Ultrasonic vocalization in the common marmoset (*Callithrix jacchus)*

A pilot study

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Ultrasonic vocalization in the common marmoset (*Callithrix jacchus)*. A pilot study.

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ABSTRACT

The common marmoset (*Callithrix jacchus*) is commonly used in biomedical research. For monitoring of their welfare in captivity, an objective measuring tool would be important. The aim of this study was to see if ultrasonic vocalizations (USV) might be a useful objective parameter in the assessment of pain and distress in these small primates. During different neutral, positive and negative trials, the occurrence of USV were measured and investigated. It was found that the common marmoset indeed produces USV and that these seem to occur mostly in stressful situations. However, it also seems to be that USV do not occur solely, but that the audible vocalizations of marmosets in some cases extend into the ultrasonic range. Vocalizations, both audible and in the ultrasonic range, may be used as an objective non-invasive and non-intrusive measurement for welfare in common marmosets.
Keywords: common marmoset, ultrasonic vocalizations, welfare

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INTRODUCTION

Animal welfare has been a subject of discussion for many years. Despite considerable effort, there is still no universally accepted definition of animal welfare (Green and Mellor, 2011). The five freedoms that were formulated by the Brambell committee in 1956 are still broadly used as a guideline for welfare assessment protocols (Green and Mellor, 2011; Ohl, 2011).

Difficulty is, that it is not always that easy to assess these five freedoms. Per example: being chased by a predator is normal species-specific behaviour for many species of animals, but this behaviour will obviously not benefit to the welfare of the chased animal (Dawkins, 2006). Another example is the experience of pain. It is broadly agreed that experiencing pain does compromise welfare. However, this ignores the biological function of a negative emotional reaction such as a pain reaction, since these have evolved to protect an individuals overall welfare (Ohl, 2011). The same is true with normal social behaviour. An animal should be free to express species-specific behaviour, for many species of animals this implies living in social groups. Nevertheless, in establishing and keeping stable social relations, 'negative' stimuli are necessary (Ohl, 2011).

Further more, to assess whether an animal is suffering from fear and chronic stress is proven to be one of the most difficult tasks. It is now widely accepted that there is no single parameter that can be used by itself and recently, more attention has come to the behaviour of animals as an important key for the assessment of animal welfare (Dawkins, 2006; Dawkins, 2004). The major advantage of using behaviour as a key to animal welfare is that it is non-invasive and furthermore, it is also non-intrusive so the animals are not disturbed while being studied for animal welfare (Dawkins, 2004). This advantage is readily important because invasive parameters can alter not only the animals' behaviour but also its physical/physiological parameters. Another possible non-invasive and non-intrusive parameter that can be measured objectively, are the vocalizations of animals, which also can give a clue about animal welfare (Arnold et al., 2011; Manteuffel et al., 2004; Dawkins, 1998).

Next to vocalizations that are audible for humans, it is known that certain species of animals produce ultrasonic vocalizations (USV) (Yu et al., 2011; Arch and Narins, 2008; Clausen et al., 2008). Despite the nearly universal ability of mammals to hear high-frequency sounds, relatively few species are known to use USV for intraspecific communication, what may be due to the fact that there are transmission limitations of high-frequency sounds, which reduces their utility as long-distance communication signals (Arch et al., 2009). However, within certain environmental and behavioural contexts, communication through USV may offer advantages, such as enhanced signal-to-noise ratio, avoidance of eavesdropping by predators or prey and increased energetic efficiency (Arch et al., 2009). The production of USV by laboratory rat and mice has been intensively studied (Takahashi, 2010; Potfors, 2007; Wöhr, 2007). Rats produce typical USV in both positive and negative situations (Burman et al., 2006; Kikusui et al., 2003). Burman et al. suggests that 22 kHz USV can induce a negative emotional state of increased anxiety in rats hearing the vocalization and could therefore be a useful indicator of welfare for rat groups (Burman et al., 2006). Williams et al. however, found that USV do not provide any more information than do audible vocalizations for assessing responses to potentially painful procedures in laboratory mice (Williams et al., 2008). It seems to be that USV do reflect the positive or negative affective state in laboratory rats but not in laboratory mice (Portfors 2007).

A few years back, it was discovered at TNO in Rijswijk that the common marmoset, *Callithrix jacchus*, also produces USV (non published data from H. van der Wiel, 2009). The possible meanings of these USV have not been studied yet, as far as we know of.

The common marmoset is a small arboreal New World primate that lives in the Atlantic coastal forests of Brazil (Pistorio et al., 2006). It is a highly social primate that lives in small family groups and has a broad vocal repertoire, which is maintained in captivity (Pistorio et al., 2006; Ziegler, 2002). Their audible vocalizations has been studied to some extend and most audible calls are classified (Bezerra and Souto, 2008; Pistorio et al., 2006; marmoset website of the University of Stirling). Osmanski and Wang discovered an auditory threshold of 36 kHz in the common marmoset (Osmanski and Wang, 2011), this is a threshold well in the ultrasonic range and therefore a possible indication that marmosets might indeed produce USV. The threshold was set at 36 kHz due to the fact that Osmanski and Wang did not go into higher frequencies than 36 kHz, so it is possible that the common marmoset actual can hear frequencies higher than this.

Because of their genetics as well as physiological and anatomical similarity to humans, the common marmoset is intensively used in biomedical research (Arnold et al., 2011). Examples are studies on multiple sclerosis, arthritis, Parkinson disease, tularaemia, anthrax and endometrosis (Arnold et al., 2011; Jagessar et al., 2011; Nelson et al., 2011; Nelson et al., 2010; Vierboom et al., 2010; Vliet et al., 2008). Since they are so frequently used in research, it is necessary to develop sensitive methods, which can detect pain and distress in these animals.

The aim of this study is to investigate whether common marmosets indeed produce USV and if so, if these USV could have a correlation with welfare. The ultimate goal would be to develop the recording of USV as a sensitive, non-intrusive and non-invasive measurement for the detection of pain and distress in the common marmoset. As a first step towards this goal, this pilot study was conducted to provide a basis for possible future studies on the USV produced by the common marmoset.

MATERIAL AND METHODS

In this study, three different types of experiments were conducted at the Biomedical Primate Research Centre (BPRC, Rijswijk, The Netherlands).

- Experiment 1: was conducted primary to see if common marmosets indeed produce USV.
- Experiment 2: was conducted to establish whether or not the USV are produced solely or in combination with audible vocalizations and if produced USV could be correlated to positive and negative situations. For this both negative and positive stimuli were used in the trials. During this experiment there were new animals moved into the housing room, the effect of this on the residents in the housing room was also recorded.
- Experiment 3: was conducted to see if the USV found in the experimental facility differ from those found in the breeding facility. Further, the reaction on a predator model was recorded to see whether USV might play a role during a predator response.

In every experiment the recording took place in the morning, with multiple recordings during every morning, while in the afternoon data was analysed. In this present study, the audible vocalizations were classified in the same way as is done at the special common marmoset website of the University of Stirling (www.marmosetcare.com).

Data in all experiments was collected using the Sonotrack™ (Metris b.v., Hoofddorp, The Netherlands). Prior to performing the experiments, the Sonotrack™ was adjusted since background noise might interfere with USV produced by the animals. To do this, the Sonotrack™ was turned on in front of an empty cage so there could be no USV from marmosets. To avoid for background noise to be recorded, the threshold was raised to 0,3 V. In this way all ultrasonic noise must be louder than this to be recorded. Since vocalizations are in the range of Volts, all USV should be recorded in this way. The number of peaks above threshold was set at 5, so 5 peaks of at least 0,3 V are necessary before an ultrasonic sound was recorded on the Sonotrack™. To be sure that only ultrasonic sound was recorded, the lower cut off frequency was set at 20 kHz. The upper cut of frequency was set at the maximum frequency the Sonotrack™ could measure; 100 kHz. Prior to every recording in a new room, the settings were checked to assure that there was not too much interference from the environment. In this way, virtually no background noise was recorded. The noise that was still recorded was either from noise from the animals jumping on the enrichment (especially when this came in contact with the iron of the cage) or from the personal computer where the Sonotrack™ was running on. This last background noise was seen as a continues bar in the screens, only apparent on the channel which stood directly by the personal computer and was no problem anymore after replacing the channel.

Data analyses

With the automatic USV counter of the Sonotrack™ was automatically given the number of USV per timeframe, the average (with minimum and maximum) frequency and time of USV per timeframe. Since there was also surrounding noise (e.g. animals jumping on enrichment which came in contact with the iron of the cage) counted as USV by the Sonotrack™, counting took place by hand with the option manual analyse ultrasound on the Sonotrack™.

Statistical analyses were performed using the software program Excel® for Windows (Microsoft Corporation, USA). Means were compared by a Student's t-test to determine significance between means. Significance level was set at $P \leq 0.05$.

Experiment 1

Subjects and housing

This experiment took place in the experimental facility. The spacious cages were equipped with branches, nest boxes and various enrichment. The cages were made of iron with on the front wire mesh where the animal caretakers could see the animals. The room temperature was kept stable at 23.2 – 26.8°C with a 12-hour light: dark cycle. All animals were fed commercial monkey pellets (Ssniff®, Soest, Germany) *ad libitum* supplemented with limited amounts of Arabic gum, fresh fruit and live insects. Water was provided *ad libitum* in drinking bottles.

Subjects for this experiment were nine pairs of female and nine pairs of male common marmosets (*Callithrix jacchus*); pairs were mostly twins. In every pair only one animal was included in the experiment. The animals used in this experiment are listed in table 1.

