# Behavioural Habituation to Human Presence in Guinea Pigs

## An Explorative study

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# Abstract


Training laboratory animals to voluntarily cooperate with husbandry and medical procedures can significantly reduce the level of stress these animals experience during these procedures. Habituation to humans and human handling is often a first step in training protocols. It is known in rabbits and rats that habituation to humans can reduce the fearfulness towards humans, which facilitates training. Currently, very little is known about behavioural habituation in guinea pigs. Thus, the objective of this explorative study was to see whether behavioural habituation towards human presence inside the cage could be observed in Dunkin Hartley guinea pigs.

For this aim five Dunkin Hartley guinea pigs (used for educational purposes) were habituated to the presence of a human inside their cage over the course of three weeks. Daily habituation sessions were performed from Monday through Friday, where an observer was present inside the cage for 10-20 minutes. Behaviour shown during the first ten minutes of the first habituation session of the week were scored from video material using focal animal sampling. The behaviours scored were active locomotor behaviour, grooming behaviour, animal-human interaction, ingestive behaviour, comfort behaviour, the amount of time spent inside or outside shelter and other locomotor behaviour. The percentage of total time spent in a certain behaviour and the latency to first display a behaviour were used in the statistical analyses.

The percentage of total time spent in ingestive behaviour was different between habituation sessions, but post-hoc analyses could not establish where the exact differences took place. No further significant statistical effects could be found. However, the percentage of total time spent underneath shelter and in other locomotive behaviour, as well as the latency to first leave shelter, showed a trend for a difference.

This explorative study implies that behavioural habituation to the presence of a human inside the cage can be observed in Dunkin Hartley guinea pigs. Behaviours of interest are ingestive behaviour, other locomotive behaviour, specifically freezing and startling, and the amount of time spent inside or outside shelter. More research will be needed to determine the exact changes in behaviour and a possible reduction in fearfulness towards humans.

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# Introduction

## History

### Domestication

Guinea pigs were domesticated thousands of years ago in the Andes mountain region, probably between 6000 – 3000 B.C. (*Kruska & Steffen, 2013; Pritt, 2012; Salomon & Morales, 1995; Wing, 1986*). They were not only used as food-source, but also in rituals, such as divination and curing practices (*Stahl, 2008*). This domestication process has led to some difference in behaviour between domesticated guinea pigs (*Cavia porcellus*) and their wild ancestor (*Cavia aparea*). Domesticated guinea pigs show more anxiety-like behaviour and less risk-taking behaviour, as seen by *Zipser et al., (2014)*. Furthermore, they show more nervousness (*Rood, 1972*) and less explorative behaviour, based on an open field test and an exploration apparatus (*Künzl et al., 2003; Zipser et al., 2014*). Lastly, domesticated guinea pigs also have more social interaction, such as social grooming *(Künzl et al., 2003; Rood, 1972; Zipser et al., 2014*), they are more tolerant and less aggressive towards conspecifics *(Künzl et al., 2003; Rood, 1972; Stahnke, 1987*), and they express overt sexual behaviour more frequently (*Künzl et al., 2003; Rood, 1972*) than wild guinea pigs. It was found that these behaviours do not vary in behavioural patterns, but rather in behavioural frequencies and thresholds (*Rood, 1972*).

### Laboratory

Nowadays, domesticated guinea pigs are mostly used as either pets or laboratory animals. The use of guinea pigs as laboratory animals is thought to have started in 1780, when Lavosier used them for the measurement of heat production *(Pritt, 2012; Wagner, 1976*). There was a peak usage of guinea pigs in laboratories in the US in the 1960s, with an estimated 2.5 million animals used per year (*Pritt, 2012*), mostly for tuberculosis research. Guinea pigs have not only been used for tuberculosis research, but also for studies regarding anaphylaxis, asthma, delayed hypersensitivity, genetics, gnotobiotics, immunology, infectious disease, nutrition, otology, pharmacology and even research in space (*Pritt, 2012; Shomer et al., 2015*). After the peak in the 1960s the use of guinea pigs in biomedical research steadily declined (*Pritt, 2012*). A strain often used in laboratories is the outbred, albino strain established by Dunkin and Hartley in 1926 (*Pritt, 2012; Wagner, 1976*). Over the years other strains have been developed, though many laboratory guinea pigs still derive from this one-hundred-year-old strain (*Pritt, 2012; Wagner, 1976*). Even though Dunkin Hartley guinea pigs have been around for such a long time, little is known about their behaviour and welfare in laboratories.

## Laboratory use and animal welfare

### Laboratory use

In 2017 approximately 530.000 animal experiments were carried out in the Netherlands. Of these tests 2.0% were performed using guinea pigs, with a total number of approximately 5.800 guinea pigs (*Nederlandse Voedsel- en Warenautoriteit, 2019*). The minimum requirements of the environment in which these animals are being kept is regulated by law. In the Netherlands these laws (*Wet op de Dierproeven*) are based on *Directive 2010/63/EU*, which, among other things, states the minimal legal requirements for the cages. The requirements for the measurements of guinea pig cages can be found in **Table 1**. Unfortunately, most of these requirements are not based on scientific research and it therefore remains uncertain if the welfare of the animals can be considered good when these minimal requirements are met. It is therefore important to look beyond these minimal requirements for the improvement of the welfare of laboratory animals.

Over the years more research about the effects of these minimal legal requirements on the welfare of the laboratory animals has become available. Positive welfare can be defined as a state in which "the animal has the freedom and capacity to react appropriately (i.e. adaptively) to both positive and potentially harmful (negative) stimuli" and is able "to reach a state that it perceives as positive" *(Ohl & van der Staay, 2012, pp. 17-18*). It is thus important for laboratory animals to be able to properly adapt to the environment in which they are being kept. There are, however, studies available that suggest that the minimal legal requirements do not meet the species-specific needs of laboratory animals (*Balcombe, 2006; Boers et al., 2002; Callard et al., 2000; Lawlor, 2002; Makowska & Weary, 2016; Olsson & Dahlborn, 2002; Sherwin, 2002; Würbel, 2001*). This can have a negative effect on not only their welfare, but also their development and biological functioning (*Castelhano-Carlos et al., 2017; Schumann et al., 2014; Sherwin, 2007*). This negative effect can result in behavioural changes, such as the development of stereotypies, automutilation or altered activity, changes in metabolism, which in turn may lead to a decrease in body weight, hormonal changes, such as an increase in blood cortisol levels, and lastly the immune system could be compromised (*Asres & Amha, 2014; Gut et al., 2018; Schumann et al., 2014; Sherwin, 2007*). Due to these changes it is possible that laboratory animals kept in suboptimal housing are no longer a representative study population and could thus compromise the outcomes and scientific validity of experiments (*Sherwin, 2007*). This highlights the importance of optimising the housing in which we keep our laboratory animals.

### Improving animal welfare

As suboptimal housing could negatively impact the welfare of the animals and the scientific validity of studies, some laboratory animal facilities offer housing that is more suitable to the behavioural needs of the animals. These alternative housing systems provide the animals with, for example, more space and shelter opportunities than the standard laboratory cages. A disadvantage of these housing systems is that the animals have more space to avoid being caught. Chasing the guinea pigs to catch them might actually be a source of stress, as they are prey animals, and makes it even harder to catch them if it causes them to flee in response to the 'predation' (*Baklová et al., 2016; Schmitz, 2017*). This might pose as an impact on their welfare. One method to overcome this potential source of stress might be to habituate the animals to the persons being present in the enclosure and train the animals, with training methods such as positive reinforcement training, to voluntarily enter a transport box.

Positive reinforcement training is already used for non-human primates, marine mammals and other mammals in both laboratories and zoos to improve care, management and welfare (*Leidinger et al., 2018; Westlund, 2014; Whittaker & Laule, 2012*). It has been shown that the application of this training significantly reduces the level of stress for these animals in husbandry and medical procedures (*Laule & Whittaker, 2007*). In available protocols that use this method one of the first steps of the protocol is habituation to humans and/or human handling (*Kemp et al., 2017; Leidinger et al., 2018; Prescott et al., 2005*). This is because overcoming fearfulness for humans through habituation can facilitate the training of the animals (*Bayne, 2003; Górecka et al., 2007; Leidinger et al., 2018; McKinley et al., 2003; Scott, 1991*).

***Table 1****. Minimal legal requirements for guinea pigs*

| | Body weight (g) | Minimum enclosure size (cm$^2$) | Floor area per animal (cm$^2$) | Minimum enclosure height (cm) |
|---|---|---|---|---|
| In stock and during procedures | <200<br>200 – 300<br>300 – 450<br>450 – 700<br>>700 | 1800<br>1800<br>1800<br>2500<br>2500 | 200<br>350<br>500<br>700<br>900 | 23<br>23<br>23<br>23<br>23 |
| Breeding | | 2500<br><br>Pair with litter. For each additional breeding female add 1000 cm$^2$ | | |

*Note.* Edited from Directive 2010/63/EU (2010).

# Habituation

## Overview

According to *Rankin et al. (2009)* habituation is defined as "a behavioural response decrement that results from repeated stimulation and that does not involve sensory adaptation, sensory fatigue or motor fatigue". It can be found in the entire Animalia kingdom (*Bolivar, 2009; Raderschall et al., 2011*) and it allows the animals to adapt to their environment by identifying repeated, harmless and irrelevant events and decreasing the responsiveness to these stimuli in favour of more important stimuli (*Ardiel et al., 2017; Blumstein, 2016; Raderschall et al., 2011; Rankin et al., 2009*). Furthermore, researchers working on habituation think that it is a prerequisite to be able to filter out these irrelevant stimuli for other forms of learning (*Rankin et al., 2009*).

The characteristics of habituation were described by *Thompson & Spencer (1966)* and were later revisited and revised by *Rankin et al. (2009)*. After this revision *Rankin et al. (2009)* defined the characteristics which can be found in **Appendix A**.

There are many internal and external factors that can influence habituation (*Bolivar, 2009*). For example, *Leussis & Bolivar (2006)* describes that factors influencing exploratory behaviour in rodents could be the arousal level, attention, fear of novelty or memory of the animal in question. Furthermore, it is known that genetics can also be an important factor influencing habituation (*Leussis & Bolivar, 2006*). Thus, habituation is not as simple as might be thought and can be quite variable between organisms.

## Effect of habituation

One study regarding the effect of behavioural habituation to humans on the behaviour of guinea pigs has been found. *Rocha et al. (2017)* looked at the effect of human handling, where the animals were gently removed from the home box and reallocated to a clean home box daily, and habituation to humans, where the animals were taken out of the cage and gently handled and stroked for 10 minutes twice a day during 10 days, on tonic immobility responses in guinea pigs. Tonic immobility is characterized as an anti-predator response and often displayed by guinea pigs during fearful situations. *Rocha et al. (2017)* found that handling decreased the duration of tonic immobility and that habituation not only decreased the duration of tonic immobility, but also increased the latency to show tonic immobility. Thus, they concluded, both habituation and handling could reduce fear of humans in guinea pigs.

