P3/J mice will show no clear habituation.

Male 129P3/J and BALB/c mice were divided over 3 habituation test designs. One group was habituated to the object recognition test arena for 4x 5 minutes, the second group was habituated once for 5 minutes and the third group was not habituated. After a familiarisation period of 24h with one object (nut or dice) in the home cage, the animals were placed in the test arena with both objects presented. A variety of behaviours were scored to investigate cognitive ability in the object recognition test.

Like we hypothesized it was found that the 129P3/J is not cognitively impaired as no differences in object memory were seen between the three test designs. As expected the 129P3/J mice did not show habituation. Like in the previous research the BALB/c mice clearly habituated to the test arena and

also showed initially higher anxiety related behaviour compared to the 129P3/J mice. The lack of habituation in the 129P3/J strain could be caused by a characteristic, or 'trait', anxiety however further research for this statement will be necessary.

Contents

Introduction

Anxiety refers to a biologically adaptive response to a potential threat. However when anxiety responses become prolonged or inappropriate, it can lead to a chronic state of non adaptive anxiety. As a consequence long lasting neuro-physiological changes can occur, finally leading to pathological anxiety disorders, which lack adaptive value. Pathological anxiety in animals can be distinguished from normal anxiety as a persistent, uncontrollable emotion triggering physiological and behavioural responses lacking adaptive value. (Ohl et al., in press) Though many animal models for anxiety measure adaptive 'state' anxiety responses (situation evoked), appropriate models for pathological anxiety have not yet been found. Reliable identification of anxiety must allow the animal to display biological relevant behaviour and considers multiple behavioural systems, which are involved in anxiety. Behavioural expression of anxiety can be assessed for example in a novel environment. According to the cognitive map theory (O'Keefe and Nadal, 1978; Leussis and Bolivar, 2006) a rodent, when placed in a new environment, starts to form an internal representation of the surrounding spatial information. Once this hippocampal 'map' is complete, exploration (measured as a change in motor activity; e.g. distance travelled, line crosses, rearing, etc.) of the environment is reduced and we consider that the rodent has habituated to the new environment. (Leussis and Bolivar, 2006) To be able to do so an animal must overcome its initial avoidance-induced behaviour inhibition. (McNaughton and Gray, 2000) Pathological anxiety can then be distinguished from state anxiety as non adaptive in terms of sensitisation (increased anxiety for one specific stimulus) or a lack of habituation (impaired coping with novelty).

In laboratory animals, highly specific (physiological) characteristics are genetically selected or genetic modifications are conducted, often ignoring the possibly complex consequences of such processes. Genetic alterations will often affect behaviour as well.(Ohl et al., in press) In this project we focus on two

inbred mouse strains, the BALB/c and the 129P3/J. The first strain is reported to exhibit strong neophobic reactions when confronted simultaneously with a familiar and a novel compartment in the free exploration test. (Griebel et al., 1993) The BALB/c strain exhibits spontaneously elevated anxiety and is suggested to be a model for pathological anxiety. (Sik et al., 2003; Belzung and Griebel, 2001; Brooks et al., 2004) The 129P3/J strain is a commonly used mouse strain for obtaining embryonic stem cells. Consequently new induced mutations are usually derived within the 129-genome. However, the 129/J sub strain shows deficits in several behavioural tasks. (Simpson et al., 1997; Montkowski et al., 1997) The behavioural dysfunctions found in these inbred strains can compromise biological functioning and probably reduces the adaptive capacity of the animals. Therefore we are interested in evaluating whether pathological anxiety (i.e. compromised habituation) can be identified in these inbred mouse strains.

In previous research with the modified hole board (mHB) (Ohl et al., 2001) it was found that after the trial of 5 minutes the male BALB/c mice demonstrated initially more avoidance behaviour towards the novel environment than the group of male 129P3/J mice. The BALB/c mice however clearly habituated after repeated exposure to the mHB whilst the 129P3/J strain did not show habituation and no changes were seen in anxiety related behaviour over time (Salomons A.R., unpublished). For testing anxiety related behaviour it is important to take related behavioural systems in to consideration. For example cognitive processes can be a potential confounding factor when assessing behavioural parameters. (van der Staay, 2006; Ohl, 2005).

In this study we want to investigate if the lack of habituation seen in the previous experiments in the mHB in the 129/J strain is caused by cognitive impairment. Therefore we perform an object recognition task *(Sik et al., 2003)* to identify object memory in the 129/J and BALB/c strain. This has the advantage that it consists of a one trial learning task which enables us to investigate effects of habituation on object recognition. Moreover commonly used cognitive tasks require a learning period which can influence habituation.

We hypothesize that the lack of habituation in the 129/J strain is not caused by cognitive impairment and we expect to see no difference between habituated groups and non habituated groups with respect to object memory. In addition we expect to see less anxiety related behaviour in the 129P3/J strain compared to the BALB/c and that the 129P3/J strain will not show habituation (as seen in the mHB).

Accordingly we expect that the BALB/c mice show more anxiety/avoidance behaviour in the non habituated groups compared to the habituated groups and that this strain is not cognitively impaired (Brooks et al., 2004).

Materials and methods

Animals

A total number of 54 male mice, 26 BALB/c and 27 129P3/J (from now on 129/J) purchased from the Jackson Laboratory (Bar Harbour, USA), 6-7 weeks old at arrival were used in this study. All mice were housed individually in Macrolon II cages with standard bedding material and several types of enrichment (a tissue, a shelter and a small amount of envirodry (shredded cardboard bedding). The animals were kept in the animal facility of the University of Utrecht where they had access to food and bottled water at libitum. All animals remained in the same room (experimental room) during the whole experimental period. Only for blood sampling and decapitation the animals were taken individually to a separate room to prevent stress in the other animals. In the experimental room the dark period (red light) started at 6.00 AM till 6.00 PM so the animals were in there active period when we preformed the object recognition task. The humidity was kept at a minimal of 45% and a constant temperature of 23 ± 1°C. Radio music was turned on constantly as background noise.