Animal	Gender
1	Female
2	Female
3	Female
4	Female
5	Female
6	Female
7	Female
8	Female
9	Female
10	Male
11	Male
12	Male
13	Male
14	Male
15	Male
16	Male
17	Male
18	Male

Table 1: Animals used in experiment 1.

Vocalization recording

Recording took place during unsedated blood sampling. This experiment was done this way because it was believed that during this procedure, the animals experience stress and pain and therefore would be more likely to produce USV.

A marmoset was taken out of his cage by using a Perspex cylinder; the animals were trained for this. This cylinder was placed at a weighing scale as a non-invasive way of assessing the body weight. After this, the marmoset was taken out of the cylinder and restrained in a blue soft leather catching glove. During blood sampling, one person restrained the animal so a second person could insert the needle (26 gauge) percutaneously into the vena saphena. Blood sampling was done by self-filling of the needle tip, this blood

was then collected by a pipette. Afterwards, pressure was applied to allow the bleeding to stop. Recording started at the moment the Perspex cylinder was placed on the weighing scale and stopped when the animal was placed back in the Perspex cylinder. During every recording, only one marmoset was recorded. Recordings were taken using two microphones placed in front of the animal.

Experiment 2

Subjects and housing

Housing and feeding regime were the same as in experiment 1. Subjects for this experiment were six male common marmosets housed in pairs or single, both pairs were twins. Table 2 contains information about the cages included in this study. The animals in cage 1 and 2 were present in the housing room during the whole experiment while the animals in cage 3 and 4 were moved into the housing room during the experiment.

Cage	Number of animals in cage
1	1
2	2
3	1
4	2

Table 2: Animals included in experiment 2.

Vocalization recording

Recordings were taken using four microphones; two on top of the cage facing downwards and two in front of the cage, standing on a cart on top of a plastic box, as seen in figure 1.

Figure 1: Trial set-up during Experiment 2

The experiment consisted of four different trials; duration of all trials was 20 minutes per trial. The animals were always restricted to the top part of the cage, unless stated otherwise. To accomplish this, an iron plate was placed in the middle of the cage, between the top and lower part of the cage. Directly after the trial the iron plate was removed so the animals could use the entire cage again. During every recording, only one pair or single marmoset was recorded at a time.

All disturbances (persons entering the room, loud cage noise and others; see table 3) and audible vocalizations during the trials were noted. On every scoring form was noted: the date, starting time of recording and cage number.

When the animals were producing a lot of noise by jumping on the enrichment (since the cages were made of iron, this happened quiet a lot), this was recorded as “loud cage noise”.

	Time
Audible vocalization in cage	
Loud shrill in cage	
Loud cage noise	
Noise in the hall way	
Someone entering the changing room	
Someone entering the room	

Marmoset drinking	
Marmoset eating	
Any other noise/disturbance	
Marshmallow presented	
Catching glove presented	

Table 3: Scoring form used in Experiment 2.

During this experiment, four different trials were conducted:

a. Neutral situation
   During this trial the animals were recorded and not disturbed.
b. Positive situation
   During this trial the animals were given a marshmallow, presented by the experimenter by hand. When there were two animals in the cage, they both received a marshmallow at the same time. Since the marmosets are trained at the BPRC with marshmallows as a reward and training with this reward goes very well, the marshmallow was chosen as a positive stimulus in this experiment.
c. Negative situation
   During this trial a blue soft leather catching glove was presented to the animals. The experimenter held the catching glove for approximately a minute in front of the cage. Nowadays the marmosets are trained to go into a Perspex cylinder by themselves in order to be caught out of the cage, this reduces the stress of being caught. Before this method, the animals used to be caught out of the cage by grapping them with the blue catching glove, what was supposed to be very stressful for the animals. The catching glove is now only used to restrain the animals when needed (e.g. in experiment 1). It is thought that because of this, the animals would still make a negative association with the catching glove, which is the case with the rhesus monkeys that are also housed at the BPRC.
d. Separation
   Prior to the trial, the animals were separated. This was done by placing the iron plate in the cage as in the neutral situation with the exception that one animal was in the top part of the cage and the other animal was kept in the lower part of the cage. During this trial, the recording set-up was adjusted. The two microphones on top of the cages facing downwards were removed and placed at the bottom of the cart for recording the animal that was kept in the lower part of the cage. The other two microphones stayed on top of the cart for recording of the animal that was kept in the top part of the cage. Since marmosets are highly social animals, separation from their cage mate was suspected to be a stressful and therefore negative situation for them. The two cages with only one marmoset were not included in this trial, since they are single housed and thus could not be separated from their cage mate.

Experiment 3

Subjects and housing

This experiment took place in the breeding facility, consisting of several breeding groups of common marmosets living in separated cages. A breeding group consisted of one breeding pair and their offspring. However, in some cages only two or three relatives were

housed while other cages consisted of a non-breeding family group where the female was on birth control (Implanon®, n.v. Organon, Oss, The Netherlands)

A cage existed of an outside and inside enclosure. The walls were made of concrete; in front of the cage there were two doors with wire mesh and in front of the wire mesh there were plastic transparent doors, see figure 2. The inside enclosure measured approximately 3x3x2 meters. In the inside enclosure the room temperature was kept stable between 23.2 – 26.8° Celsius. To prevent that the outside temperature would influence the inside temperature, access to the outside enclosure was through plastic flaps. The animals were free to choose between the inside and outside enclosure. They were fed commercial monkey pellets (Ssniff®, Soest, Germany) *ad libitum*, supplemented with limited amounts of Arabic gum, fresh fruit and live insects. Water was provided *ad libitum* via drinking nipples.

Figure 2: The inside enclosure of a cage at the breeding facility.

Cages recorded were chosen at random. Included cages are listed in table 3.

Cage	Number of animals in cage	Remarks
1	2	
2	3	
3	10	
4	10	
5	2	Female on Implanon®
6	11	Fighting in the family group
7	3	
8	7	Female on Implanon®
9	3	
10	9	New born animals (9 days old)
11	5	
12	11	Female on Implanon®
13	8	New born animals (recorded at 1 and 2 days old)

14	5	One animal separated from the others because of fighting in the group
15	3	Female on Implanon®
16	3	
17	12	

Table 3: Animals used in Experiment 3.

In cage 10 and 13 there were newborn animals in the cage. In cage 10, these animals were nine days old at the moment of recording. The newborn animals in cage 13 were twice recorded, during the first recording these animals were one day old and during the second recording they were two days old.

The family group in cage 6 was already fighting during some time prior to recording. At the moment of recording this family group was still fighting. In cage 14, there was also fighting in the group but these animals were separated prior to recording. The cage was divided in two equal halves; at the right side one animal was kept and the other animals were kept in the left side of the cage to stop the fighting in this group.

Vocalization recording

During every recording, only one cage was recorded at a time. Recordings were taken using four microphones, all in front of the cage. Two were placed on the bottom of the cart where the Sonotrack™ stood on, the other two were placed on the top of the cart on plastic boxes, as seen in Figure 3. In this way the whole inside enclosure was recorded. Since it was winter during recording, the marmosets seldom went outside. During recording, the plastic doors in front of the wire mesh doors were opened to optimize recording.

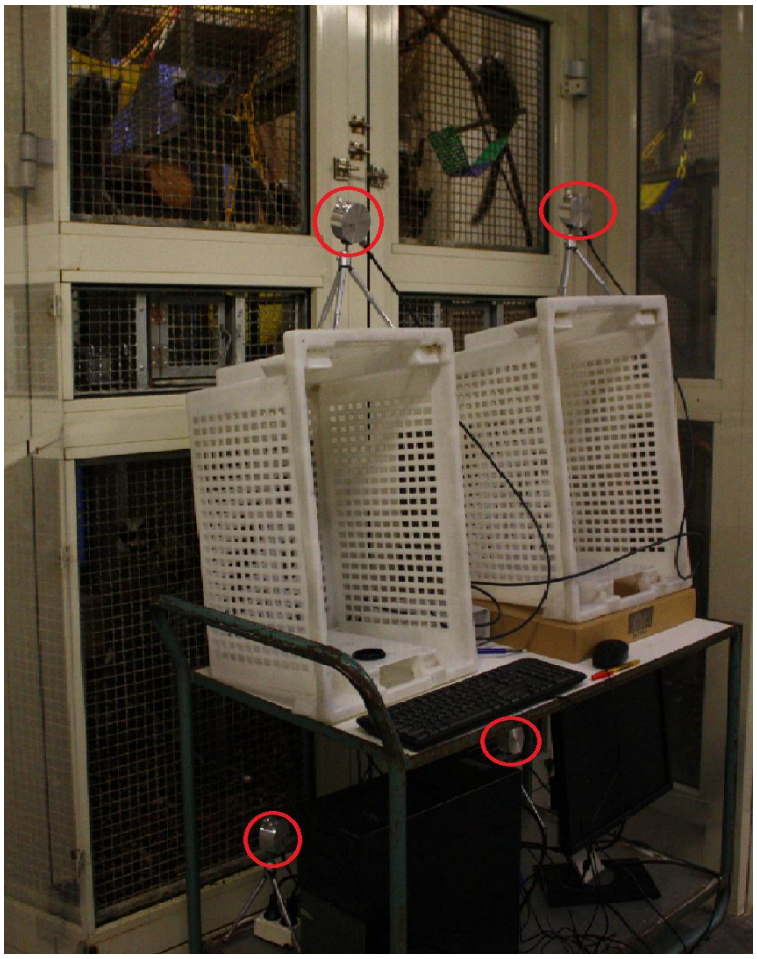

Figure 3: Trial set-up during Experiment 3

Two different trials were conducted:

1. Neutral
   During this trial the animals were recorded for 30 minutes and were not disturbed.
2. Stuffed animal
   Prior to recording, a stuffed animal was placed as a negative stimulus inside the cage. After approximately 5 to 7 minutes, the stuffed animal was removed. Recording in total was 30 minutes. It is known that captive, naïve marmosets react on snake models in a way very similar to wild marmosets reacting on a living snake (Cagni et al., 2011; Emile and Barros, 2009; Clara et al., 2007). Since marmosets react on the presence of a snake with mobbing calls (Clara et al., 2007), it was here investigated whether USV also play a role during these mobbing calls.
   a. Snake
      The snake (see Figure 4) was placed in the cage to see if this would evoke a predator response.
   b. Cow
      The cow (see Figure 4) was placed in the cage as a control to establish whether the reaction on the snake was indeed a predator response or just a response to a novel object in the cage.