There are also several studies available regarding rabbits and rats, which look at the effect of (neonatal) handling or human presence on fear towards humans. *Cloutier et al. (2012)* found that exposure to a passive hand, tickling (tickling the rat's nape, followed by the ventral surface, to mimic playful rough-and-tumble behaviour of rats) and restraint between the age of 57 to 74 days all caused rats to be less fearful towards humans in comparison with minimally handled rats. In rabbits studies it has been found that habituation to human contact through handling, both in the neonatal phase and in adulthood, can reduce the fearfulness towards humans (*Anderson et al., 1972; Bilkó & Altbäcker, 2000; Dúcs et al., 2009; Pongrácz et al., 2001; Swennes et al., 2011*). *Bilkó & Altbäcker (2000)* even found that the effects of neonatal handling can last for at least 5-6 months. Furthermore, *Dúcs et al. (2009)* found that it is not necessary to actually handle the animals during the neonatal period to gain this effect. The mere presence of humans is enough to induce a reduction in fearfulness to humans in rabbits.

To the author's knowledge no other studies regarding behavioural habituation in guinea pigs are available. There is, however, a myriad of studies available regarding the behavioural habituation of other rodents. One of the circumstances in which behavioural habituation has been studied in these species is a novel environment. Several behaviours have been found to reflect behavioural habituation towards a novel environment in these species. First of all, it was found that the avoidance of unprotected areas can decrease over time in mice (*Salomons et al., 2010a; Salomons et al., 2010b*). Secondly, there can be a change in activity over time in rats and mice (*Bolivar, 2009; Bolivar et al., 2000; Rojas-Carvajal et al., 2018; Salomons et al., 2010a; Salomons et al., 2010b; Terry, 1979*). This change can vary, depending on the genetic background and individual differences. Thirdly, the grooming behaviour of mice and rats has been found to increase over time (*Rojas-Carvajal et al., 2018; Salomons et al., 2010a*). Lastly, it was found in mice that an increase in exploration behaviour of the new environment can be seen (*Salomons et al., 2010a; Salomons et al., 2010b*), whereas in rats a decrease was found (*Rojas-Carvajal et al., 2018*) . Thus, in mice and rats, behavioural habituation towards a novel environment was seen as a decrease in anxiety-related behaviour over time, a change in activity, an increase in grooming behaviour and a change in explorative behaviour.

As mentioned above, there are very few studies which focus on specific behaviours linked to behavioural habituation towards humans in guinea pigs. In light of this absence, other types of studies were examined. As has been described earlier, behavioural habituation towards humans can be seen as a decrease in fearfulness towards humans. Thus, to identify other behaviours that might show behavioural habituation towards humans, one could look at studies investigating the behavioural stress response of the animals and behaviour towards predators. Studies regarding stress in guinea pigs have looked at stress in a social context, such as social conflict (*Haemisch, 1990*), social buffering (*Hennessy et al., 2008; Maken & Hennessy, 2009*), social stress (*Sachser & Lick, 1989, 1991*) and isolation (*Hennessy et al., 2004*). The behaviours investigated in these studies were changes in sociopositive behaviour, agonistic behaviour and aggression between familiar or unfamiliar conspecifics. As the behaviours studied are intraspecies social behaviours, it is very difficult to extrapolate them to interspecies social behaviours, such as behaviour towards humans. With regard to non-social behaviours, *Anthony et al. (1959)* found that Dunkin Hartley guinea pigs usually jumped at the start of noise stress and remained inactive during the noise stress. Over the course of four weeks, with daily exposure to noise stress, this did not change. *Rood (1972)* observed that *Cavia spp.* frequently yawned when in stress situations. A recent study found that guinea pigs showed a higher frequency of hiding, an increase in startling, more locomotion and explorative behaviour and less time spent eating when a human was present (*Gut et al., 2018*). All of these behaviours could show behavioural habituation when the animals are repeatedly exposed to humans.

It is highly likely that humans are a source of fear for guinea pigs (*Rocha et al., 2017*). Guinea pigs might also regard humans as a predator and could thus respond accordingly. Domestic guinea pigs can respond to predators with fleeing, vigilance or freezing (*Baklová et al., 2016; Rood, 1972*). The definition of these behaviours can be seen in **Table 2**. *Baklová et al. (2016)* found that domestic guinea pigs showed mostly vigilance in the presence of an unfamiliar human, but would also freeze. These behaviours could also show behavioural habituation when the animals are exposed to humans.

***Table 2.*** *Definitions of fleeing, freezing and vigilance, as used by Baklová et al. (2016) and Rood (1972)*

| | |
|---|---|
| Fleeing | the individual runs trying to escape from the stimulus |
| Freezing | alert posture with freezing and extended front legs and eyes directed towards the stimulus |
| Vigilance | guinea pig staying immobile in a crouched posture |

*Note.* Edited from Baklová et al. (2016) and Rood (1972)

## Purpose of the study

This study is an explorative study to see whether behavioural habituation towards human presence inside the cage could be observed in Dunkin Hartley guinea pigs. It was hypothesized that behavioural habituation towards humans would be reflected by a difference in active locomotor behaviour; an increase in grooming behaviour, animal-human interaction and ingestive behaviour; and a decrease in the time spent underneath shelter, comfort behaviour and other locomotor behaviour. To test these hypotheses, first an ethogram was developed to score the behaviours shown by the guinea pigs. Thereafter these behaviours were scored on video data obtained from habituation sessions with the guinea pigs and the change in behaviours over sessions was investigated. During these habituation sessions an observer was present inside the cage without actively interacting with the animals.

# Material & methods

## Ethical statement

This experiment is non-invasive, since only behavioural observations are done. As such it is not an animal experiment according to the European Directive 2010/63/EU.

## Subjects

Dunkin Hartley guinea pigs (strain HsdDhl:DH) from Envigo were used in this study (n=5). The females (n=3) were 2.3 years old and had an average weight of 1.10 kg (0.98 – 1.21 kg). The males (n=2) were 0.9 years old and had an average weight of 1.20 kg (1.19 – 1.21 kg). The animals were used for educational purposes at the Central Laboratory Animal Research Facility of Utrecht University (location GDL), to teach students how to correctly handle the animals. The female animals had been in the facility from the age of 8 weeks and the males had been in the facility from the age of 10 weeks. To be able to individually identify the animals they were marked. For this the Kerbl Top Marker was used in the colours Green and Blue, resulting in one of the female guinea pigs not being marked.

## Housing and husbandry

The animals were housed in a room also containing cages with rabbits and guinea pigs used for another experiment. People involved with these other experiments would occasionally enter the room to carry out tasks. The cages, in which the guinea pigs were kept, had concrete floors and walls consisting partly of wood and partly of fencing. The fencing enabled the animals in adjacent cages to not only hear and smell each other, but also see and possibly touch (nose-nose). The animals were kept in a 12:12 hour light/dark cycle (dark period from 19:30 – 07:30) at 19,4 – 20,1 °C and a relative humidity of 64±5%.

The female animals were housed together in a 193x164x300 cm (lxwxh) cage and the males were housed together in a 193x160x300 cm cage. The cages were adjacent to one another. They both had autoclaved straw as bedding and had access to two shelters inside the cage. Two types of plastic shelter were used. The large shelter (40x64x64 cm) had a light colour and small holes in the roof and had one entrance. The small shelter (25x32x116 cm) consisted of a grated top with open sides and slowly sloped upwards to a maximum height of 25 cm. The location of the shelters inside the cage would vary, as they were moved after the cages were cleaned. As further enrichment they had an autoclaved willow branch. The cages are shown in **Figure 1** and **Figure 2**, respectively.

They were fed ad libitum with guinea pig pellets (Special Diet Services, UK), autoclaved hay and water. During this experiment there were also one or two food bowls present with either dried and crushed peas or Critical Care (Oxbow, Omaha, Nebraska, USA), as the animals were being habituated to eating foods other than the hay and pellets. The days these foods were present inside the cage can be found in **Appendix B**.

In the morning (8:00-9:00) and the afternoon (15:00-16:00) the hay, pellets and water were replenished if needed, the room was swept and the light was checked. Every Wednesday the cages were cleaned, and the animals were weighed by the animal caretaker. Throughout the entire experimental period radio music was turned on as background noise.


***Figure 1.*** *Cage lay-out of the female guinea pigs.*


***Figure 2.*** Cage lay-out of the male guinea pigs.

# Study design

## Habituation

This experiment was conducted within a project that aims to improve the welfare of the guinea pigs used for educational purposes present at the Faculty of Veterinary Medicine, Utrecht University. The animals were used for practicals in which students were taught how to handle and fixate the animals. The guinea pigs were used five times for practicals during the experiment. Due to the aim of the project and the low number of animals available, this study can be considered an explorative study. During this study the rabbits in the same room, which were also used for educational purposes, were habituated at the same time as the guinea pigs.

The sequence in which the cages were habituated, the location of the observer inside the cage and the observer inside the cage were semi-randomized. All possible sequences were determined and afterwards randomized using the RAND() function in excel. If a cage had the same position in the sequence three times on a row, two rows would be swapped manually. On some occasions the sequence of the observer had to be altered, due to availability of the observers. The sequence in which the cages were habituated was randomized to avoid that some cages had more time to habituate to the observer during every session, as they could already see, smell and hear the observer as they were habituating other cages. The observer and the location of the observer inside the cage were randomized to avoid conditioning of the animals.

The experiment took place over a 6-week period and was divided in two stages. Stage 1 was the preparatory phase and stage 2 was the habituation phase.

## Stage 1 – Preparatory phase

These two weeks were used to prepare and set-up the equipment for the experiment and to establish a protocol. This meant that over the course of these two weeks a researcher would occasionally be present in the room.

To establish a protocol a literature review was done to find previously used habituation protocols. There were only a few available protocols for habituation of rats and rabbits to human contact (and handling) and, to the author's knowledge, only one for guinea pigs. The article involving guinea pigs used habituation sessions of 10 minutes, twice a day for 10 days (*Rocha et al., 2017*). One article involving rats used 2 weeks of daily, short habituation sessions and another involving rabbits consisted of a 4-week period with 6 short habituation sessions a week and afterwards one session every other day (*Leidinger et al., 2018; Verwer et al., 2009*). Based on this information, it was anticipated that the habituation would take approximately 2 to 4 weeks and that the protocol would encompass one habituation session a day for 5 consecutive days a week (Mon – Fri). The observers would determine weekly whether the habituation had to be continued. This was done because it is not possible to predetermine how long the animals need to habituate, as habituation can be influenced by many different factors, such as genetics, and can vary between individuals (*Boleij et al. 2012; Bolivar, 2009; Wirz et al., 2015*).

To determine the most suitable time of day for the habituation a literature review was done to find the activity period(s) of guinea pigs. From studies in wild guinea pigs it is known that they are mostly active during dusk and dawn and thus are crepuscular (*King, 1956; Rood, 1972*). However, laboratory studies have indicated that guinea pigs kept indoors in conditions of relatively uniform temperatures, humidity and light have no decisive diurnal or nocturnal period of activity, but rather show a pattern of intermittent activity and rest throughout the day (*Nicholls, 1922; Pellet & Béraud, 1967*). As such there was no specific period of activity in which habituation should be conducted and habituation would take place either during the morning or during the afternoon. The time of day was alternated between habituation sessions to avoid conditioning of the animals.