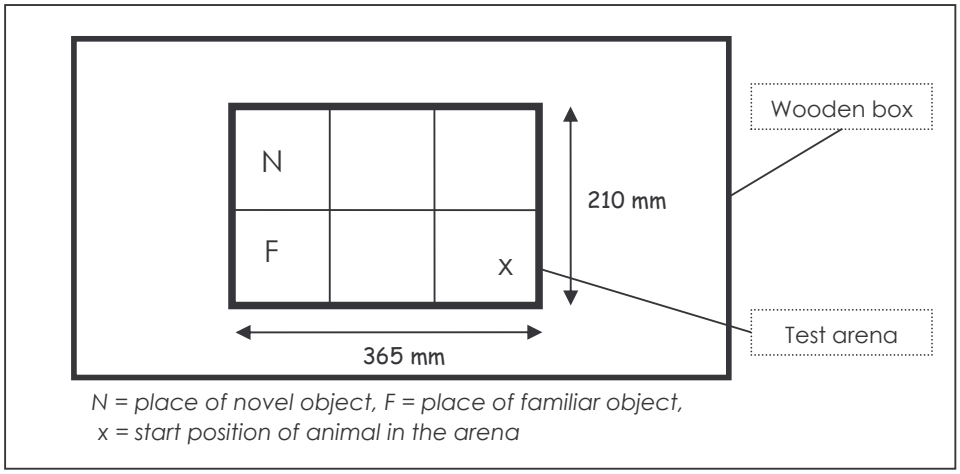
Object Recognition Task

The apparatus was a test arena (Macrolon II cage, 365 x 207 x 140 mm) without any bedding placed in an open wooden box so the animals were minimally distracted by anything outside the test arena. The floor of the test arena was divided in 6 equal squares by black lines to monitor the locomotion of the animal (see figure 1).

All experiments were conducted under red light between 9 AM and 2 PM (active period). The objects to be discriminated were a dice and a nut, both objects were considered too heavy to be displaces by the animal. After each trial the test arena and the objects were cleaned with water and paper towels to remove droppings and urine.

Figure 1

Apparatus object recognition task



N = place of novel object, F = place of familiar object,
x = start position of animal in the arena

To discriminate the behaviour in habituated and non habituated animals the animals were divided over 3 test designs (n=9 per strain; see figure 1).

Figure 2

Protocol object recognition task.

	Day 0	Day 1	Day 2
Design 1 (n= 18; 9 BALB/c and 9 129P3/J)	Familiar object placed in home cage (nut or dice, randomised)	Familiar object taken out home cage	Object recognition task
Design 2 (n= 17; 8 BALB/c and 9 129P3/J)	Familiar object placed in home cage (nut or dice, randomised)	Familiar object taken out home cage 5 min habituation in test arena	Object recognition task
Design 3 (n = 18; 9 BALB/c and 9 129P3/J)	Familiar object placed in home cage (nut or dice, randomised)	Familiar object taken out home cage. 4x 5 min habituation in test arena (interval 1 hour)	Object recognition task

Animals in test design 1 were not allowed to habituate to the test arena. Animals in design 2 were allowed to habituate once for 5 minutes an the animals in test design 3 were allowed to habituate 4 times for 5 minutes with an interval of 1 hour. The habituation took place 24h prior to the object recognition task.

All animals were allowed to familiarize with one object for 24 hours (nut or dice randomly chosen) two days before testing. After the familiarisation period this object was considered familiar for the animal. During the object recognition task the animals were exposed to both objects. The duration of the object recognition task was 10 minutes, during this period a trained technician observed and scored the behaviour of the animals using the computer programme 'Observer 4.1' (Noldus Information Technology, the Netherlands). Behaviours were categorized in two properties: events (shortly lasting actions, frequency of events, e.g. jump) and state (long lasting actions, e.g. grooming). The scored behaviours are listed below in figure 3.

Figure 3

List of behaviours with descriptions scored in the object recognition task.

Name	***Description***
Field	Animal is picked up by tale and gently placed in the test arena (x)
Exploration familiar object	Directing the nose at a distance ≤ 2cm to the familiar object and/or touching it with the nose
Exploration novel object	Directing the nose at a distance ≤ 2cm to the novel object and/or touching it with the nose
Stretched-attend posture	Stretched/elongated body posture (hind paws planted): forward elongation of head and shoulders followed by retraction to original position
Immobility	Being motionless: animal quietly lies/sits on the floor
Jumping	Animal jumps, non of the 4 paws touches the floor
Escape attempt	Vertical active escape attempt with all paws leaving the cage bottom or upright body with all paws rapidly moving against cage side/bottom
Rearing	Upright posture with forelegs moving into the air (≥45°) or leaning against the wall of the enclosure
Stiffing	Mobile or immobile olfactory exploration of the environment
Grooming	Auto-grooming; mouse scratches or licks fur, washes face or licks genitalia
Defecation	Defecation
Urination	Urination
Line crossing	Mouse crosses one of the lines marked on the arena floor
Other	Unspecified behaviour in open field

Blood sampling

Blood samples were taken 6 days prior to and 30 minutes post the object recognition task. The first sample (basal sample) was used to set a baseline for the stress hormone corticosterone in the plasma. In the second sample the plasma corticosterone level was measured again to determine the stress response after behavioural testing. This was done 30 minutes post testing because than the peak of the corticosterone level in the blood can be measured. (Fluttert et al., 2000) To obtain the blood samples a small incision was made in the tail vain (1/3 from the tail base), the drops of blood were collected in a lithium-heparin coated tube (50µl). This was done in a separate room to prevent stress by the smell of blood in the other animals.

Brain Removal

For immunocytochemical analysis and further research in the future the brains were removed. After removal the brains were quickly frozen by immersing in cold 2-Methyl-butane and stored in small tin cans at -80°C.

Statistical analysis

Statistical analyses were performed using the program SPSS 12.0. Continues data (plasma corticosterone, latency and relative duration of the behavioural parameters) were summarized as means with standard deviation (SD). Discrete data on the ordinal scale (frequencies of behavioural parameters) were presented as median with interquartile range (IQR). The Kolmogorov-Smirnov test was used to investigate the normality of the continues data. A two-way ANOVA was performed with strain and design as main factors. Homoscedasticty was tested by Levene's test. Results were considered significant when the P-value was <0,05.

When two-way ANOVA showed significant differences Post hoc analysis were done. For the continues data the non paired Student-t test, and for the ordinal data the Mann-Withney U test were preformed.