Figure 4: Stuffed animals used in trial 2a and 2b of Experiment 3.

During recording a similar scoring form was used as in experiment 1, as seen in Table 4. On every scoring form was noted: the date, starting time of recording, cage number, animals present in the cage and special remarks when present (per example: new born animals, unstable family group etc.). When the animals suddenly jumped around in the cage for no apparent reason, this was noted as 'unrest in cage'.

	Time
Audible vocalization in cage	
Unrest in cage	
Loud cage noise	
Person entering the room	
Any other noise/disturbance	

Table 4: Scoring form used in Experiment 3.

RESULTS

Experiment 1

During this experiment there were USV recorded from almost every animal, only one animal did not produce USV during recording. The number of USV per minute recorded per animal can be seen in figure 5.

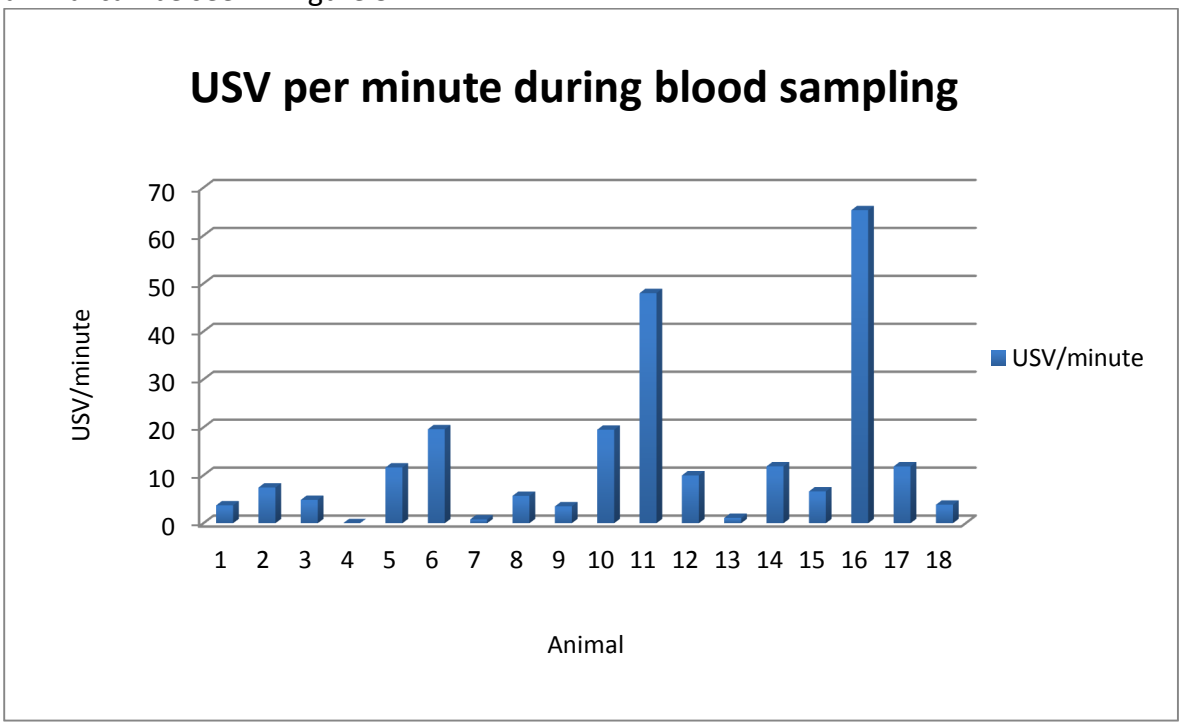

Figure 5: USV per minute per animal

On average, the males produced more USV per minute then did the females, but this was not significant (P = 0.11). In some animals blood sampling did not succeed in one try. This was due to either difficulty to find the blood vessel or when the blood was clotted too soon so there was not enough blood collected and the needle had to be inserted for a second or third time. The animals were the blood sampling did succeed in one try, produced more USV then the animals where the blood sampling did not succeed in one try, this was true for both the females and males, as can be seen in figure 6. This difference was again not significant (P = 0.88).

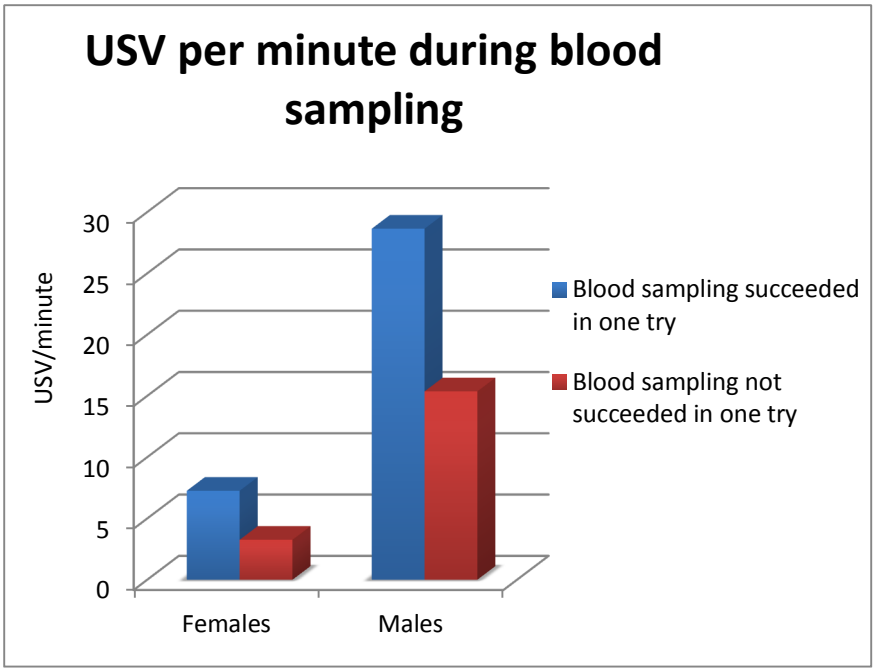

Figure 6: Average USV/minute with blood sampling succeeded in one try and with multiple tries.

During the blood sampling, different types of USV were found. These can be seen in figure 7, 8 and 9.

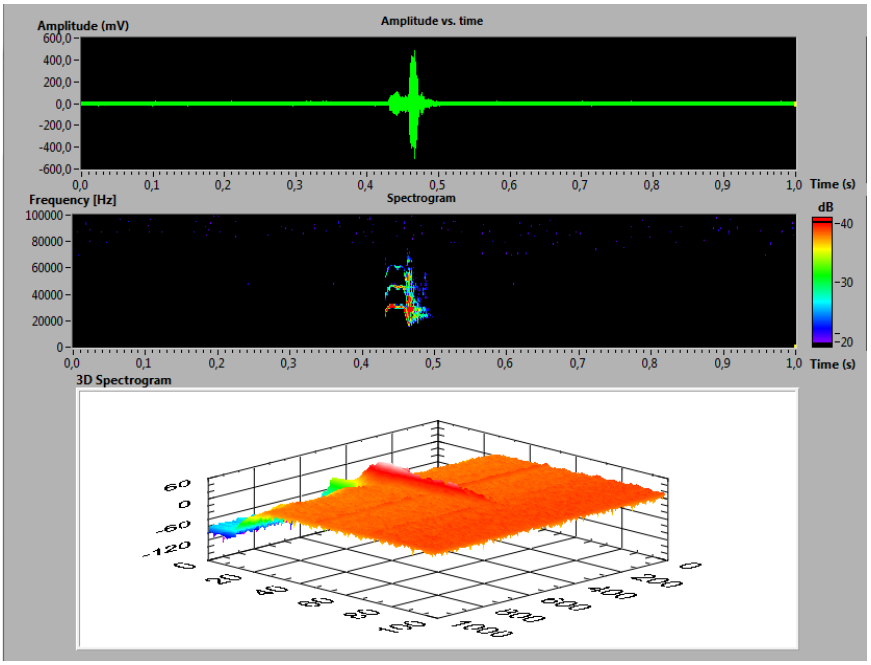

Figure 7: An example of USV recorded in experiment 1.