To decide the duration of the habituation sessions two observers sat outside the cages at the end of week 2 and timed how long it took for all the guinea pigs to come out from underneath the shelter. Within 10 minutes all the animals had moved about and left shelter. This time was used in the protocol as the minimum time the observer would be inside the cage during a habituation session. During the first week of habituation this time was doubled to give the animals more time to start moving about and to prevent the animals from learning that if they remained still the observer would leave.

The final protocol used was the following: the predetermined observer would enter the cages in the predetermined order and sit down on the predetermined location inside the cage. The observer would remain in this location for 10-20 minutes without actively interacting with the animals, which meant no eye contact or approaches with for example hands. During the session the observer would write down striking behaviours or what happened outside of the view of the camera (e.g. the shelters). When the 10-20 minutes were over the observer would slowly get up, as to not scare the animals, and immediately leave the cage. Every habituation session was recorded, see 'Video recording'.

### Stage 2 – Habituation phase

The schedule used for the habituation sessions can be seen in **Table 3**. As can be seen the rotation between observers started in week 5. This was done to ensure that the animals did not just habituate to one person being present in the cages. The rabbits present in the same room as the guinea pigs were also observed and habituated, but the data is not presented in this study.

## Video recording

Two *Bascom®* cameras had a top view of the cages. One Tracer® camera was used to record the behaviour of the male guinea pigs underneath the large shelter. The cameras were turned on when the observer entered the room and kept recording until all four cages (2 guinea pig cages and 2 rabbit cages) had their habituation session according to the predetermined order. The upper camera of the male guinea pigs had to be alternated with a camera for the rabbit. This meant that the habituation session of the male guinea pigs was recorded, but the behaviour during part of the habituation of other cages was not recorded. The cameras were turned off after the last cage had their habituation session.

## Behavioural scoring

First an ethogram was developed and based on several existing ethograms and our own observations (*Gut et al., 2018; Peter & Kunkel, 1963; Rood, 1972*). The ethogram was divided into several behavioural groups, which were used to score the behaviours. The detailed ethogram as developed can be found in **Appendix C**. The groups that were used to score the behaviour were the underlined behaviours below:

1. Individual behaviour
    a. <u>Ingestive behaviour</u>
    b. <u>Elimination</u>
    c. <u>Marking</u>
    d. <u>Active locomotor activity</u>
    e. <u>Inactive locomotor activity</u>
    f. <u>Other locomotor activity</u>
    g. <u>Comfort behaviour</u>
    h. <u>Grooming</u>
2. Animal-Environment Interaction
    a. <u>Explorative behaviour</u>
3. Social behaviour
    a. <u>Sociopositive behaviour</u>
    b. <u>Socionegative defensive behaviour</u>
    c. <u>Socionegative offensive behaviour</u>
4. Animal-Human Interaction
    a. <u>Explorative behaviour</u>

The habituation sessions were scored through video coding using Noldus Observer XT 12, using continuous recording and focal sampling (*Altmann, 1974*). The first 10 minutes of every first habituation session of the week were scored by a trained observer. The intra-observer reliability ranged between 0.91-1.0 as measured by Pearson's correlation coefficient.

## Outcomes

To explore which behaviours might be used as an indication of habituation, the following behaviours were used as outcomes in this study:

- Percentage of total time spent inside the shelter and the latency until the first exit from the shelter if animals started the habituation session inside the shelter.
- Percentage of total time spent in active locomotor behaviour or inactive locomotor behaviour, including the latency until the first active locomotor activity.
- Percentage of total time spent in other locomotive activity and the latency until the first other locomotive behaviour.
- Percentage of total time spent grooming and the latency until the first grooming behaviour.
- Percentage of total time spent in comfort behaviours shown and the latency until the first comfort behaviour.
- Percentage of total time spent with human exploration and the latency until the first human exploration.

## Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, version 25.0.0.1. If a behaviour was not displayed during the first 10 minutes of the habituation session, the latency was set at 600 seconds (total time scored). The data was first investigated for gaussianity using the Kolmogorov-Smirnov test. All data that showed a non-parametric distribution were log transformed. If, after log transformation, the data revealed a parametric distribution, a Friedman's ANOVA was used on the log transformed data. As post-hoc tests the Wilcoxon signed-rank test with Bonferroni correction was used. If the data was non-parametric, a One-Way Repeated Measures ANOVA was used on the non-log transformed data. Before running this ANOVA, it was investigated whether the data met the assumption of spherity using Mauchly's test. *P*-values of ≤ 0.05 were considered statistically significant and *P*-values between 0.1 – 0.05 were considered a trend.

***Table 3.*** *The schedule used for the habituation sessions.*

| | **Cage sequence** | **Observer** | **Location of observer** | **Time of day** | **Time in cage (min)** |
|---|---|---|---|---|---|
| *Week 3* | | | | | |
| Monday | KL-CV-KR-CM | 1 | L | Morning | 20 |
| Tuesday | CV-KR-KL-CM | 1 | R | Afternoon | 20 |
| Wednesday | KL-CM-KR-CV | 1 | M | Morning | 20 |
| Thursday | CV-CM-KR-KL | 1 | L | Afternoon | 20 |
| Friday | KR-CV-CM-KL | 1 | R | Morning | 20 |
| *Week 4* | | | | | |
| Monday | KR-KL-CM-CV | 1 | R | Morning | 10 |
| Tuesday | KL-KR-CV-CM | 1 | M | Afternoon | 10 |
| Wednesday | KL-CV-CM-KR | 1 | L | Morning | 10 |
| Thursday | CV-CM-KR-KL | 1 | M | Afternoon | 10 |
| Friday | CV-KL-CM-KR | 1 | L | Morning | 10 |
| *Week 5* | | | | | |
| Monday | CM-KR-CV-KL | 1 | R | Morning | 10 |
| Tuesday | CM-KL-CV-KR | 2 | L | Afternoon | 10 |
| Wednesday | KL-KR-CM-CV | 3 | R | Morning | 10 |
| Thursday | CV-KL-KR-CM | 4 | M | Afternoon | 10 |
| Friday | KR-KL-CV-CM | 1 | M | Morning | 10 |
| *Week 6* | | | | | |
| Tuesday | KL-CM-CV-KR | 1+3 | R | Morning | 10 |

*Note.* CV = female guinea pigs; CM = male guinea pigs; KL = rabbit cage 1; KR = rabbit cage 2; L = front left; R = front right; M = centre front.

# Results

## Descriptive statistics

In **Figure 8-14**, which can be found in **Appendix D**, an overview of the data of all four habituation sessions per animal is shown. The behaviour 'Human Interaction' occurred too rarely to compare between sessions (n=2) and will therefore not be described.

## Gaussianity

The outcomes of the Kolmogorov-Smirnov test and the Q-Q plots indicated that none of the behavioural categories scored in all four of the habituation sessions were parametric. After log transformation only the latency and percentage of total time for active locomotor behaviour and the percentage of total time for grooming were parametric. The other behaviours were found to be non-parametric.

## Behaviours

An overview of the outcomes from the One-way repeated measures ANOVA's and the Friedman's ANOVA's are shown in **Table 4**.

### Active locomotor behaviour

Mauchly's test indicated that the assumption of sphericity was true for the latency of active locomotor behaviour, $\chi^2(5) = 5.79$, $p = 0.354$. The results show that there was no significant difference between habituation sessions in the latency for active locomotor behaviour, $F(3, 12) = 1.98$, $p = 0{,}171$. Mauchly's test indicated that the assumption of sphericity had been violated for the percentage of total time spent on active locomotor behaviour, $\chi^2(5) = 14.03$, $p = 0.022$, therefore degrees of freedom were corrected using Huynh-Feldt estimates of sphericity ($\varepsilon = .41$). The results show that there was no significant difference between habituation sessions in the percentage of total time spent on active locomotor behaviour, $F(1.24, 4.96) = 3.58$, $p = 0{,}116$.

### Comfort

The latency for comfort behaviour did not significantly change between the habituation sessions, $\chi^2(3) = 4.90$, $p = 0.179$. The percentage of total time spent on comfort behaviour did not significantly change between the habituation sessions, $\chi^2(3) = 3.88$, $p = 0.275$. Comfort behaviour was not often observed. On average it was scored 0,75 times per guinea pig per habituation session, with a mean duration of 0,56 seconds.

## Grooming

The latency for grooming did not significantly change between the habituation sessions, $\chi^2(3) = 1.50$, $p = 0.682$. Mauchly's test indicated that the assumption of sphericity had been violated for the percentage of total time spent grooming, $\chi^2(5) = 22.11$, $p = 0.001$, therefore degrees of freedom were corrected using Huynh-Feldt estimates of sphericity ($\varepsilon$ = .53). The results show that there was no significant difference between habituation sessions in the latency for grooming, $F(1.58, 5.05) = 1.72$, $p$ = 0,249.

## Inactive locomotor behaviour

The latency for inactive locomotor behaviour did not significantly change between the habituation sessions, $\chi^2(3) = 4.09$, $p = 0.252$. The percentage of total time spent in inactive locomotor behaviour did not significantly change between the habituation sessions, $\chi^2(3) = 5.40$, $p = 0.145$.

## Ingestive behaviour

The latency for ingestive behaviour did not significantly change between the habituation sessions, $\chi^2(3) = 3.59$, $p = 0.310$. The percentage of total time spent in ingestive behaviour did significantly increase over the habituation sessions (see **Figure 3**), $\chi^2(3) = 9.44$, $p = 0.024$. Wilcoxon tests were used to follow up this finding. A Bonferroni correction was applied and so all effects are reported at a 0.0083 level of significance. It appeared that the percentage of total time spent in ingestive behaviour did not significantly change between session 1 and session 2, $T = 2$, $p = 0.593$; session 1 and session 3, $T = 3$, $p = 1.000$; session 1 and session 4, $T = 0$, $p = 0.043$; session 2 and session 3, $T = 0$, $p = 0.180$; session 2 and session 4, $T = 0$, $p = 0.043$; session 3 and session 4, $T = 1$, $p = 0.080$.

## No shelter

The latency for the guinea pigs to leave the shelter ('no shelter') did not significantly change between the habituation sessions, $\chi^2(3) = 6.59$, $p = 0.086$. The percentage of total time spent in no shelter did not significantly change between the habituation sessions, $\chi^2(3) = 6.57$, $p = 0.087$. Both the latency and percentage of total time for no shelter show a trend for a difference between the habituation sessions, which can be seen in **Figure 4** and **Figure 5**.

## Other locomotor behaviour

The latency for other locomotor behaviour did not significantly change between the habituation sessions, $\chi^2(3) = 2.58$, $p = 0.461$. The percentage of total time spent in other locomotor behaviour showed a trend for a difference between the habituation sessions, $\chi^2(3) = 6.35$, $p = 0.096$, see **Figure 6**.