The discrimination was considered as index measures of discrimination between the novel and the familiar object (Sik et al., 2003) and calculated as followed:

$$DI = \frac{(n - f)}{(f + n)}$$

DI= Discrimination index

n= total exploration time novel object (s)

f = total exploration time familiar object (s)

Results

Two-way ANOVA revealed no significant differences in the discrimination index ($p > 0,05$). *See appendix 1, figure 4.* However we also performed a paired t-test to examine if the animals could distinguish between the novel and the familiar object. These results showed significant differences in all three designs for BALB/c though no differences were found for 129/J between the novel and the familiar object. *See appendix 1, figure 5 and 6.*

The latency time to the familiar and the novel object revealed a significant difference revealed in two-way ANOVA within the designs. Post hoc analysis of de BALB/c data showed significant differences in the latency to the familiar object between design 1 and 3 and to the novel object between design 1 and 2 and design 1 and 3. *See appendix 1, figure 7.* No significant differences were found between the designs within the 129/J strain. *See appendix 1, figure 8.*
Two-way ANOVA revealed a significant difference in the number of stretched attends within the strains and designs. Post hoc analysis showed a significant difference within the BALB/c and the 129/J strain between design 1 and 3. A significant difference was also found within design 1 between the two strains. *See appendix 1, figure 9.* Statistical analysis for the number of defecations revealed a significant difference within strain for al three designs. *See appendix 1, figure10.*

The locomotor activity was determined by the number of line crossings in the test arena. Two-way ANOVA revealed a significant difference in strain and design. Post hoc testing showed a significant difference between the two strains in design 2 and 3. A significant difference was also found among design 1 and 3 within the BALB/c strain. No significant differences were found within the 129/J strain. *See appendix 1, figure 11.*
The number of "sniffings" and "rearings" can be seen as indicators for exploratory behaviour. Both sowed a significant difference in strain in the Two-way ANOVA. Post hoc Mann Whitney U test revealed a significant difference

between the two strains in design 2 for both behavioural parameters. *See appendix 1, figure 12 and 13.* All relevant behavioural parameters and data are collected in one table. *See appendix 1, figure 14.*

Discussion

With this study we have demonstrated that the lack of habituation in male 129/J mice is not caused by cognitive impairment. The discrimination index (DI) for instance showed no significant difference between the two strains over the three designs. However, the 129/J shows a negative relative discrimination in design 3, which indicates a higher interest in the familiar object, but this negative value could be caused by the high variation within each design or by possible outliers. After a closer look at our data we found two outliers in the 129/J strain (design 2 and design 3). When the data is corrected for the outliers the DI graph shows no negative value for the 129/J strain in design 3. This indicates that the outlier caused the negative value and the 129/J strain in design 3 in general does not show more interest in the familiar object as was found in the BALB/c strain. As expected still no significant differences were found in de DI for both strains. *See appendix 2, figure 15.* In the corrected exploration duration data of the objects less variation was found in the 129/J strain, however still no significant differences were found.

When these outliers are excluded in the statistical analysis, this leads to different significant outcomes for several parameters. For instance, the latency to the familiar and novel object in the 129/J strain: in two-way ANOVA analysis over the original data of both latency to the novel and the familiar object a significant difference was found within the designs. The same statistical analyses over the corrected data also revealed a significant difference within the designs. In addition a significant difference to the familiar object between the two strains was found. Subsequent analysis showed new significant differences in the 129/J strain between design 1 and 3. *See appendix 2, figure 18 and 19.* The latency to the novel object decreased as the 129/J strain spent more time in the test arena which indicated possible habituation. This is in contrast to the results found in the mHB *(Salomons A.R.,*

unpublished), although no habituation was seen in other parameters such as "sniffings", "rearings" and line crossings (see discussion below).

Line crossings and immobility can be considered indicators for locomotor activity. After data correction of number of line crossings by excluding the outliers a significant difference was no longer found within the BALB/c strain between design 1 and 3. The significant difference within design 2 between the two strains remained and a significant difference within design 3 was revealed. *See appendix 2, figure 20.* This confirms outcomes of other studies which reported a lower locomotion activity of various 129 strains. (Montkowski et al., 1997; Tang and Sanford, 2005) In addition the higher locomotion activity of the BALB/c strain compared to the 129/J strain found in this study confirms the outcome of the findings in the previous research. *(Salomons A.R., unpublished)* Two-way ANOVA testing over the corrected immobility data no longer showed a significant difference in design, only in strains. Again post hoc testing does not reveal significant differences.

The difference found between the two strains within the 3 designs after correction by excluding the two outliers, "rearings" and "sniffings" were no longer significant. *See appendix 2, figure 21 and 22.* These two parameters can be considered indicators for exploratory behaviour. As we hypothesized no significant differences in the designs were found, since we expected no habituation in the 129/J strain.

The outliers did not affect any of the other parameters. As we hypothesized the BALB/c strain showed initially higher anxiety related behaviour (number of stretched attends) compared to the 129/J strain. Even a significant difference is seen between the two strains within design 1. These results confirmed the outcomes of the mHB, showing a clear strain difference in anxiety related behaviour. As expected the BALB/c strain habituated and therefore showed les stretched attends in the habituated group (design 3). However, remarkable is the significant difference found within the 129/J strain between

design 1 and 3. This could indicate possible habituation. Though as mentioned before no habituation was seen in the other parameters of the 129/J strain. The number of defecations can also be used as an indicator for anxiety. *(Barone et al., in press)* However in both the mHB and the object recognition task, a higher number of defecations were seen in the BALB/c compared to the 129/J independent of habituation designs. This indicates that the high number of defecations in BALB/c can be a characteristic of this strain.

Although the blood samples have not been analysed yet as well as the frozen brains a statement can be made by use of the behavioural data. In conclusion the data of the various parameters confirm the hypothesis that the lack in habituation seen in male 129/J mice is not caused by cognitive impairment. Clear habituation was seen in several parameters for BALB/c strain as expected. The lack of habituation can be caused by a characteristic, or 'trait', anxiety however further research for this statement will be necessary.