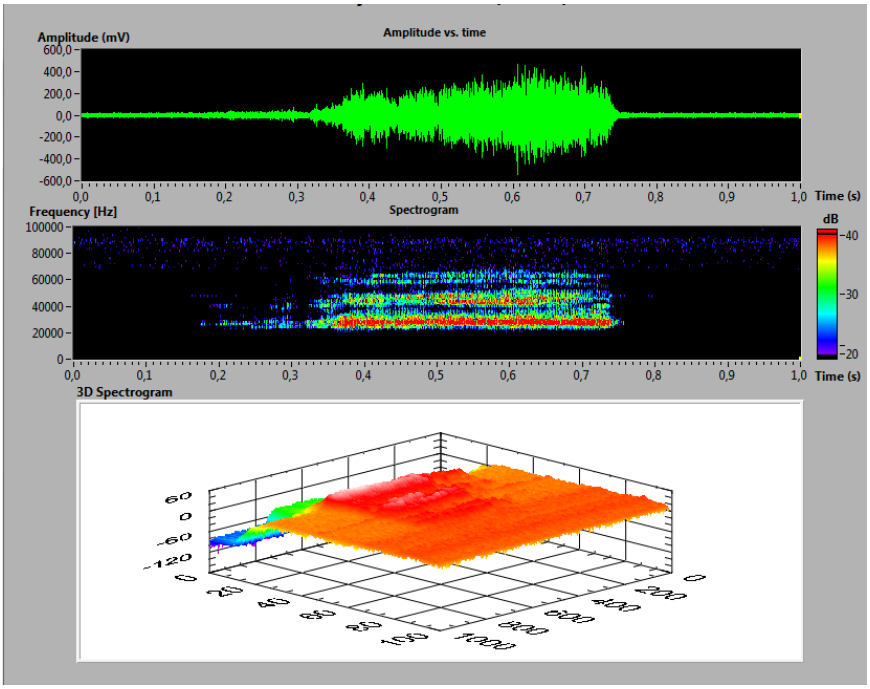

Figure 8: An example of USV recorded in experiment 1.

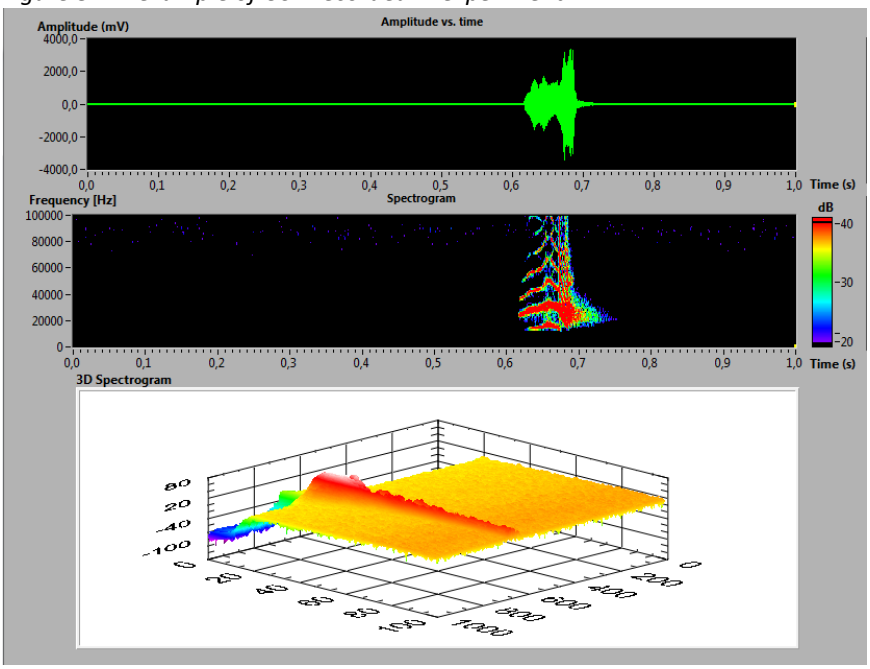

Figure 9: An example of USV recorded in experiment 1.

All the USV shown above consist of multiple frequency levels, the one in figure 6 shows a tendency to go down in frequency, the USV in figure 7 seems to stay equal and the USV in figure 8 shows a tendency to go up in frequency.
All animals produced audible vocalizations during this experiment, mostly chatter and screams.

Experiment 2

The average USV per minute and average audible vocalizations per minute per trial and cage can be seen in figure 10 till 13.

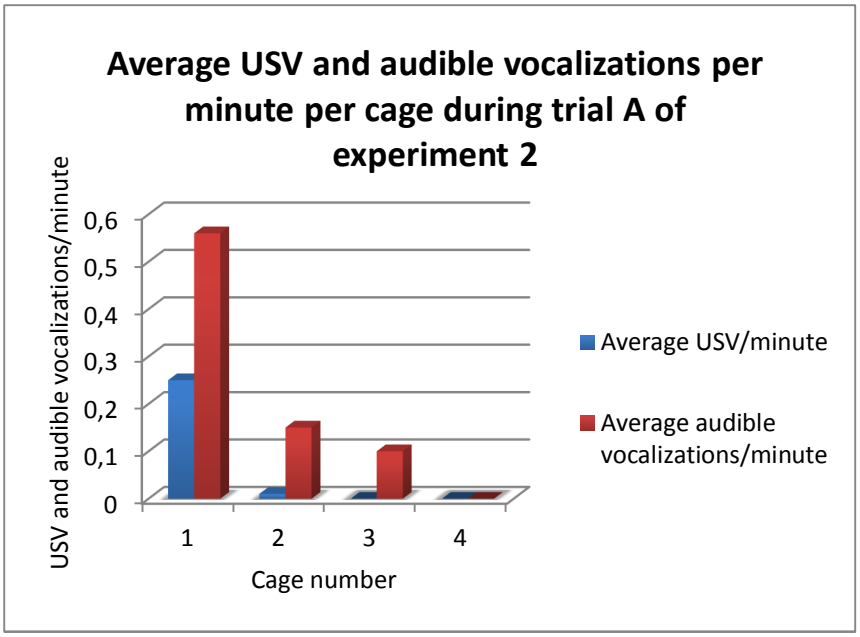

Figure 10: USV and audible vocalizations per minute during trial A of experiment 2.

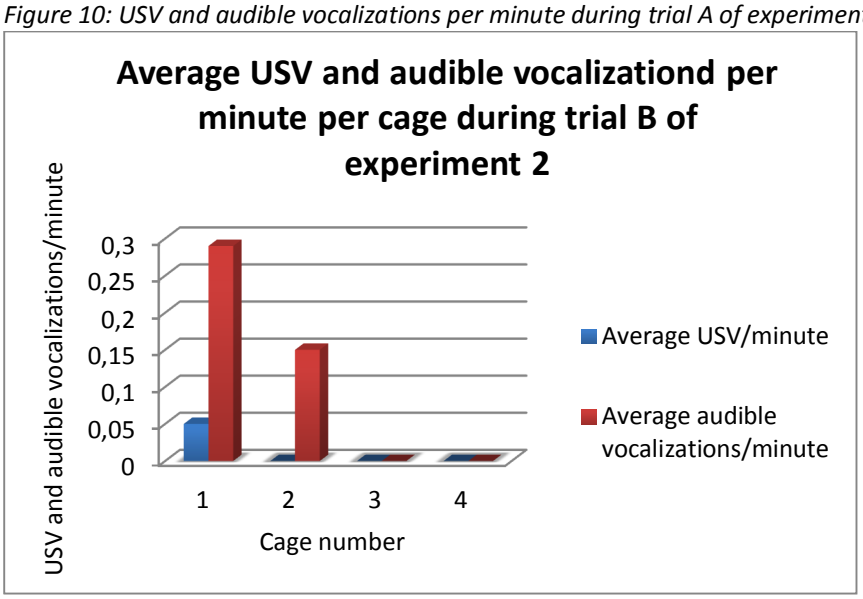

Figure 11: USV and audible vocalizations per minute during trial B of experiment 2.

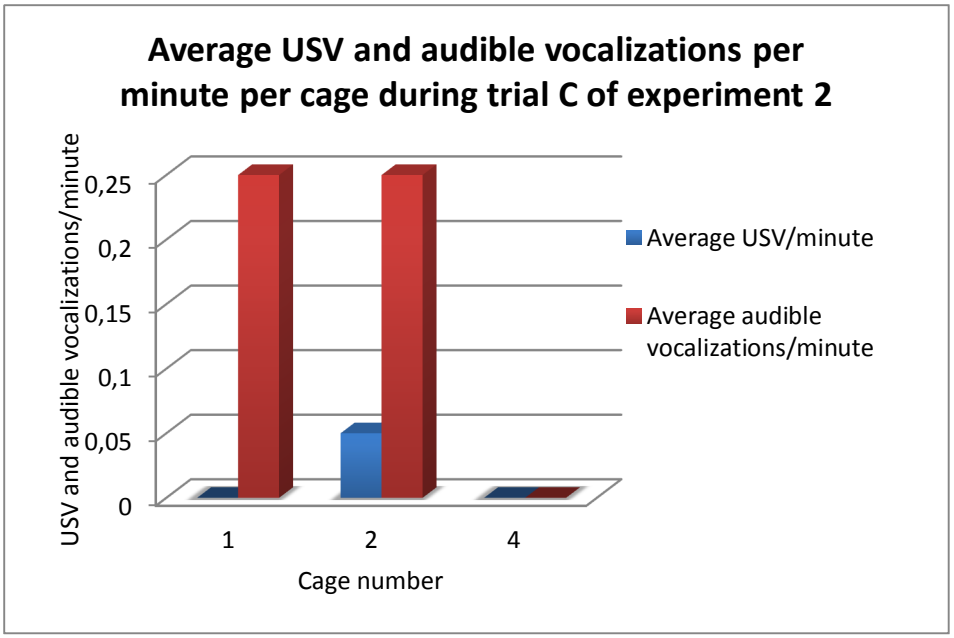

Figure 12: USV and audible vocalizations per minute during trial C of experiment 2.