## Shelter

The latency for the guinea pigs to enter the shelter ('shelter') did not significantly change between the habituation sessions, $\chi^2(3) = 5.40$, $p = 0.145$. The percentage of total time spent underneath shelter showed a trend for a difference between the habituation sessions, $\chi^2(3) = 6.57$, $p = 0.087$, see **Figure 7**.

***Table 4.*** *An overview of the outcomes from the One-way repeated ANOVA's and the Friedman's ANOVA's for all the behavioural categories.*

| **Behaviour** | | **F value** | **$\chi^2$ value** | **df** | **p value** |
|---|---|---|---|---|---|
| Active locomotor behaviour | Latency | 1.98 | | 3, 12 | 0.171 |
| | Percentage of all time | 3.58 | | 1.24, 4.96 | 0.116 |
| Comfort behaviour | Latency | | 4.90 | 3 | 0.179 |
| | Percentage of all time | | 3.88 | 3 | 0.275 |
| Grooming | Latency | | 1.50 | 3 | 0.682 |
| | Percentage of all time | 1.72 | | 1.58, 5.05 | 0.249 |
| Ingestive behaviour | Latency | | 3.59 | 3 | 0.310 |
| | Percentage of all time | | 9.44 | 3 | 0.024 |
| Inactive locomotor behaviour | Latency | | 4.09 | 3 | 0.252 |
| | Percentage of all time | | 5.40 | 3 | 0.145 |
| No shelter | Latency | | 6.59 | 3 | 0.086 |
| | Percentage of all time | | 6.57 | 3 | 0.087 |
| Other locomotor behaviour | Latency | | 2.58 | 3 | 0.461 |
| | Percentage of all time | | 6.35 | 3 | 0.096 |
| Shelter | Latency | | 5.40 | 3 | 0.145 |
| | Percentage of all time | | 6.57 | 3 | 0.087 |

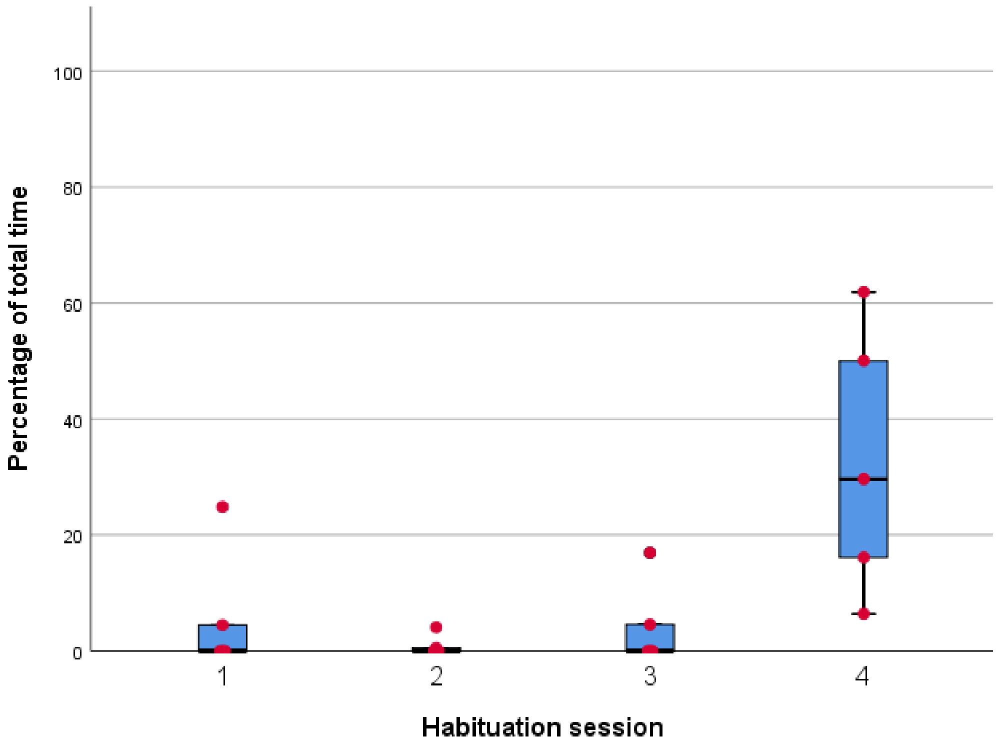


***Figure 3***. *Changes in the latency and percentage of total time for the behaviour 'Ingestive behaviour' per guinea pig across the four habituation sessions.*

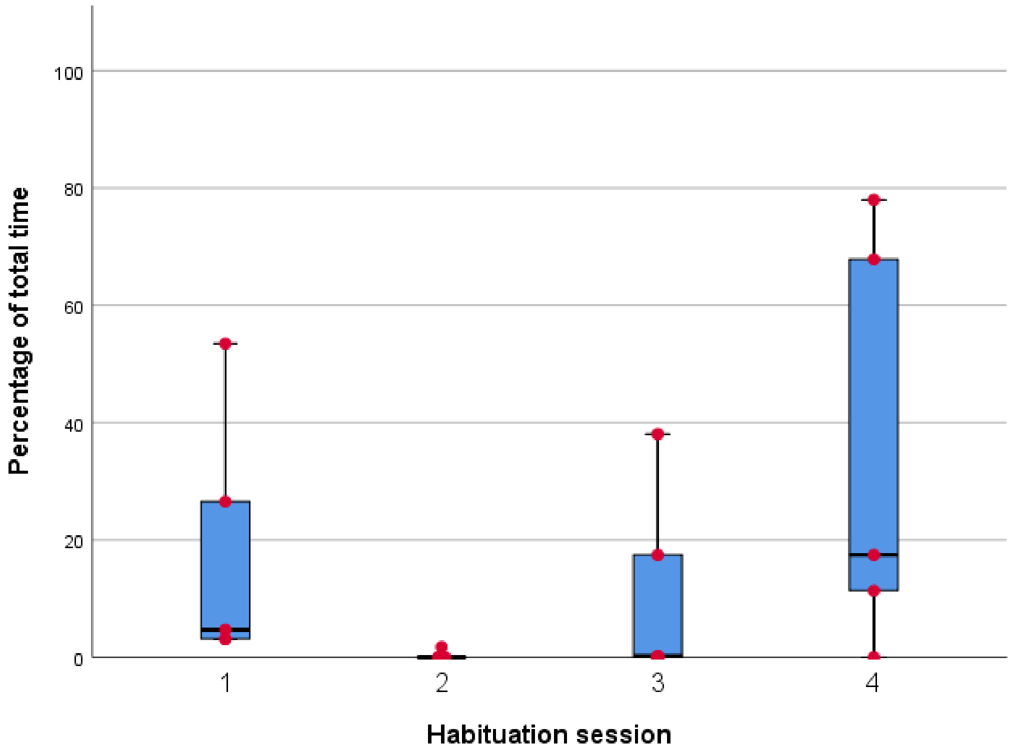


***Figure 4***. *Changes in the percentage of total time for the behaviour 'No shelter' per guinea pig across the four habituation sessions.*

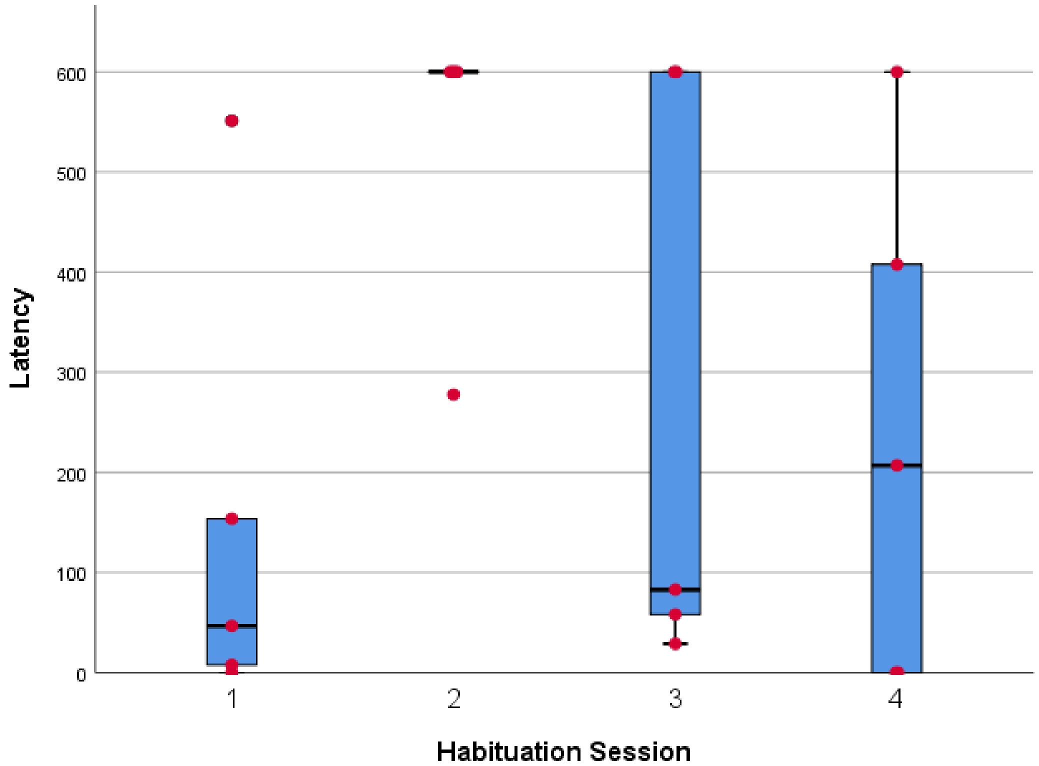


***Figure 5**. Changes in the latency for the behaviour 'No shelter' per guinea pig across the four habituation sessions.*