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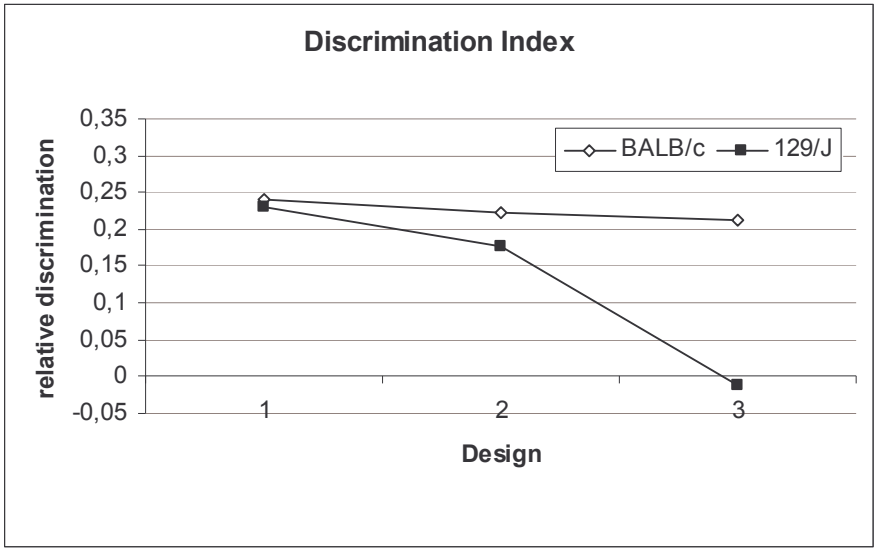
Appendix

Appendix 1; Results

Appendix 2; Discussion

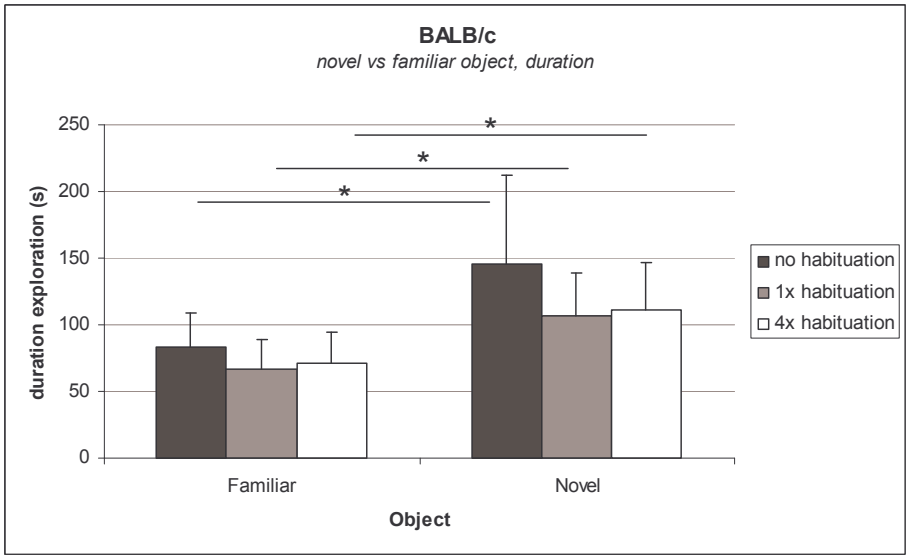
Appendix 1; Results

Figure 4



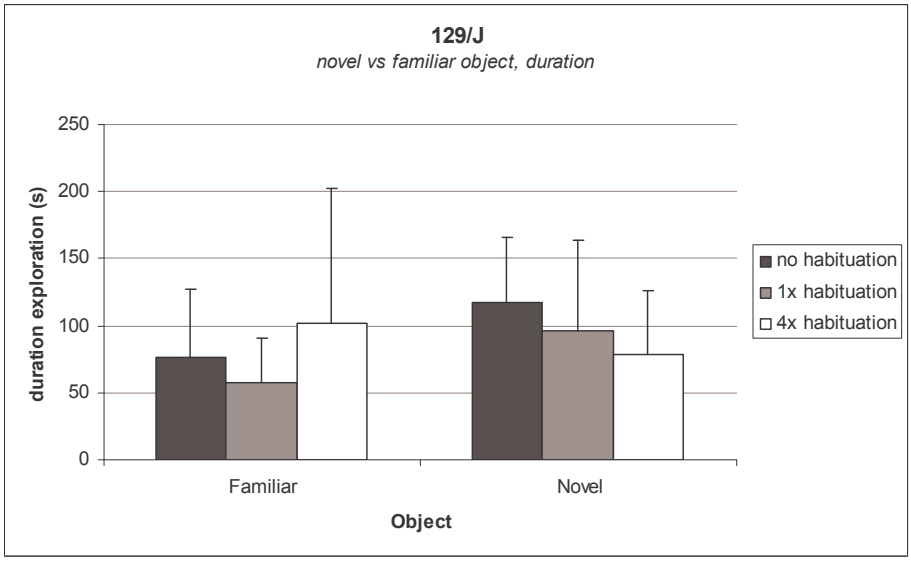
No significant difference was found in the discrimination index ($p>0,05$). However remarkable is the negative value of the 129/J strain in design 3 (see discussion).

Figure 5



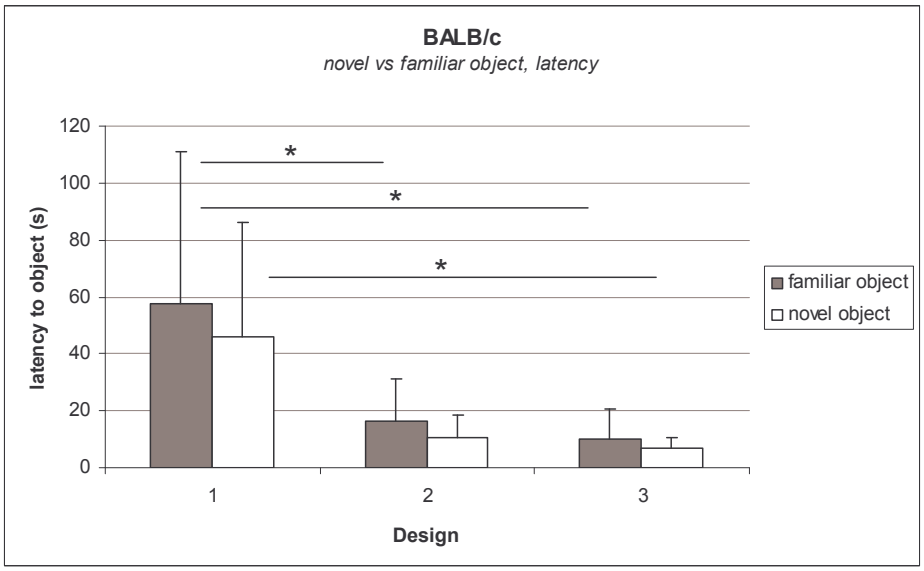
Significant difference was found in the duration exploration data between the novel and the familiar object within the 3 habituation designs (*=p<0,05).