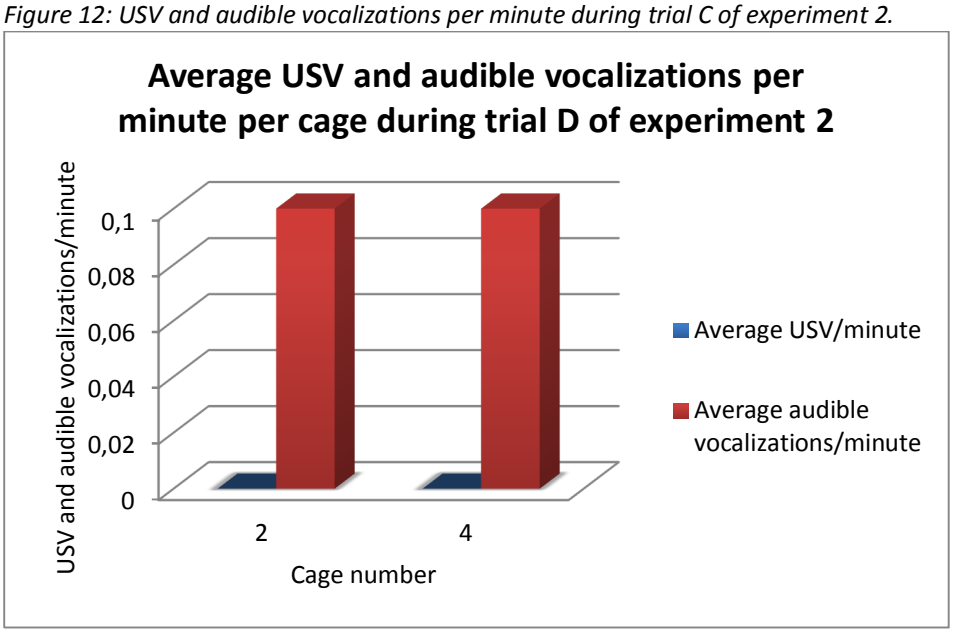

Figure 13: USV and audible vocalizations per minute during trial D of experiment 2.

As can be seen in the figures above, no USV were recorded when there was no audible vocalization during recording. Furthermore, every USV that was recorded came simultaneously with an audible vocalization. On the other side, not in every recording with audible vocalizations there were USV recorded. In figure 14 the averages per trial in total can be seen. No significant differences were found between the trials.

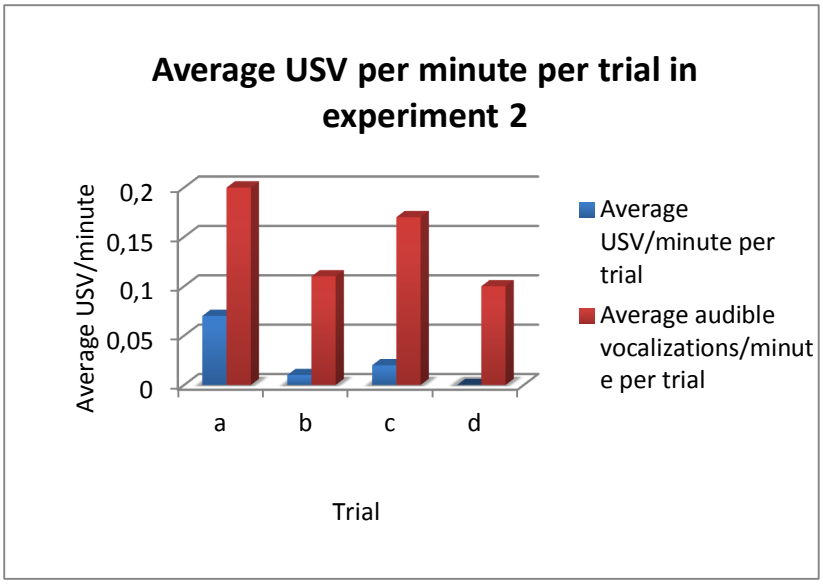

Figure 14: Average USV per minute per trial in experiment 2.

During this experiment, it was seen that with three different audible vocalizations, different types of USV were recorded. With a loud shrill, an ek call and a tsik-ek call there were USV recorded. The different types of USV recorded can be seen in figure 15, 16 and 17.

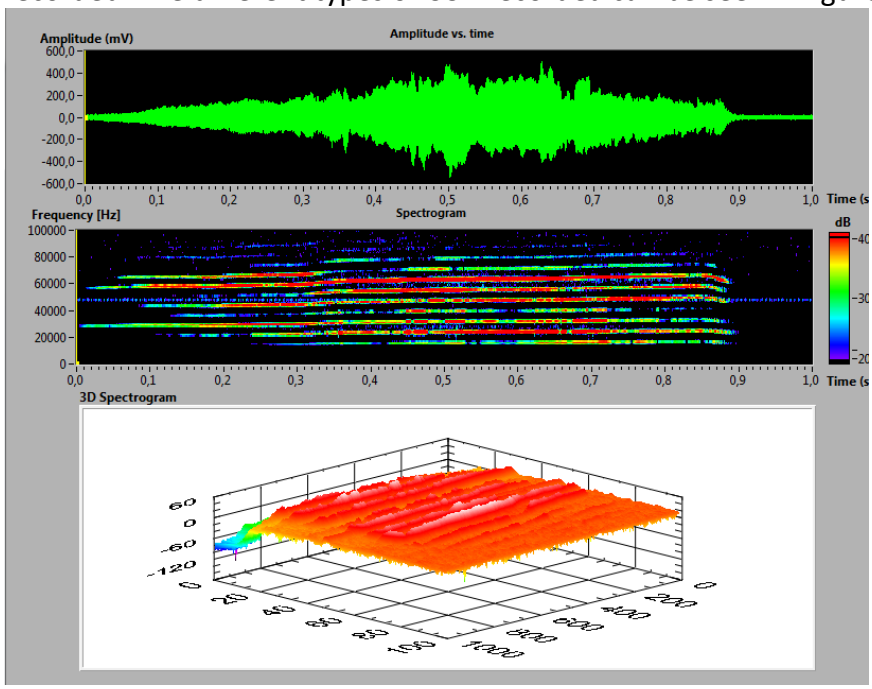

Figure 15: An example of an USV recorded with simultaneously a loud shrill.

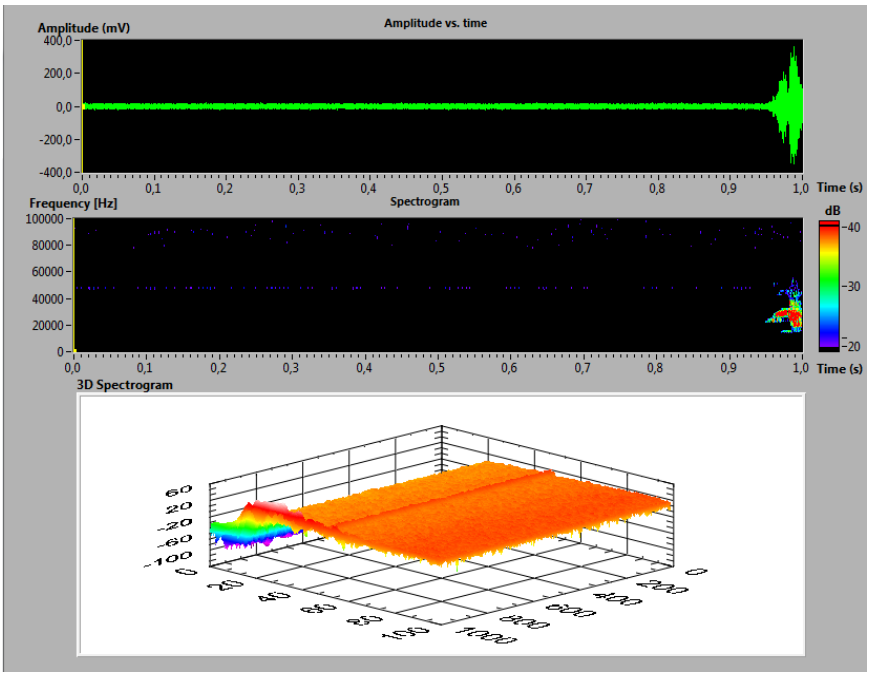

Figure 16: An example of an USV recorded with simultaneously an ek call during presenting the blue catching glove.

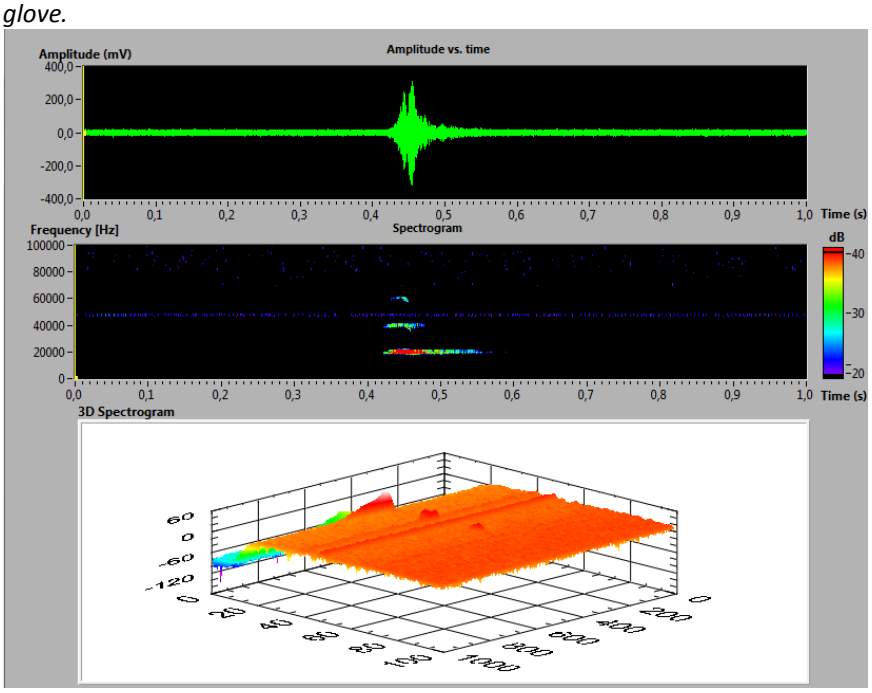

Figure 17: An example of an USV recorded with simultaneously a tsik-ek call during presenting the bottom of a boot.