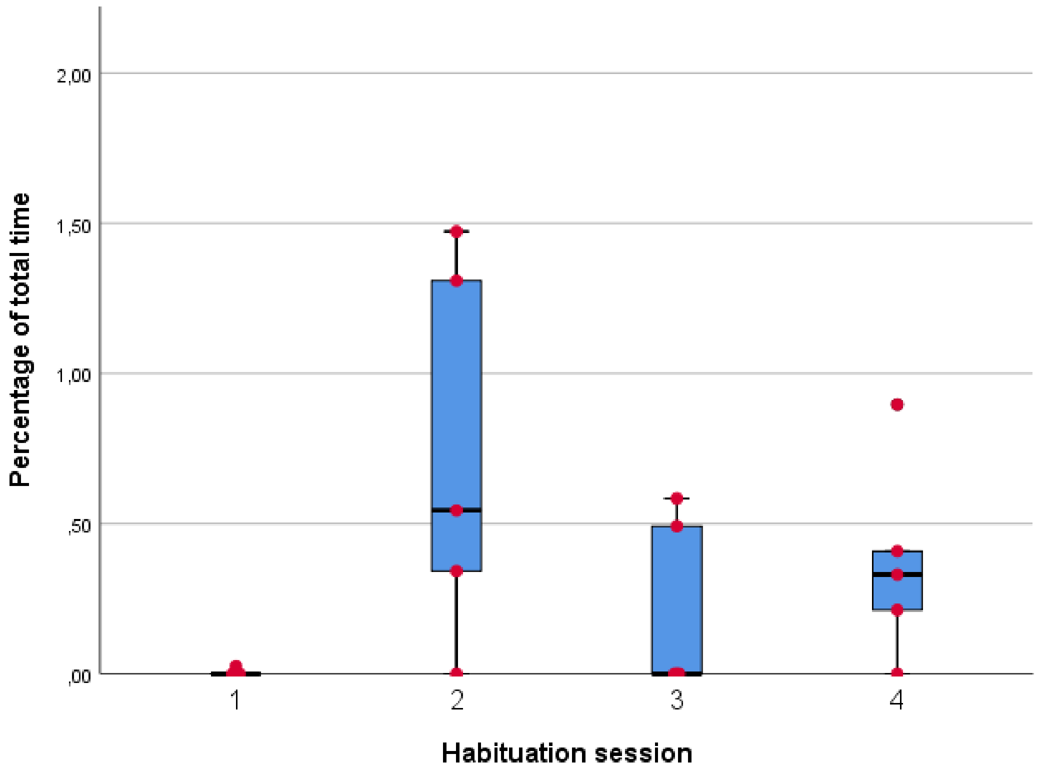


***Figure 6**. Changes in the percentage of total time for the behaviour 'Other locomotor behaviour' per guinea pig across the four habituation sessions.*

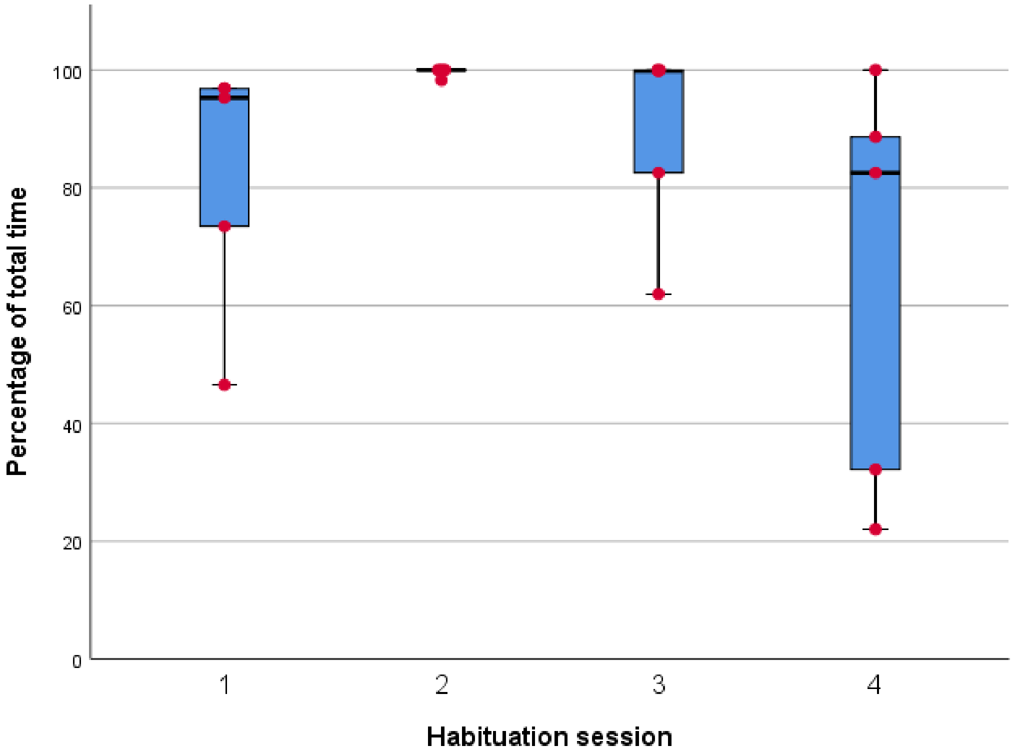


***Figure 7***. *Changes in the percentage of total time for the behaviour 'Other locomotor behaviour' per guinea pig across the four habituation sessions.*

# Discussion

## Results

This explorative study was conducted to see whether behavioural habituation towards human presence inside the cage could be observed in Dunkin Hartley guinea pigs. The Dunkin Hartley guinea pigs used in this study showed a significant increase in the percentage of total time spent in ingestive behaviour over the habituation sessions. Furthermore, they showed trends for differences between habituation sessions for the percentage of total time spent inside and outside shelter, the latency to leave shelter for the first time and the percentage of total time spent in other locomotive behaviour. This implies that behavioural habituation to human presence inside the cage can be observed in Dunkin Hartley guinea pigs.

Although a significant statistical effect in percentage of total time spent in ingestive behaviour was found, post-hoc analyses could not establish where the exact differences took place. **Figure 3** implies that most of the ingestive behaviour occurred during habituation session 4. One must keep in mind, however, that this habituation session took place on a different day and time compared to the other habituation sessions. So, it may be that the guinea pigs were observed during a different time in their routine, which could have caused the behavioural differences observed between the habituation sessions. Nonetheless, when the notes of the observers were checked, no indication could be found that the guinea pigs used to eat more during the morning habituation sessions than during the afternoon habituation sessions. Although this is subjective, the different time of day is not expected to have caused the behavioural habituation observed (*Nicholls, 1922; Pellet & Béraud, 1967*).

It is possible that inhibition of food intake is a behavioural stress response of the guinea pig, as it has been observed that mice and rats respond to acute stressors, such as restraint (*Calvez et al., 2011; Harris et al., 2002a; Harris et al. 2002b; Jeong et al., 2013; Rybkin et al., 1997; Tabarin et al., 2007*), forced swimming (*Diane et al., 2008*), social defeat (*Bhatnagar et al., 2006*) and immobilisation (*Ricart-Jané et al., 2002*), with a decrease in food intake. In a previous study, involving animal-assisted therapy, it was found that guinea pigs would show an increase in the amount of time spent not eating while a human was present, compared to the control setting without a human present (*Gut et al., 2018*). Thus, the significant increase in ingestive behaviour in the guinea pigs that was observed in this study could possibly be a reflection of the reduced stress response towards human presence.

Apart from ingestive behaviour, no significant statistical effects could be found. There were, however, some behaviours that showed a trend. One of these behaviours was the amount of time spent underneath shelter. The guinea pigs seemed to seek more shelter during habituation sessions 2 and 3, as compared to habituation session 1, while seemingly going out of shelter more often during habituation session 4. This indicates a positive trend in the amount of time spent outside shelter. There also seemed to be a difference between habituation sessions in the latency to exit shelter. Especially during habituation session 2 the latency increased considerably. Both the increase in the amount of time spent underneath the shelter during habituation session 2 and 3, and the considerable increase in the latency to first leave shelter during habituation session 2, are rather unexpected. It appears that, possibly, a confounding effect had occurred during or before habituation session 2 and 3, which influenced the behavioural response of the guinea pigs. However, it is currently unknown what this might have been. The trends for differences found in the time spent underneath or outside shelter and the latency to first leave shelter imply habituation of anxiety-like behaviour in guinea pigs.

In mice it was found that both the avoidance of unprotected areas in a novel environment and the latency to first enter the unprotected area would decrease over time (*Salomons et al., 2010a; Salomons et al., 2010b*). This avoidance of unprotected areas is seen as an indication of anxiety (*Bailey & Crawley, 2009; Lezak et al., 2017; Salomons et al., 2010a; Salomons et al., 2010b*). In rabbits it was found that animals that had not been habituated to humans would maintain a relatively stable latency to first leave shelter over time (*Anderson et al., 1972*). Furthermore *Gut et al. (2018)* found that guinea pigs would show a higher frequency of hiding when a human was present during animal-assisted therapy, as compared to the control setting without a human present. The overall positive trend in the amount of time spent outside shelter seems to be as is expected from research done with mice. The increase in the latency to first leave shelter, on the other hand, is unexpected. Whether the trends truly indicate an increase or decrease in the amount of time spent inside or outside shelter or the latency to first leave shelter has yet to be determined with more research.

For future research it is recommended to use a more standardised set-up. It would be wise not to use the animals for any other experiment or purpose that requires contact with humans while they are still in the habituation experiment. During this study the animals were also used for educational purposes, which resulted in some habituation sessions having to take place at different times than planned. Furthermore, it is possible that, if the animals had a practical before the habituation session, this influenced their behavioural response during the habituation session. So, minimizing contact between humans and the animals during the experiment, apart from the habituation sessions, is advised.

Another behaviour showing a trend is the percentage of total time spend in other locomotive behaviour. Overall, there was a positive trend. During habituation session 2 the behaviour shown was rather unexpected, as was seen for the behaviours 'shelter' and 'no shelter'. This further indicates that, possibly, a confounding effect had occurred during or before this habituation session, since, compared to other habituation sessions, a considerable increase in other locomotive behaviour was seen during this habituation session. In the ethogram used in this study the behavioural group 'other locomotive behaviour' consisted of freezing, startling and jumping. However, jumping was never observed during the study. So, the seen trend reflects the changes in the behaviours freezing and startling. One would expect the amount of time spent in other locomotive behaviour to decrease over time, not increase, as freezing and startling are behaviours shown during an aroused state.

*Gut et al. (2018)* found that guinea pigs would show more startling when a human was present during animal-assisted therapy, compared to the control setting without a human present. Furthermore, *Baklová et al. (2016)*, which studied anti-predator behaviour in domestic guinea pigs, saw that domestic guinea pigs would mostly show an increase in vigilance in response to the presence of an unfamiliar human, but also showed freezing. Hence, the trend for difference in time spent in other locomotive behaviour could indicate habituation of anti-predator behaviour in guinea pigs. Whether the trend truly indicates an increase in the amount of time spent in other locomotive behaviour, has yet to be determined with more research.

No significant differences or trends for differences were found for other behaviours, in contrast to what was expected based on research done with rabbits and rodents. However, the behavioural response of Dunkin Hartley guinea pigs may vary greatly from these other species, as very little is known about their behaviour. For this reason, further research into the behaviour of Dunkin Hartley guinea pigs is necessary.

For future research the use of naïve animals, such as animals that just came from the breeder, should be considered. The animals used in this study had already been at the facility for at least several months and had also been used for numerous practicals. So, it is likely that they were already less frightened of humans at the beginning of the study. It could be that the presence of a human inside the cage, without active interaction or movement, was not perceived as stressful or dangerous enough by these guinea pigs to elicit clear behavioural habituation. With naïve animals a more evident behavioural response might be observed. If this is not the case, it could be that a different, stronger trigger is needed. A possibility would then be to take the animals out of the cage and gently handle and stroke them for 10 minutes twice a day, as was done by *Rocha et al. (2017)* with guinea pigs. Or they could be taken out of the cage and be placed on a handler's lap for 5 minutes while they are being stroked every other day, as was done by *Verwer et al. (2009)* with rabbits.