Figure 6



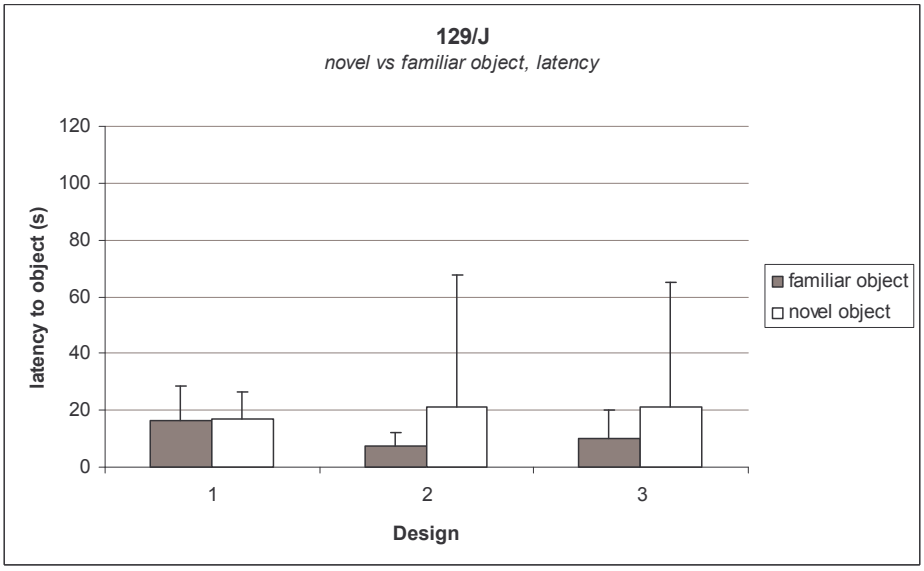
No significant difference was found in duration exploration between the novel and the familiar object within the 3 habituation designs. However design 3 (4x habituation) presents high variations within the familiar object data (see discussion).

Figure 7



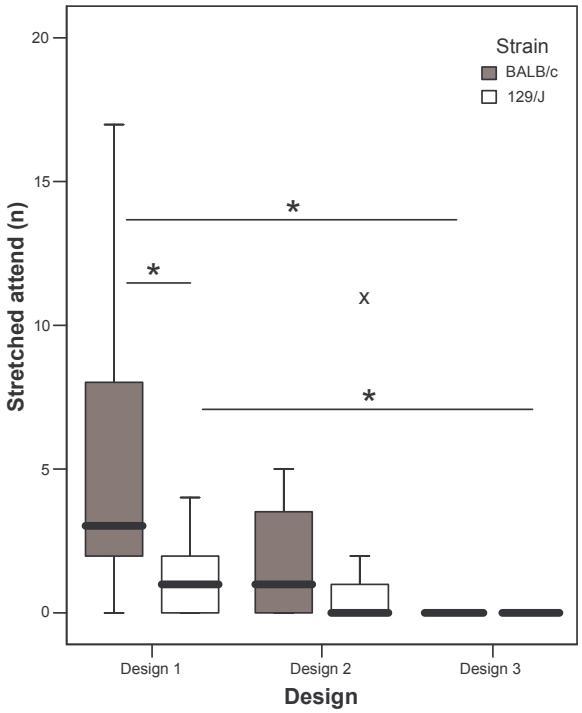
Significant difference (*=p<0,05) was found in latency of the BALB/c strain to familiar object between design 1 and 2 and design 1 and 3. And in the latency to the novel object data a significant difference (p<0,05) was found between design 1 and 3.

Figure 8



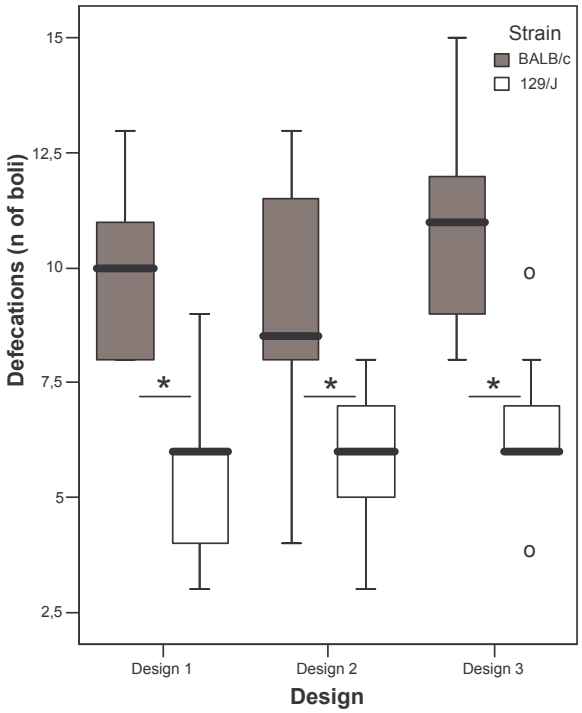
No significant difference (p>0,05) was found in latency of the 129/J strain to the familiar of novel object. Notable is the high variation within the novel object data in design 2 and 3 (see discussion).

Figure 9



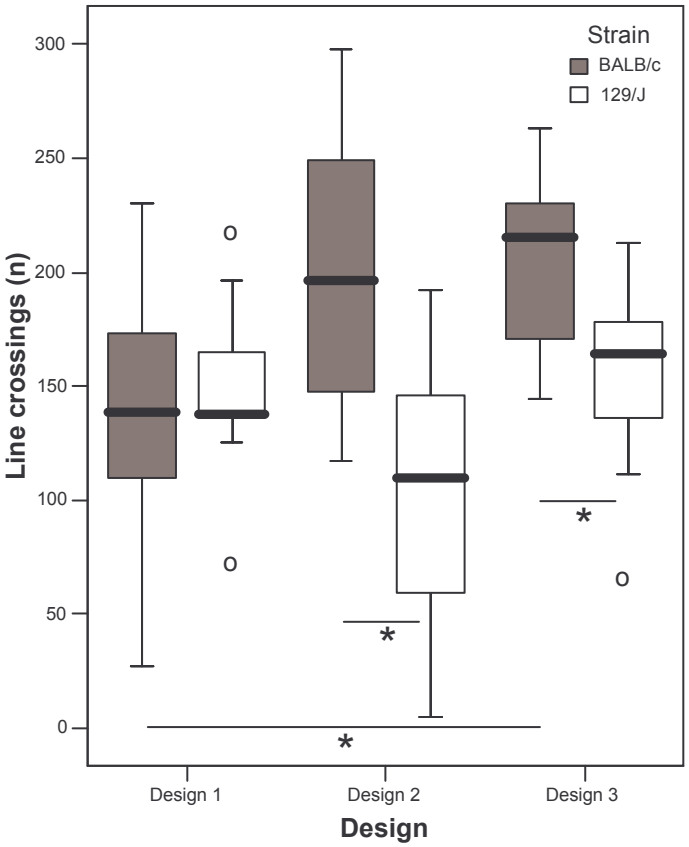
A significant difference (*=p<0,05) was found for the BALB/c and 129/J strain between design 1 and 3. A significant difference was also found between the two strains in design 1.
The statistical analysis program detected one data outlier in design 2 of the 129/J strain (x= value>3*IQR).