Again, all USV seem to consist of multiple frequency levels. The USV in figure 15 and 17 seem to stay equal in frequency while the frequency levels in figure 16 seem to melt together at the end of the call. Unfortunately, there was no clearer picture of this USV, since there was only one USV recorded during presenting of the catching glove.

The loud shrills were all observed in cage 1, which contained a single housed marmoset. The ek vocalization was found in cage 2 when the catching glove was presented to the animals. The tsik-ek calls were only found when the animals saw the bottom of the boot. Because it was not known that the animals reacted on this, it was not clear whether this was a neutral, negative or positive stimulus. Since this would influence the USV per

minute during the trials, the results of the USV per minute recorded when the animals saw the bottom of a boot are shown separately in figure 18.

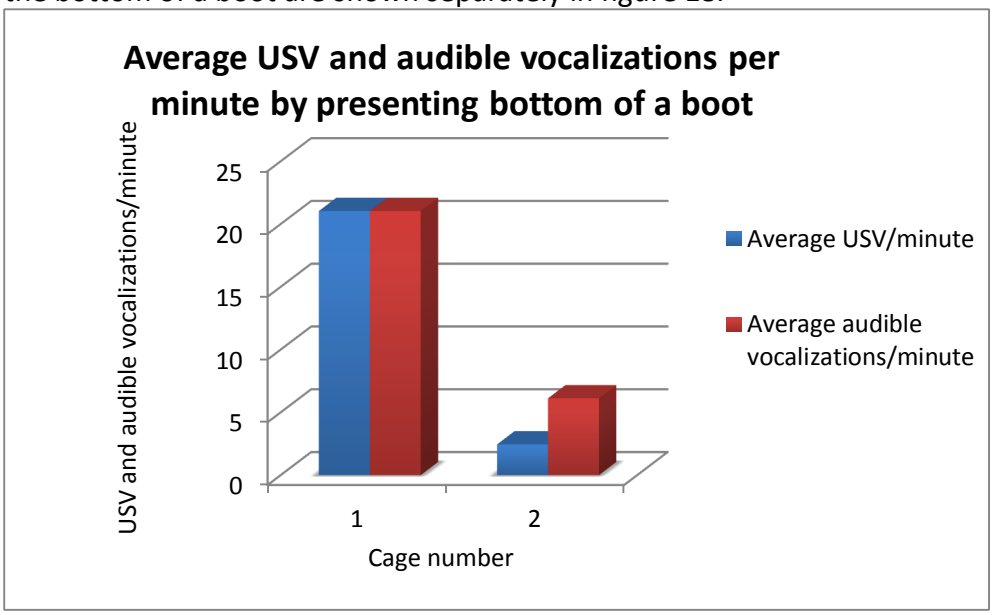

Figure 18: Average USV and audible vocalizations per minute by presenting bottom of a boot during experiment 2.

No USV were recorded during the actual presenting of the marshmallow. In one instance, the animals in cage 2 reacted on the marshmallow with only an audible vocalization (not specified). The USV recorded in trial B were during a loud shrill without the presence of a marshmallow from the animal in cage 1. All animals did come in front of the cage to collect the marshmallow.

During presenting of the catching glove, only one USV was recorded and only one audible vocalization was heard. These came simultaneously from one animal in cage 2. The other cages did not react on the catching glove with audible vocalizations or USV. The animals all stopped moving when the glove was presented and stared at the glove.

With the separation, no USV were recorded. In both cages, the animal that was kept in the lower part of the cage reacted on the separation with two audible vocalizations (not specified). These were not accompanied by USV. The animal that was kept in the top part in both cages did not produce any audible vocalizations during the separation. After the animals were placed back together, a neutral trial was conducted. In these trials no USV were recorded. The animals in cage 2 began grooming each other; the animals in cage 5 produced only audible vocalizations (not specified).

The animals in cages 1 and 2 were present in the experimental facility during the whole duration of experiment 2. The animals in cages 3 and 4 were brought to the experimental facility when the other two cages were already a couple of times recorded. Cages 1 and 2 were therefore also recorded in a neutral trial during the moving in of the animals in cage 3 and 4. These four cages were the only cages that contained animals during this experiment in this housing room of the experimental facility.

The animals in cage 2 did not produce any USV or audible vocalization; they kept very quiet and stared the whole time at the new arrivals (they were placed in cages opposite cage 1 and 2). After recording cage 2, cage 1 was also recorded. This marmoset showed an increased scent marking behaviour but no USV were recorded. This marmoset did produce audible vocalizations (not specified) and showed an increased jumping around in the cage.

Experiment 3

All cages, except cage 2, were used in trial 1; only 3 cages were used in trial 2, these cages were all used in trial 2a and 2b.

The recorded number of USV found in trial 1 can be seen in figure 19. There were five cages with quiet high USV per minute (cage 5, 6, 10, 15 and 17). In cage 5 there were a lot of tsik vocalizations, these marmosets were very skittish. In cage 6 there was fighting in the family group, the animals kept jumping around and were very vocal during recording. In cage 10, there were newborn animals of nine days old. The whole family group stayed in the back except for one marmoset that was almost the whole time in front of the cage, giving loud shrills calls towards other cages. Almost all the recorded USV in this cage where from this animal during the loud shrills. In cage 15 and 17, nothing special happened. The animals in both of these cages were very vocal during recording.

In four cages there were no USV recorded. In cage 7 the animals were most of the recording time at the back of the cage and were very skittish when they did come in front. They did groom each other at the back of the cage during recording. The animals in cage 11 were also very skittish and they all went to the outside enclosure at the end of the recording time. In cage 9 and 12, the animals were not skittish and did come in front of the cage a lot.

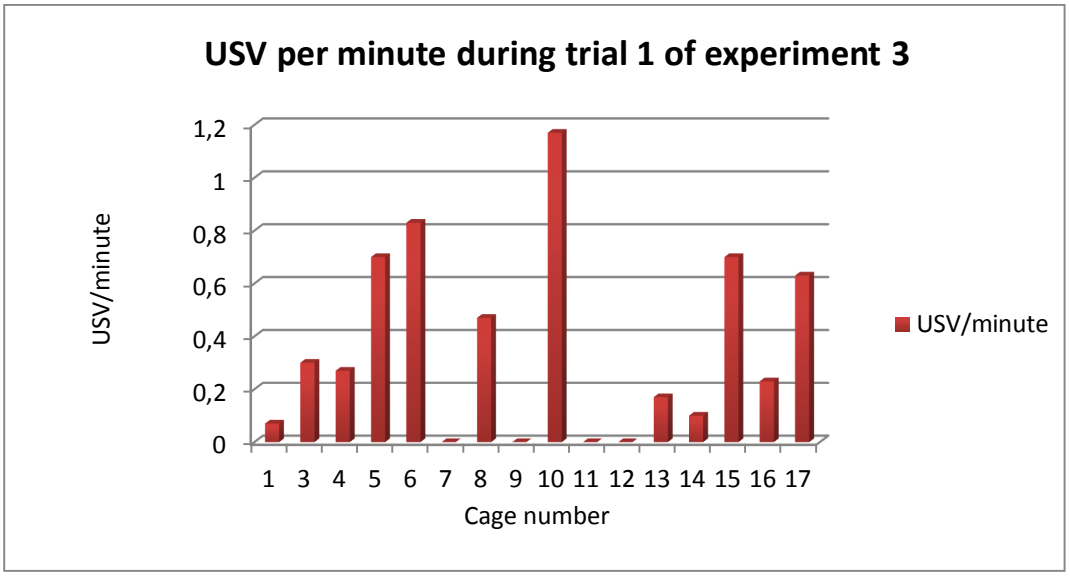

Figure 19: USV per minute recorded during trial 1 in experiment 3.

When comparing the average production of USV from cages with only two or three animals with that from cages with more then three animals, no significance can be found (P = 0.57). Comparison between family groups with new born animals (< 4 months of old) and family groups with no new born animals (all animals in the group older then 4 months) shows no significance (P = 0.38). Also no significance was found between groups where the female was on birth control and groups where the female was not on birth control (P = 0.48).
Some examples of USV recorded during trial 1 of experiment 3 are shown in figures 20 till 27.