Additionally, an extra parameter could be added to further explore animal-human interaction. In this study animal-human explorative behaviour was not seen often enough to be used in the statistical analysis. However, notes of the observers indicate that they did seem to think the animals would interact considerably more with them over time. Although the notes of the observers are subjective, they do imply that there is a possibility the method used was not suitable to measure animal-human interaction. Apart from direct contact between animals and humans, one could also look at the amount of time spent in close proximity to one another. It has been observed in rabbits and rats that animals that were handled by humans would spend more time in close proximity to humans than non-handled animals (*Anderson et al., 1972; Cloutier et al., 2012*). Thus, the amount of time spent in close proximity to humans, possibly measured using a grid of tape on the floor, could be used as an extra parameter for animal-human interaction.

Lastly, it is advised to perform a more detailed analysis of the different behaviours. This means using the individual behaviours, rather than the behavioural groups, for the analysis.

## Limitations

One of the limitations of this explorative study is the small sample size, which causes the study to have a low power. Additionally, habituation can be influenced by many different factors, such as genetic background, epigenetic factors and environmental factors (*Bolivar, 2009; Crabbe et al, 1999; van der Staay et al., 2010; Leussis & Bolivar, 2006; Salomons et al., 2010a; Salomons et al., 2010b*). It is therefore difficult to extrapolate the outcomes found in this study to other guinea pigs. Although the results cannot easily be extrapolated, they do indicate that it might be possible to observe behavioural habituation to humans in Dunkin Hartley guinea pigs. For future research it is advised to use a larger sample size to increase the power of the study.

Another limitation is that the study was not blinded, as the observer scoring the video data did this in a non-randomised order and was also the main observer during the habituation sessions. Furthermore, the study could only be semi-randomised due to, for instance, schedule conflicts of the observers. This could have caused observer bias.

Additionally, it is possible that marking the animals could have influenced their behaviour and thus the results. It has been found that marking Harris sparrows with dye (*Rohwer, 1977*) and Zebra finches with coloured plastic leg bands (*Burley et al., 1982*), can alter the way they interact with one another. In rats it was found that tail-marking might influence their individual behaviour and their behaviour towards humans (*Burn et al., 2008*). Findings of this study also indicated that rats avoid the odour of some types of marker pen ink. To the author's knowledge, no studies regarding the effect of marking guinea pigs with dye are available. However, as guinea pigs have colour vision (*Jacobs & Deegan, 1994*) and seem to have a good sense of smell (*Niimura et al., 2014; Ribeiro et al., 2014*), it is possible that marking them with dye might have a behavioural effect. So, we cannot be certain that the behaviour they displayed during this study is their 'normal' behaviour in these situations.

Lastly, the camera set-up made it difficult to properly see all the behaviour displayed by the guinea pigs. They could hide inside shelters, underneath heaps of hay or behind other obstacles. Because of this, 'Not Visible' was scored quite often and it was difficult to distinguish between freezing and inactivity. Distinguishing between these two behaviours is important, as they indicate different motivational systems. Freezing is often seen in response to an alarm call of a conspecific or when the animal is alarmed due to the presence of a possible predator, and is thus characterised as an anti-predator behaviour (*Baklová et al., 2016; Rood, 1972*). It indicated that the animal is in an aroused state. Inactivity, on the other hand, was scored when the animal was resting and in a calm state. Hence, it is possible that the behaviours observed in this study do not give a true representation of the actual behaviour displayed. For future research a different camera set-up, where it is possible to always see the guinea pigs, is advised. To achieve this, several side views instead of a top view could be used. In addition, it is important to have a full view of what happens underneath the shelters. A test area with less obstacles, such as heaps of hay, might be needed to accomplish this. The guinea pigs would first need to be habituated to this area, as to prevent behavioural habituation in response to a new environment during the experiment. An overview of all the recommendations for future research can be found in **Box 1**.

| Recommendations for future research |
|---|
| • Use a different camera set-up, with a side view and a view underneath the shelters<br>• Use attest area with fewer obstacles<br>• Use naïve animals<br>• Possibly use a different, stronger trigger<br>• Use a more detailed analysis of individual behaviours<br>• Add the amount of time spent in close proximity to a human, measured using a grid of tape on the floor, as an extra parameter for human-animal interaction<br>• Use a more standardised experiment |

***Box 1****. An overview of all the recommendations for future research.*

## Advice for facilities housing laboratory guinea pigs

As mentioned in the introduction, training the animals to voluntarily cooperate with husbandry and medical procedures with positive reinforcement training can significantly reduce the level stress of these animals during these procedures (*Laule & Whittaker, 2007*). Doing this can improve the welfare of the laboratory guinea pigs and can be seen as a form of refinement. Habituation to humans and human handling plays an important role in the training process. This is because overcoming fearfulness for humans through habituation can facilitate training the animals (*Bayne, 2003; Górecka et al., 2007; Leidinger et al., 2018; McKinley et al, 2003; Scott, 1991*).

Currently, little is known about behavioural habituation in guinea pigs. After more extensive study of this subject, the development of a habituation protocol for guinea pigs could be started. When developing this protocol, one could look at the protocol used by *Rocha et al. (2017)*, or protocols used for rabbits, such as *Dúcs et al. (2009)* and *Verwer et al. (2009)*, as guidelines. Furthermore, it might be important to think about when to start with the habituation of the guinea pigs when developing this protocol. In rabbits it has been found that the first week postpartum is the sensitive period for habituation to humans (*Bilkó & Altbäcker, 2000*). If the rabbits were habituated to humans during that first week, they would later on show a lack of fear towards humans. Such a sensitive period for the habituation to humans and human handling has not yet been found in guinea pigs, nor has a socialisation period been found. So, for developing a good habituation protocol for guinea pigs, it is important to investigate whether such a sensitive period exists in guinea pigs. Lastly, a developed protocol will need to be tested on guinea pigs, to see whether it gives the desired outcome.

# Conclusion

This explorative study implies that behavioural habituation to the presence of a human inside the cage can be observed in Dunkin Hartley guinea pigs used for educational purposes. Behaviours of interest for further investigation are ingestive behaviour, other locomotive behaviour, specifically freezing and startling, and the amount of time spent inside or outside shelter. More research will be needed to determine the exact changes in behaviour and a possible reduction in fearfulness towards humans.

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# Appendix A – The 10 Characteristics of Habituation

| | |
|---|---|
| 1. | "Repeated application of a stimulus results in a progressive decrease in some parameter of a response to an asymptotic level. This change may include decreases in frequency and/or magnitude of the response. In many cases, the decrement is exponential, but it may also be linear; in addition, a response may show facilitation prior to decrementing because of (or presumably derived from) a simultaneous process of sensitization." |
| 2. | "If the stimulus is withheld after response decrement, the response recovers at least partially over the observation time ("spontaneous recovery")." |
| 3. | "After multiple series of stimulus repetitions and spontaneous recoveries, the response decrement becomes successively more rapid and/or more pronounced (this phenomenon can be called potentiation of habituation)." |
| 4. | "Other things being equal, more frequent stimulation results in more rapid and/or more pronounced response decrement, and more rapid spontaneous recovery (if the decrement has reached asymptotic levels)." |
| 5. | "Within a stimulus modality, the less intense the stimulus, the more rapid and/or more pronounced the behavioral response decrement. Very intense stimuli may yield no significant observable response decrement." |
| 6. | "The effects of repeated stimulation may continue to accumulate even after the response has reached an asymptotic level (which may or may not be zero, or no response). This effect of stimulation beyond asymptotic levels can alter subsequent behavior, for example, by delaying the onset of spontaneous recovery." |
| 7. | "Within the same stimulus modality, the response decrement shows some stimulus specificity. To test for stimulus specificity/stimulus generalization, a second, novel stimulus is presented and a comparison is made between the changes in the responses to the habituated stimulus and the novel stimulus. In many paradigms (e.g. developmental studies of language acquisition) this test has been improperly termed a dishabituation test rather than a stimulus generalization test, its proper name." |
| 8. | "Presentation of a different stimulus results in an increase of the decremented response to the original stimulus. This phenomenon is termed "dishabituation." It is important to note that the proper test for dishabituation is an increase in response to the original stimulus and not an increase in response to the dishabituating stimulus (see point #7 above). Indeed, the dishabituating stimulus by itself need not even trigger the response on its own." |

| | |
|---|---|
| 9. | "Upon repeated application of the dishabituating stimulus, the amount of dishabituation produced decreases (this phenomenon can be called habituation of dishabituation)." |
| 10. | "Some stimulus repetition protocols may result in properties of the response decrement (e.g. more rapid rehabituation than baseline, smaller initial responses than baseline, smaller mean responses than baseline, less frequent responses than baseline) that last hours, days or weeks. This persistence of aspects of habituation is termed long-term habituation." |

*Note.* Edited from *Rankin et al. (2009)*

# Appendix B – Availability of Crushed Peas and Critical Care

| | **Female Guinea pigs** | | **Male Guinea pigs** | |
|---|---|---|---|---|
| | *CP* | *CC* | *CP* | *CC* |
| *Week 3* | | | | |
| Monday | | | | |
| Tuesday | X | | X | |
| Wednesday | X | | X | |
| Thursday | X | | X | |
| Friday | X | | X | |
| *Week 4* | | | | |
| Monday | X | | X | |
| Tuesday | X | | X | |
| Wednesday | X | | X | |
| Thursday | X | | X | |
| Friday | X | X | X | |
| *Week 5* | | | | |
| Monday | X | X | X | |
| Tuesday | X | X | X | X |
| Wednesday | X | X | X | X |
| Thursday | X | X | X | X |
| Friday | X | X | X | X |
| *Week 6* | | | | |
| Monday | X | X | | |
| Tuesday | X | X | | |

*Note.* The days crushed peas (CP) and/or Critical Care (CC) were present inside the cages.