Figure 10



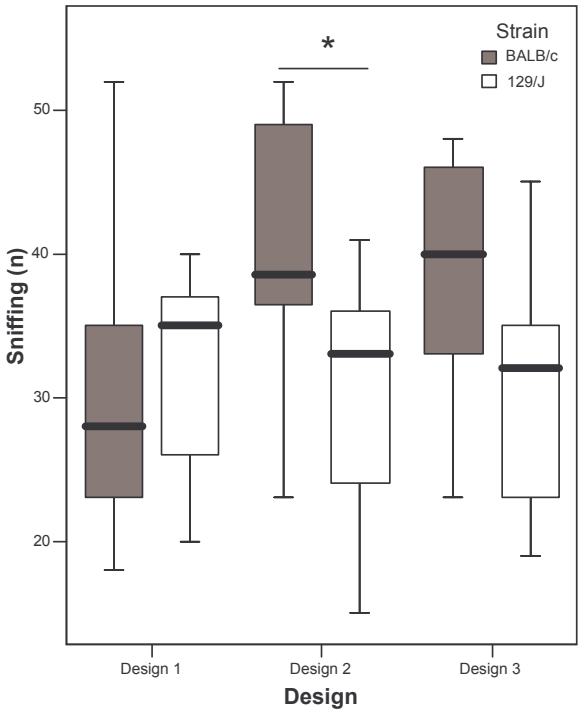
Within all 3 designs a significant difference (*=p<0,05) was found between the two strains.
The statistical program SPSS detected two data outliers in design 3 of the 129/J strain (o= value>1,5*IQR).

Figure 11



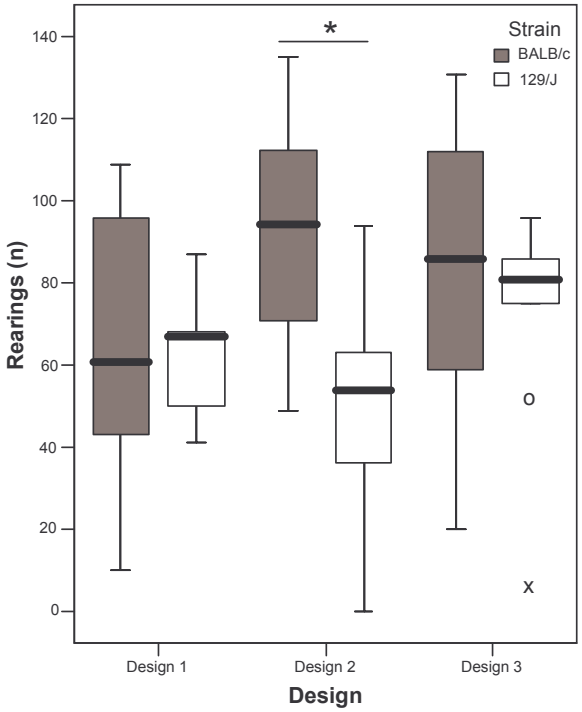
The difference between the number of line crossings was significant (*=p<0,05) within the BALB/c strain between design 1 and 3. A significant difference was also found between the two strains in design 2 and 3.
Outliers in the data of the 129/J strain were detected by SPSS in design 1 and 3 (o= value>1,5*IQR)).

Figure 12



Due to outliers a significant difference (*=p<0,05) was found between the two strains within design 2 (see discussion).

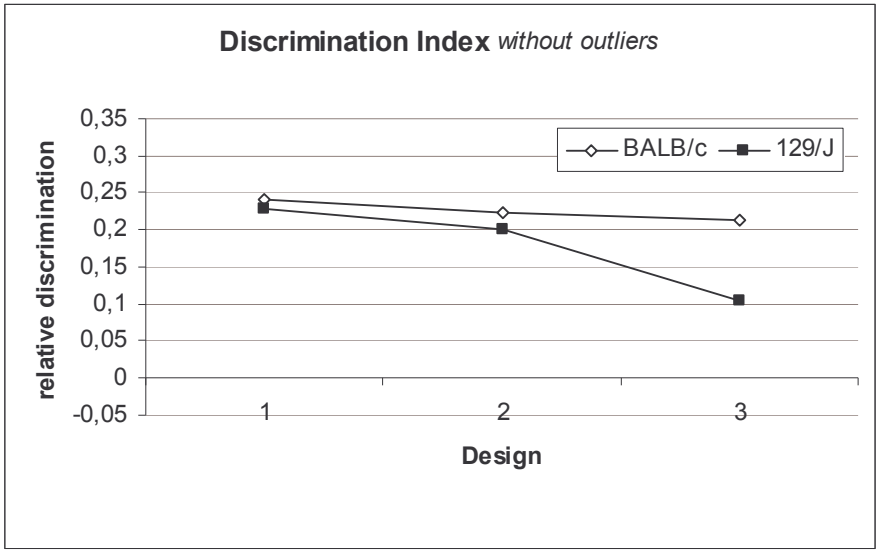
Figure 13



Due to outliers a significant difference (*=p<0,05) was found between the two strains within design 2 (see discussion).
The statistical analysis program detected two outliers in design 3 of the 129/J strain (o= value>1,5*IQR; x= value>3*IQR)