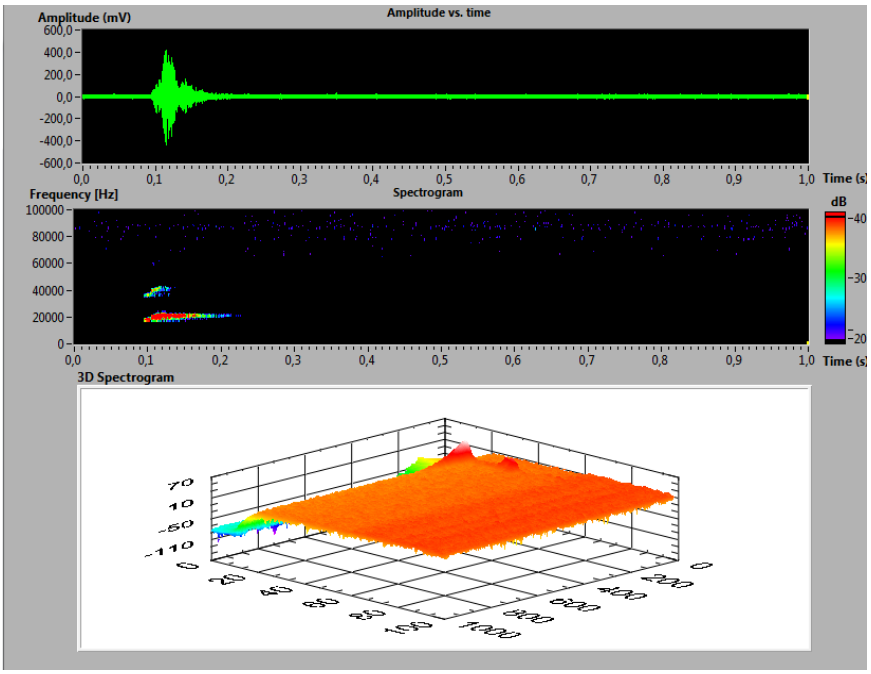

Figure 20: USV recorded from cage 1, nothing special happened.

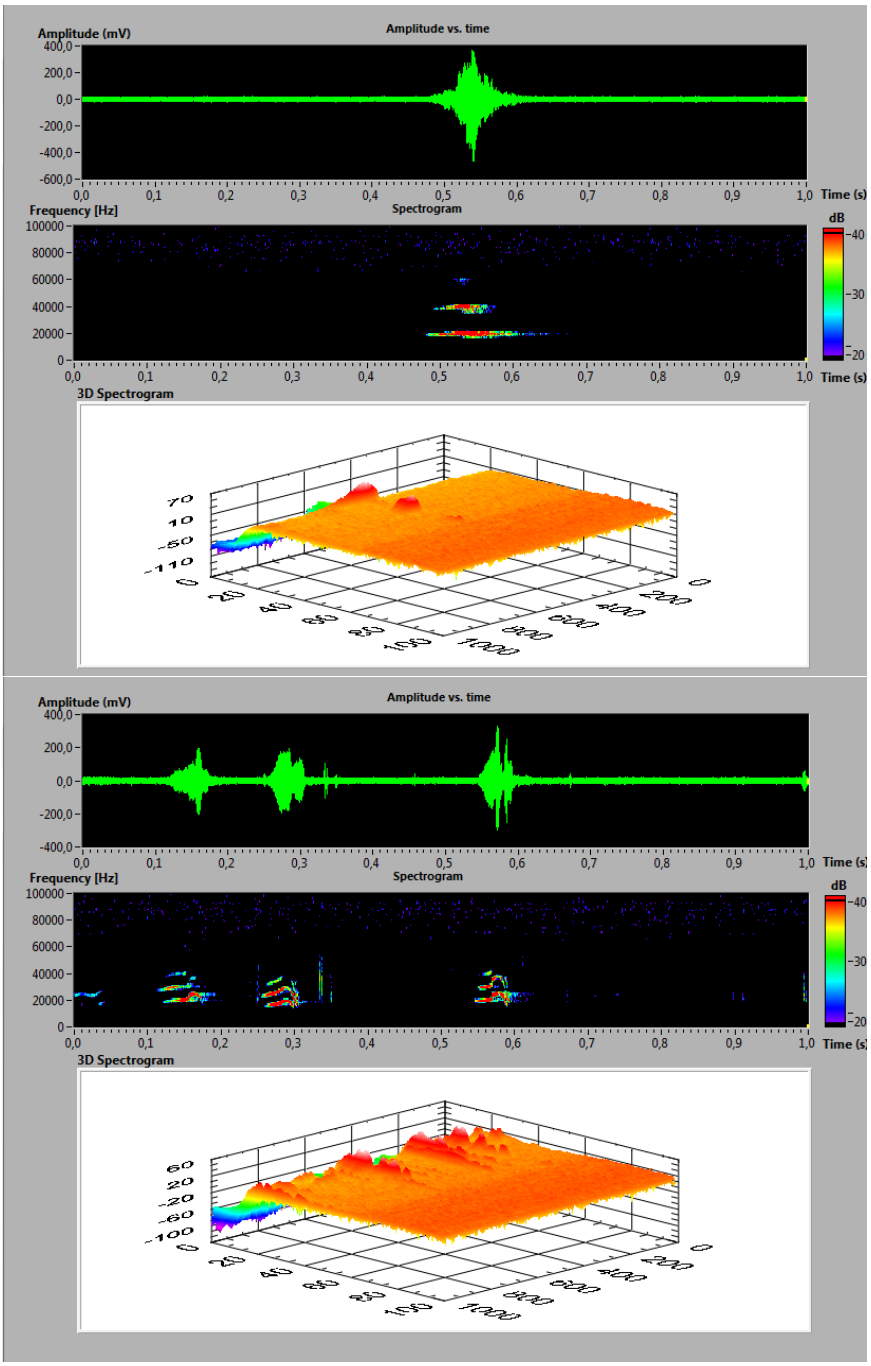

Figure 21: Two different types of USV recorded from cage 3, nothing special happened.

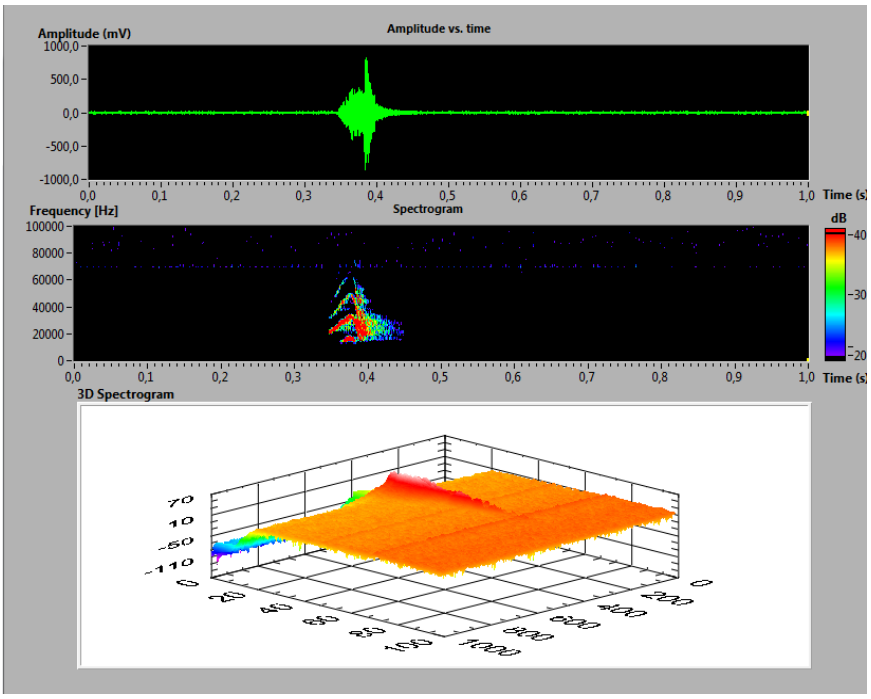

Figure 22: USV recorded from cage 5 during a tsik vocalization.

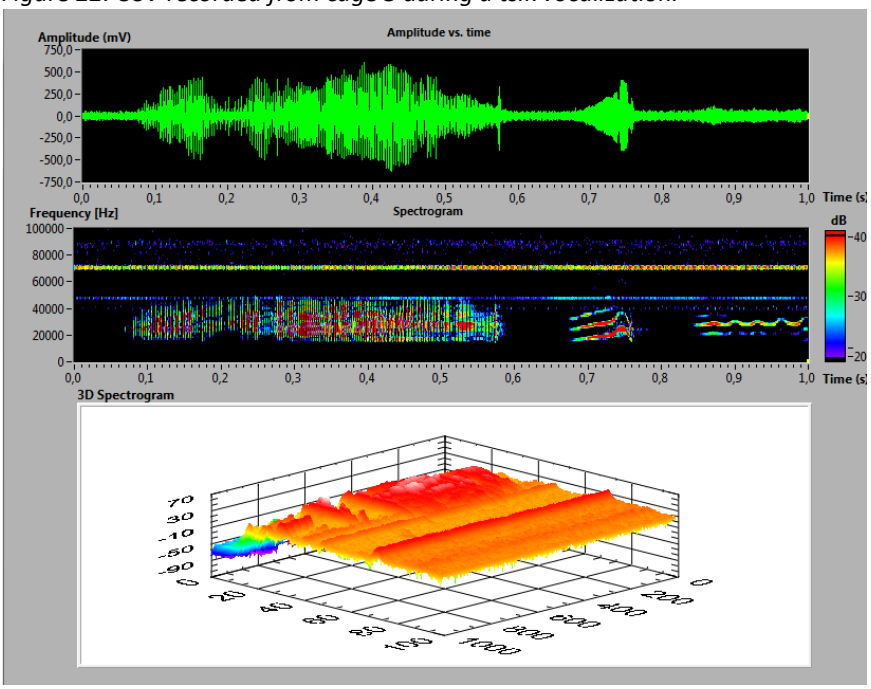

Figure 23: USV recorded during fighting in cage 6.