# Appendix C – Ethogram

| *Behavioural categories* | *Behavioural groups* | *Observed behaviours* | *Definitions* |
|---|---|---|---|
| ***Individual behaviour*** | **Ingestive behaviour** | Eating | The animal consumes food |
| | | Cecography | A sitting animal lowers its head to its anal region, then raises it and makes chewing motions |
| | | Drinking | Drinking from either a bowl or a water bottle |
| | **Elimination** | Urinating | The animal slightly spreads the hindlimbs, slightly elevates its perineal region and urinates |
| | | Defecating | The animal freezes and squats slightly to defecate |
| | **Marking** | Perineal drag | The animal squats and pulls its hindquarters forward, dragging the perineum across the ground. |
| | | Supracaudal glands | Side to side rubbing of the rump on objects. |
| | **Active Locomotive behaviour** | Locomotion | Moving from one place to another in the cage; 2 or more paws take steps |
| | **Inactive locomotive behaviour** | Resting | The animal lays on the ground with the stomach facing down or on its side, while relaxing or sleeping |
| | | Standing still | Standing on at least 3 feet while staying in one spot |
| | **Other locomotive behaviour** | Freezing | Freezing in place; cessation of all movements for a timespan of more than 1 second |
| | | Jumping | Sudden locomotion directed mostly upwards, not caused by a trigger |
| | | Startling | Sudden and jerky movement directed mostly upwards due to a trigger |

| | | | |
|---|---|---|---|
| | **Comfort behaviour** | Sneezing | Sudden and involuntary burst of air pressed from lungs through nose at a high velocity. |
| | | Coughing | Burst of air pressed from lungs through throat and mouth. |
| | | Yawning | Deep inhalation of air combined with wide opened mouth. |
| | | Shaking | Rapid side to side movement of body or part of body (example head). |
| | | Stretching | Straightening or extending the entire body or part of the body. |
| | **Grooming** | Face wipes | The front paws are licked and the proximal surface drawn across the face from the eye or ear to the tip of nose. Both forelimbs may be used alternately or together or only one may be used. |
| | | Scratching | Rapid strokes of the hindfoot scratched the head, back and side. |
| | | Nibbling and licking | Any part of the body behind the nape may be cleaned by nibbling with the incisors or licking with the tongue |
| | | Nosing | The sides and back may be groomed by rubbing the nose through the hair with posteriorly directed head movements. |
| | | Combing | Combing through the hair of the abdomen with the claws of the front feet. |
| | | Rolling | The animal laid on its side, rolled onto its back and then back on its side, typically several times in succession |
| ***Animal-Environment Interaction*** | **Explorative behaviour** | Gnawing objects | Gnawing on objects in cage using teeth without eating. |
| | | Pushing objects around | Displacing objects by pushing them with nose. |
| | | Digging | Displacing litter with several strokes of the front and back limbs. |

| | | | |
|---|---|---|---|
| ***Social behaviour*** | **Sociopositive behaviour** | Nose-nose | Two encountering animals touch noses and sniff each other |
| | | Body sniff | Sniffing a part of another animals' body |
| | | Social grooming: Nibbling | Two animals sitting together may nibble each other's pelage or an animal may approach and nibble another |
| | | Social grooming: Chin-rub | The groomer rubs the back of the animal being groomed with the underside of its head using jerky side-to-side movements |
| | **Socionegative defensive behaviour** | Retreating | Sudden locomotion away from another animal with an increase in distance of more than one body length. |
| | | Head-up | Head is thrown back and nose is pointed straight upwards. |
| | | Kicking | Using one or both hind feet, directed toward another animal |
| | | Head-thrust | Thrusting of head towards another animal |
| | | Facing | Turning around to face an animal at the rear in a sudden motion. |
| | **Socionegative offensive behaviour** | Stand-threat | Curved body posture is displayed by two animals toward each other. |
| | | Attack-lunge | Short run or jump towards another animal. |
| | | Chasing | Pursuing an animal which is moving away. |
| | | Pawing | Foot motions same as in digging but with faster strokes. |
| ***Animal-Human Interaction*** | **Explorative behaviour** | Sniffling | Repeatedly drawing air up nose to detect a small, directed at a human or a part of a human |
| | | Body contact | Contact with a human initiated specifically by guinea pig |
| | | Gnawing | Gnawing of a human or part of a human, including clothing or writing material carried by the human |

*Note.* Based on *Gut et al., 2018; Peter & Kunkel, 1963; Rood, 1972*.

# Appendix D – Overview of the data

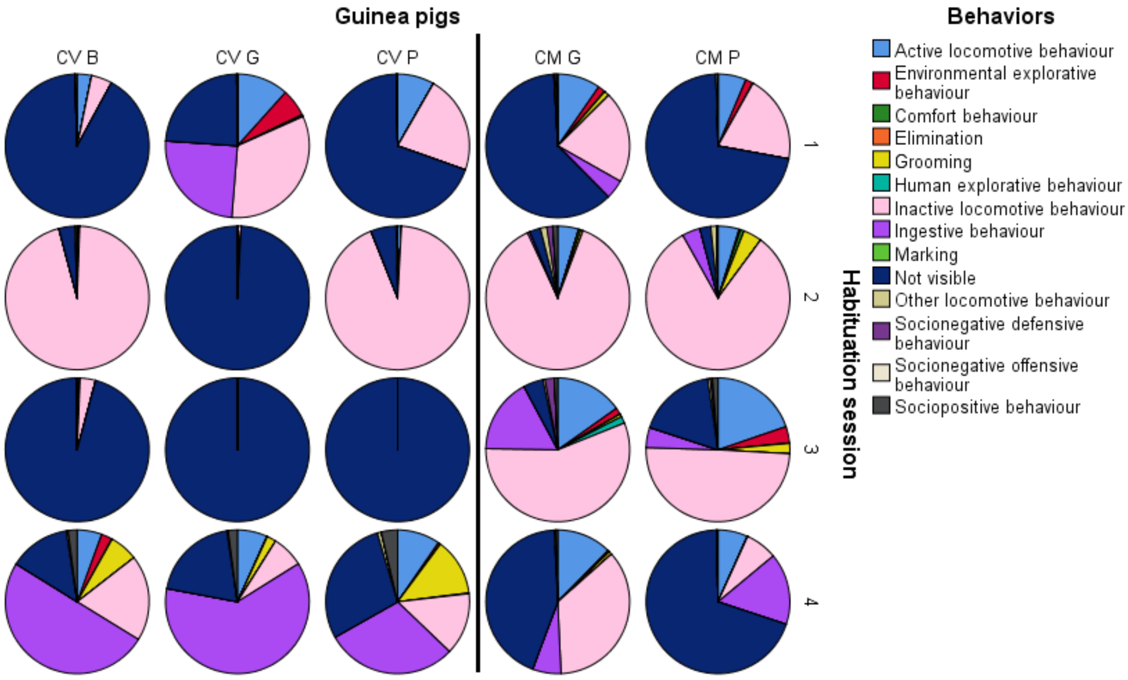


***Figure 8.*** *An overview of the percentage of total time spent per behaviour for all four habituation sessions and all five of the animals. CV B = the female guinea pig without marking; CV G = the female guinea pig with green marking; CV P = the female guinea pig with blue marking; CM G = the male guinea pig with green marking; CM P = the male guinea pig with blue marking.*

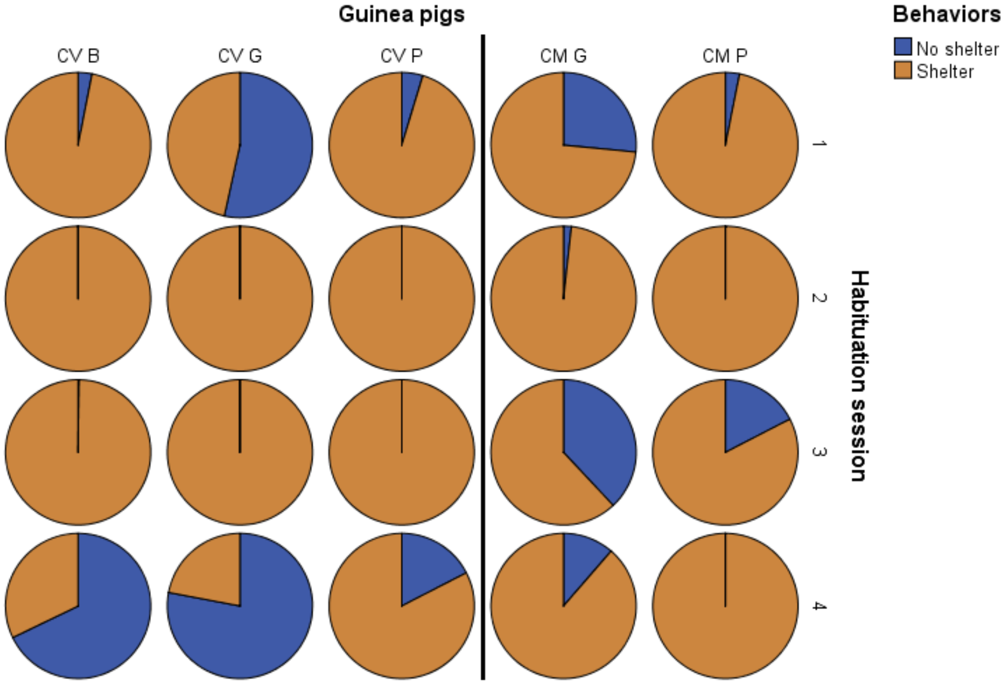


***Figure 9***. *An overview of the percentage of total time spent inside or outside shelter for all four habituation sessions and all five of the animals. CV B = the female guinea pig without marking; CV G = the female guinea pig with green marking; CV P = the female guinea pig with blue marking; CM G = the male guinea pig with green marking; CM P = the male guinea pig with blue marking.*

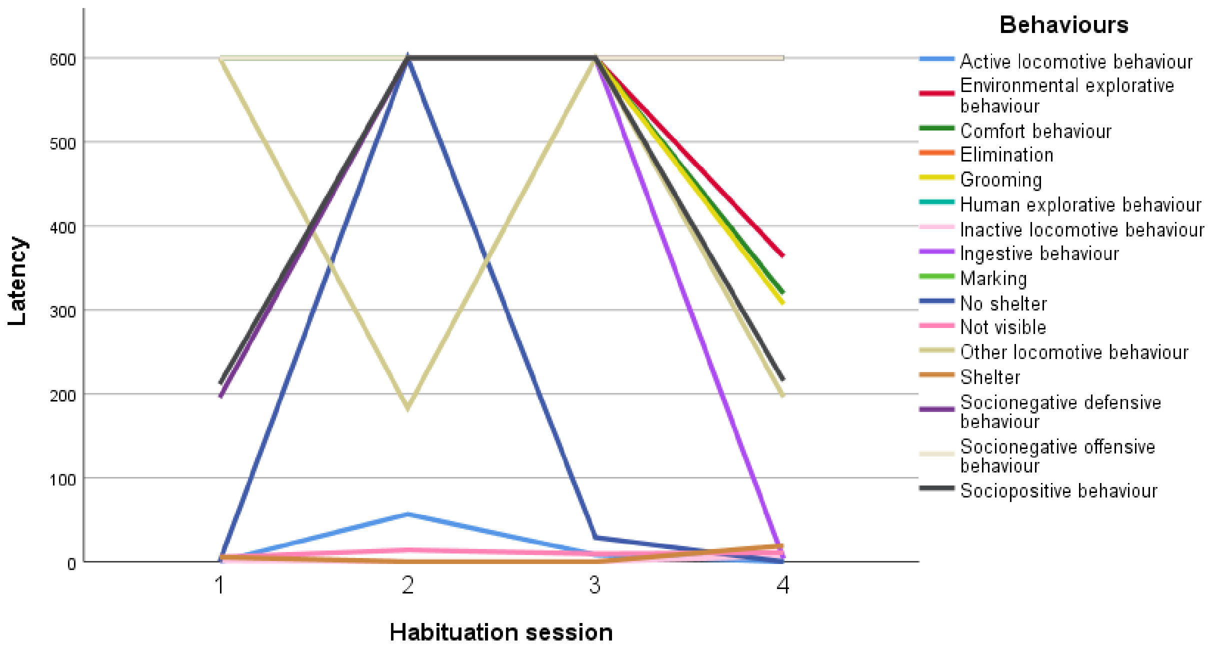


***Figure 10***. *An overview of the latencies per behaviour for all four habituation sessions for guinea pig CV B (the female guinea pig without marking).*