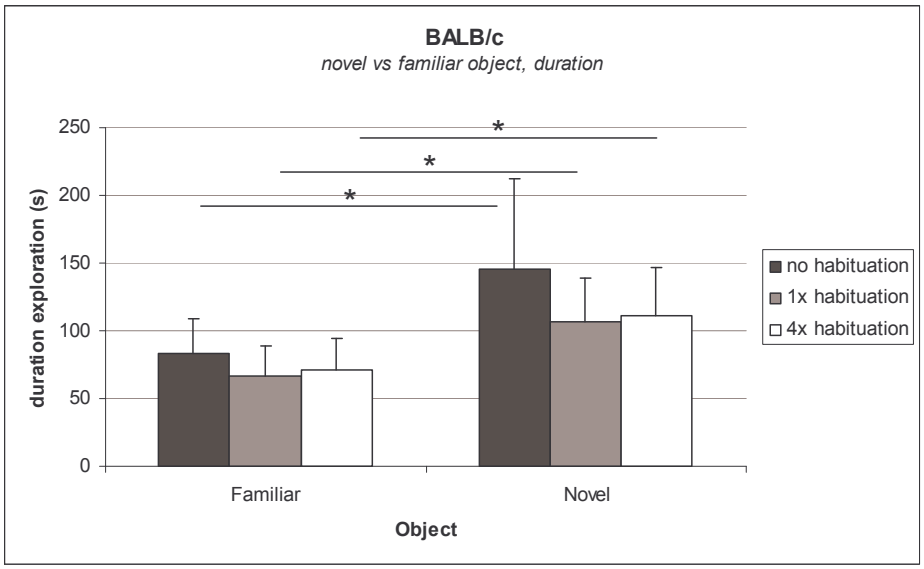
Appendix 2; Discussion

Figure 15



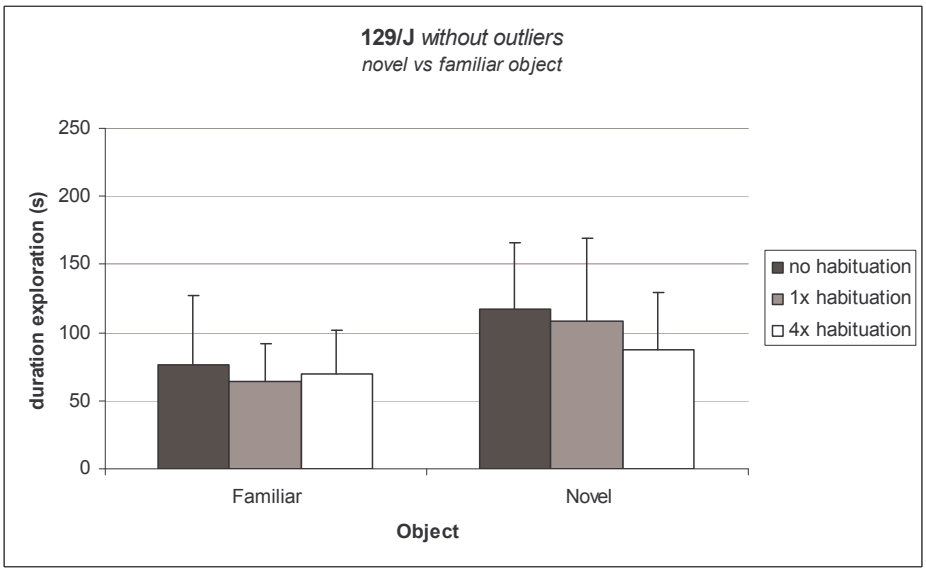
After data correction by excluding two outliers in the 129/J strain, again and as expected no significant difference was found in the discrimination index ($p>0,05$).

Figure 16



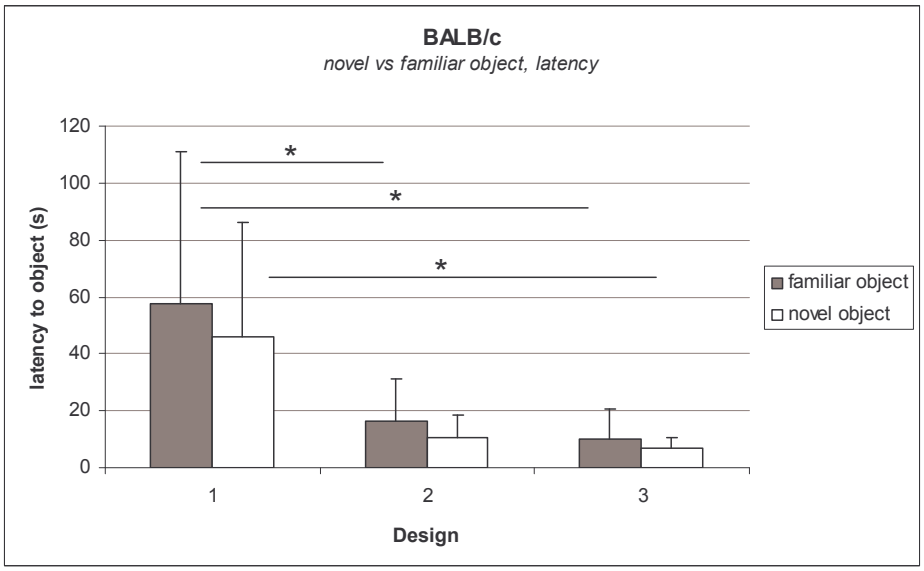
After data correction in the 129/J strain, still no significant difference between the strains was found. The previous found significant difference (*=p<0,05) between the designs in the BALB/c strain remained unaffected.