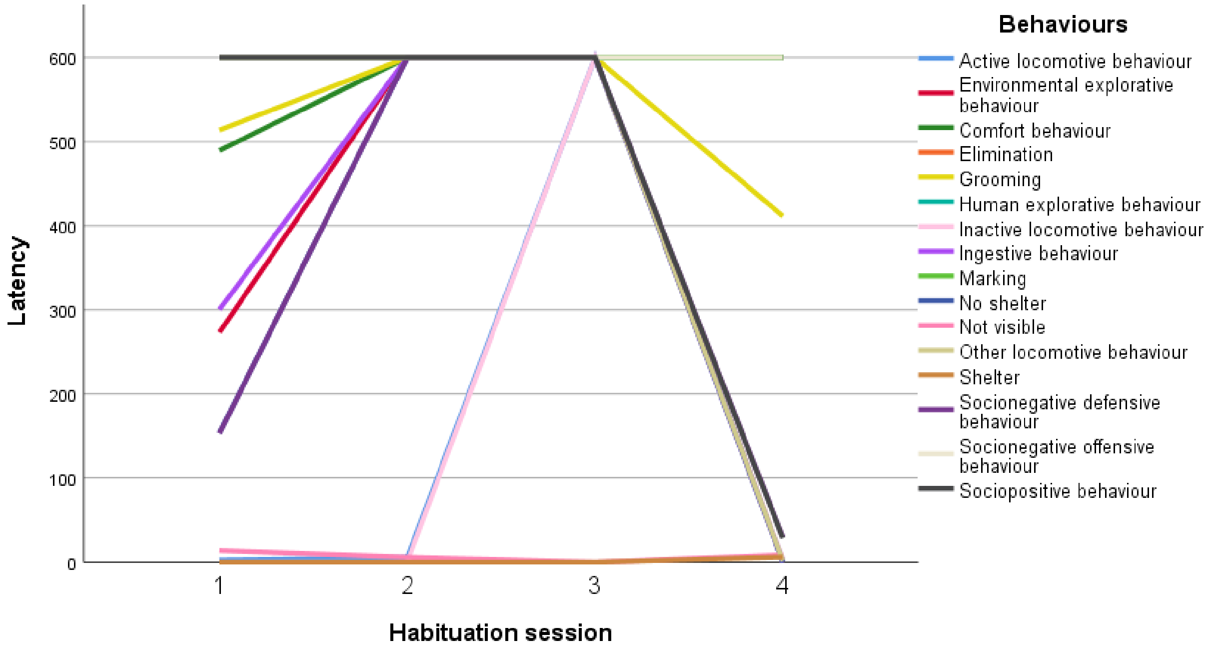


***Figure 11***. *An overview of the latencies per behaviour for all four habituation sessions for guinea pig CV G (the female guinea pig with green marking).*

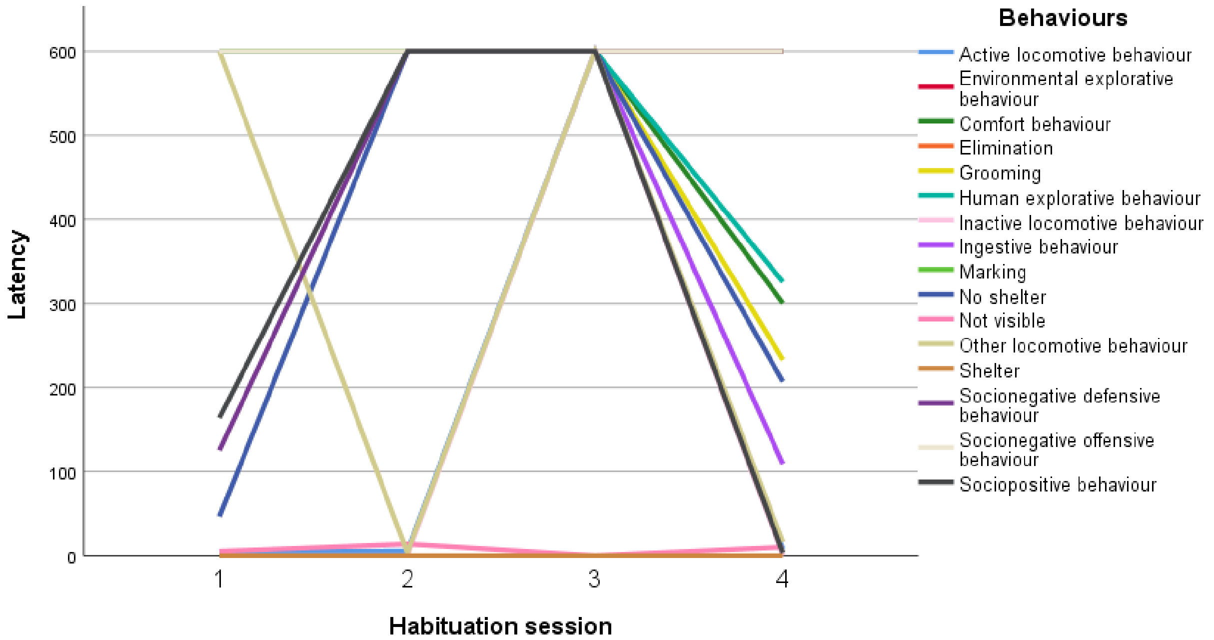


***Figure 12***. *An overview of the latencies per behaviour for all four habituation sessions for guinea pig CV P (the female guinea pig with blue marking).*

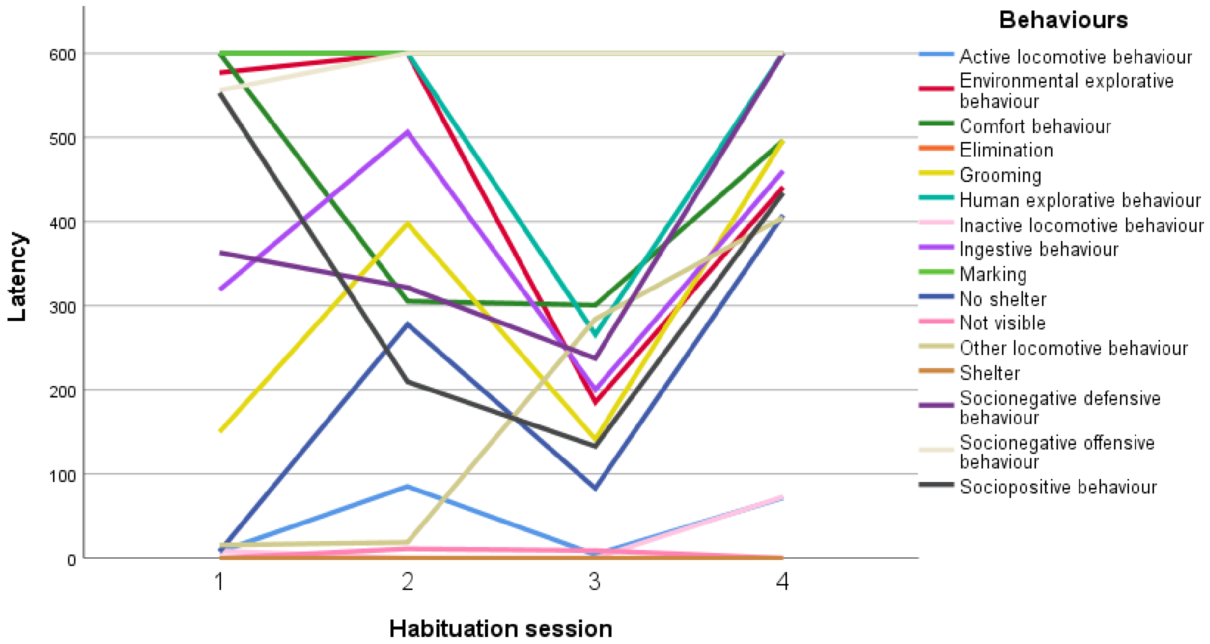


***Figure 13****. An overview of the latencies per behaviour for all four habituation sessions for guinea pig CM G (the male guinea pig with green marking).*

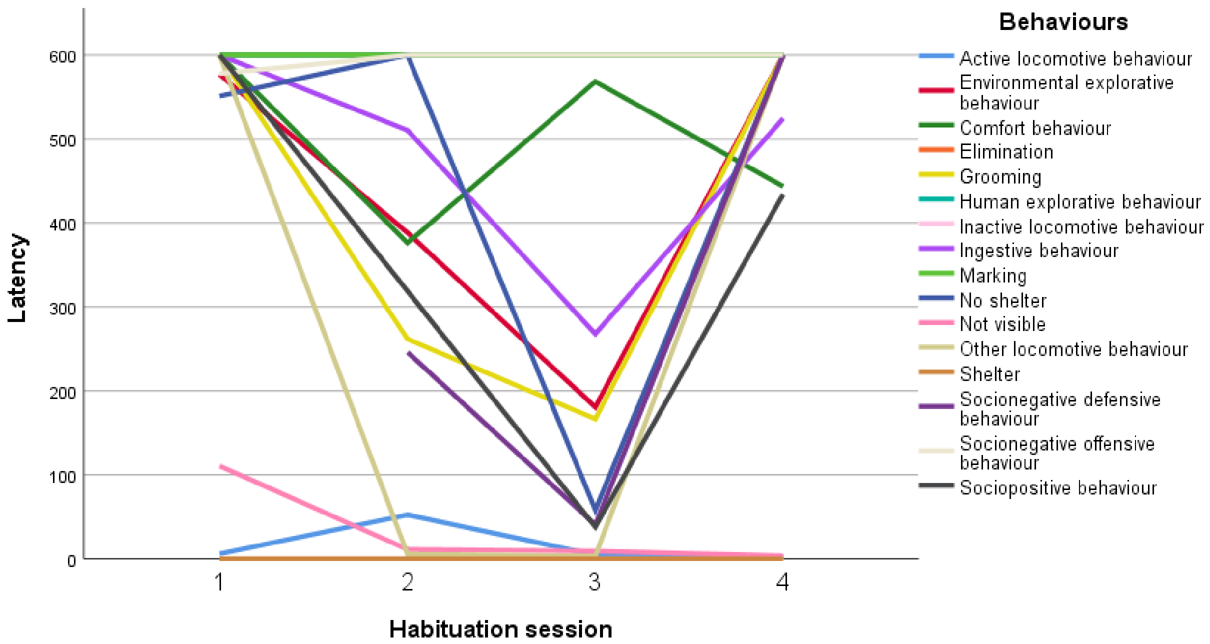


***Figure 14****. An overview of the latencies per behaviour for all four habituation sessions for guinea pig CM P (the male guinea pig with blue marking).*