Figure 17



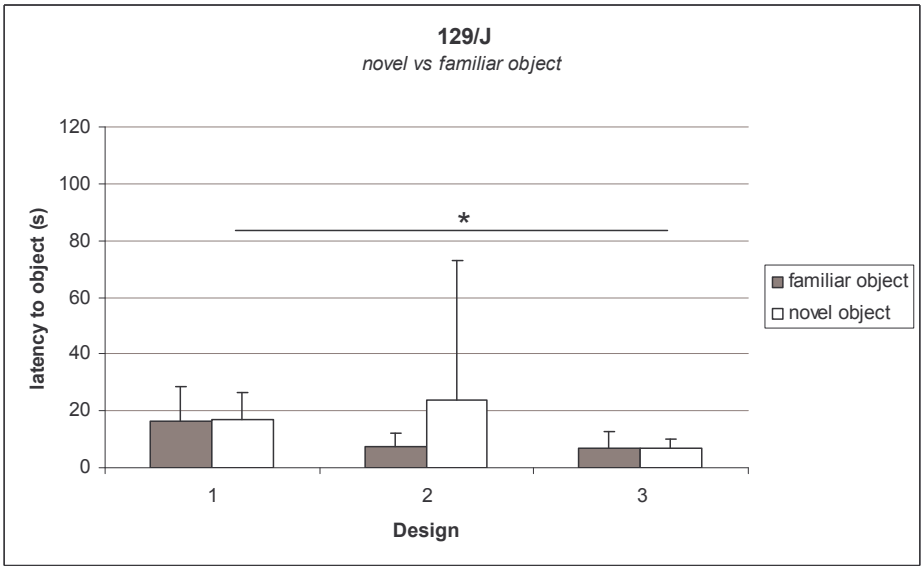
By correcting the exploration duration data, the variation in design 3 decreases, especially in the exploration duration of the familiar object. As expected still no significant difference (p>0,05) was found.

Figure 18



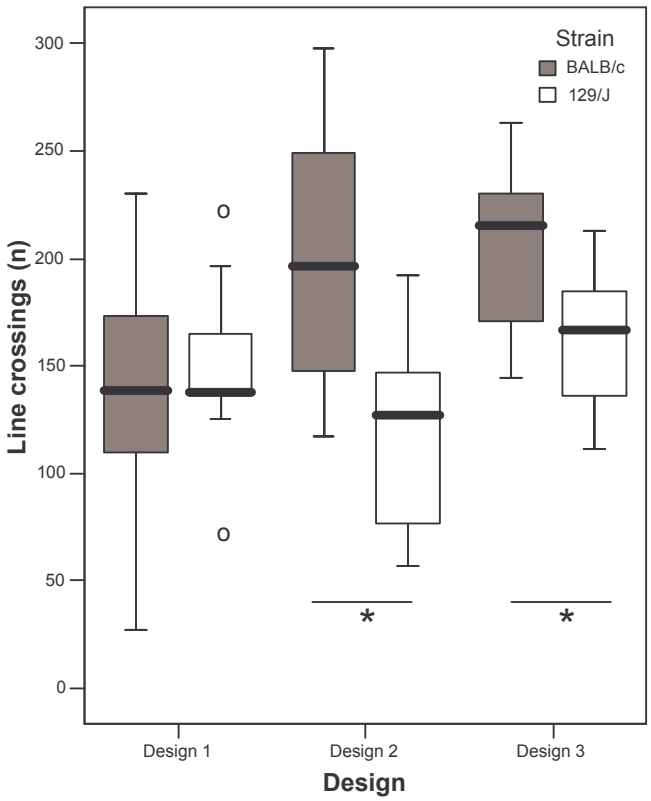
After data correction the previous found significant differences (*=p<0,05) within the BALB/c strain remained the same.

Figure 19



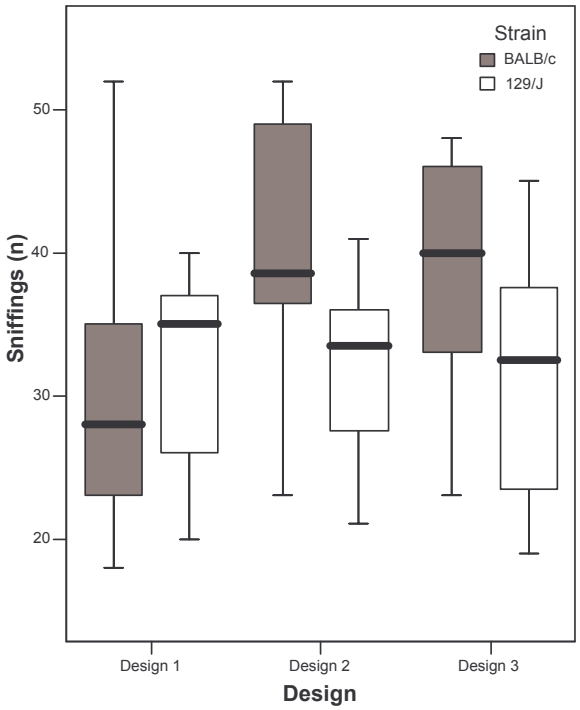
The corrected data of the 129/J strain revealed a significant difference (*=p<0,05) in the latency to the novel object between design 1 and 3.

Figure 20



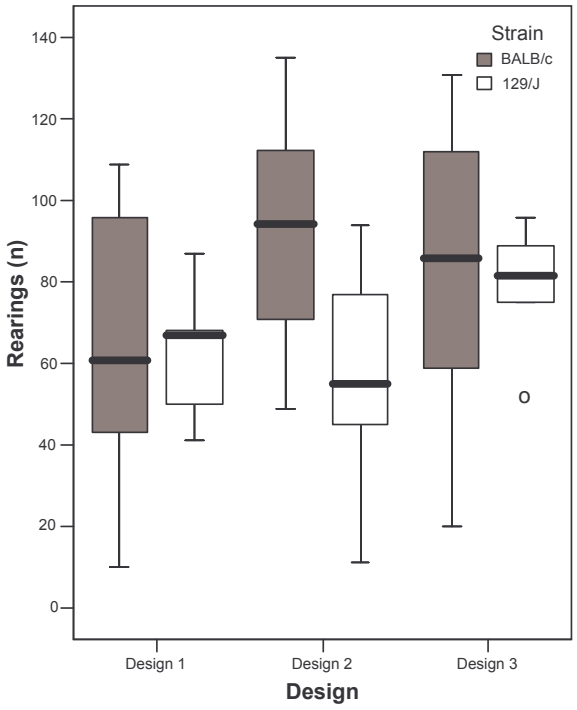
The statistical analysis over the corrected data did no longer reveal a significant difference (p>0,05) within the BALB/c strain between design 1 and 3. The significant strain difference (*=p<0,05) in design 2 and 3 remained.
SPSS detected in the corrected data two outliers in the 129/J strain in design 1 (o= value>1,5*IQR).

Figure 21



After data correction the significant difference (p>0,05) in the number of sniffings was no longer found.

Figure 22



After data correction the significant difference (p>0,05) in the number of rearings was no longer found.
The statistical analysis program detected one data outlier in design 3 of the 129/J strain (o= value>1,5*IQR)