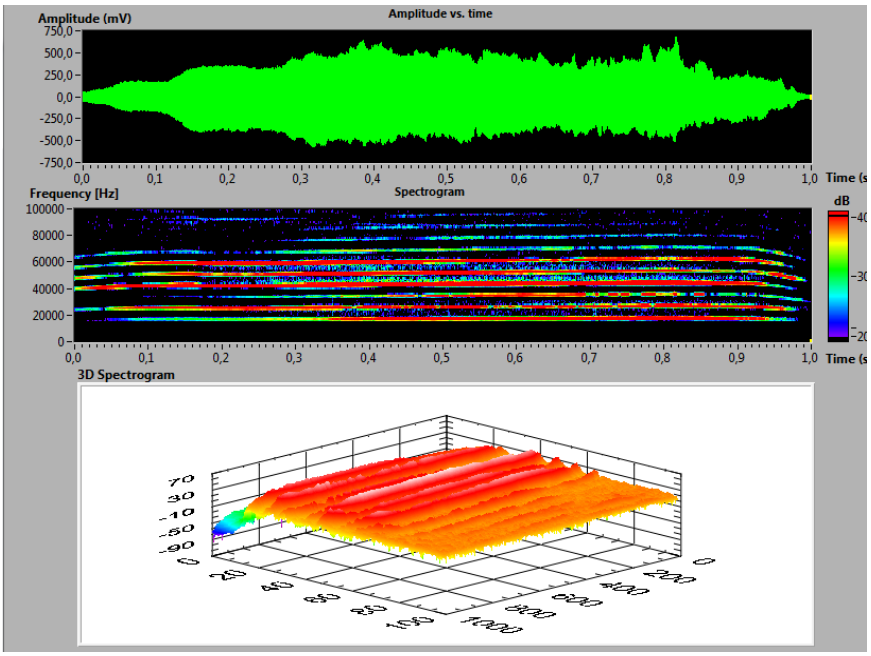

Figure 24: USV recorded during a loud shrill from a marmoset in cage 10.

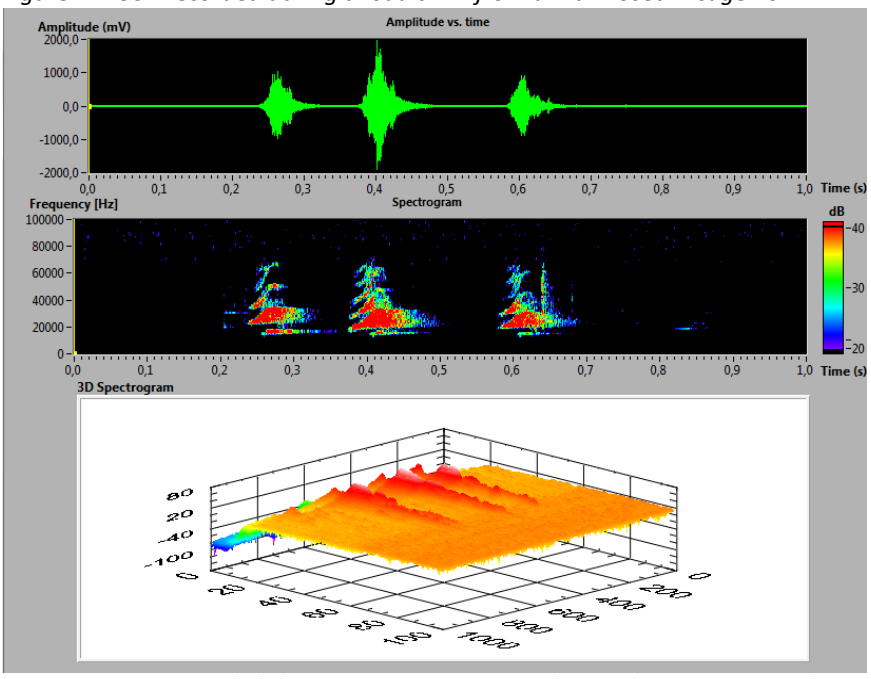

Figure 25: USV recorded during unrest in cage 13, the newborns were one day old.

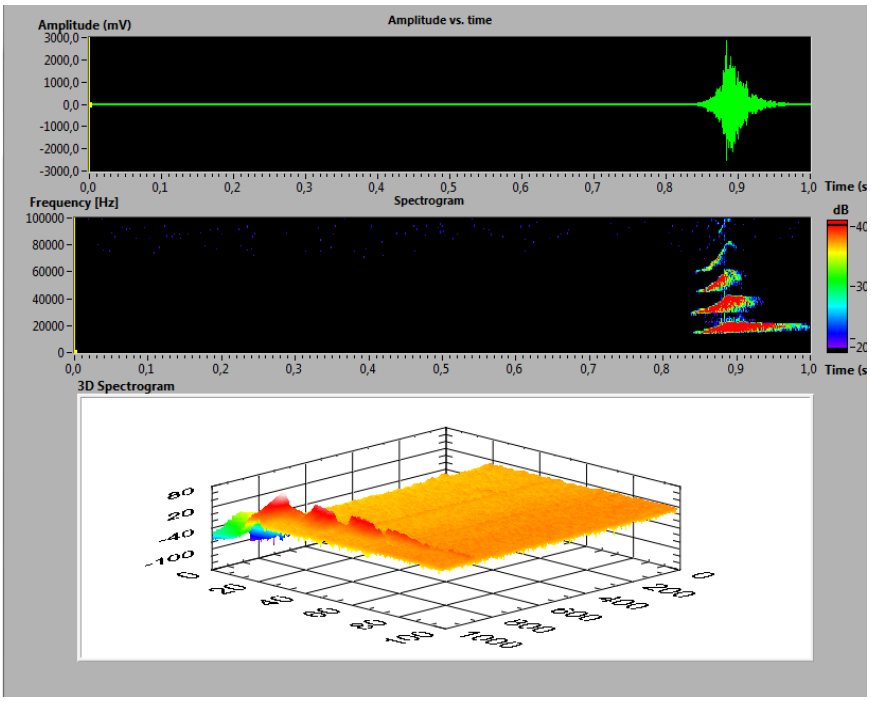

Figure 26: USV recorded during unrest in cage 14. In this cage one adult marmoset is separated from the others in this cage due to fighting in this family group.

Again all USV shown above consist of multiple frequency levels. The USV in figure 20, the top one of figure 21 and the one in figure 24 seem to stay equal in frequency. The lower USV of figure 21 and the USV in figures 22, 25 and 26 show a tendency to go up in frequency. Figure 23 shows three different types of USV, all in one second. From the first USV in this figure it is hard to tell what the frequency is doing, it seems to be that it is staying equal. The frequency of the second USV seem to go up at first and at the end of the USV go down while the third one shows a more wavy pattern. The USV that were recorded during unrest in the cage (figures 25 and 26) seem to resemble each other.

The USV per minute recorded during trial 2a and 2b can be seen in figure 27. For these trials, cages 2, 3 and 8 were used. Cage 2 and 3 were confronted with the stuffed snake first and afterwards with the stuffed cow while cage 8 was confronted with the stuffed cow first and afterwards with the stuffed snake.

As can be seen, all cages reacted on the snake more then they did on the cow. With the snake present in the cage, the animals in all three cages reacted on this with mobbing calls, staring at the object and did not approach the object closely but stayed at a certain distance and kept the object in sight. During the cow present in the cage, all cages reacted first with single tsik vocalizations and these vocalizations were less intense then with the snake present in the cage. At first they were staring at the cow, but after some time they did not pay that much attention to it anymore. One animal even sat on the wire mesh with it's back towards the cow and his tail almost touching it. All cages reacted more on the cow then on the snake. Cage 8 did react more on the cow than did cage 2 and 3, but cage 8 also reacted on the snake more than did cage 2 and 3.

No significance was found between the different events, the difference between a snake and a cow present in the cage was nearly significant (P = 0.06); the lack of significance may be due to the small sampling size.

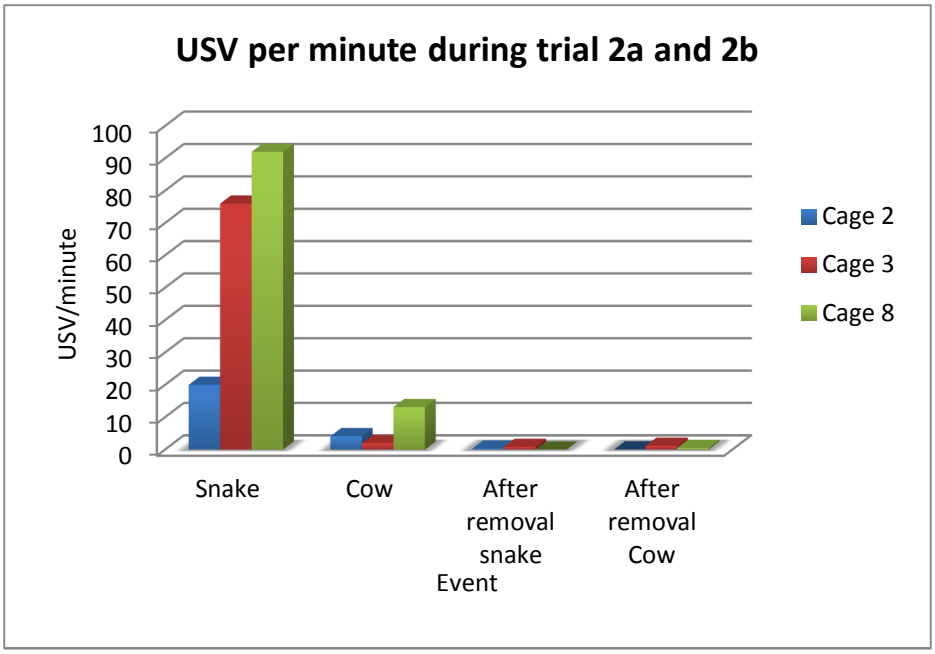

Figure 27: USV per minute during trial 2a and 2b of experiment 3.

An example of USV recorded during the snake present in the cage can be seen in figure 28. Figure 29 shows an example of USV recorded during the cow present in the cage. Both examples were recorded from cage 1.

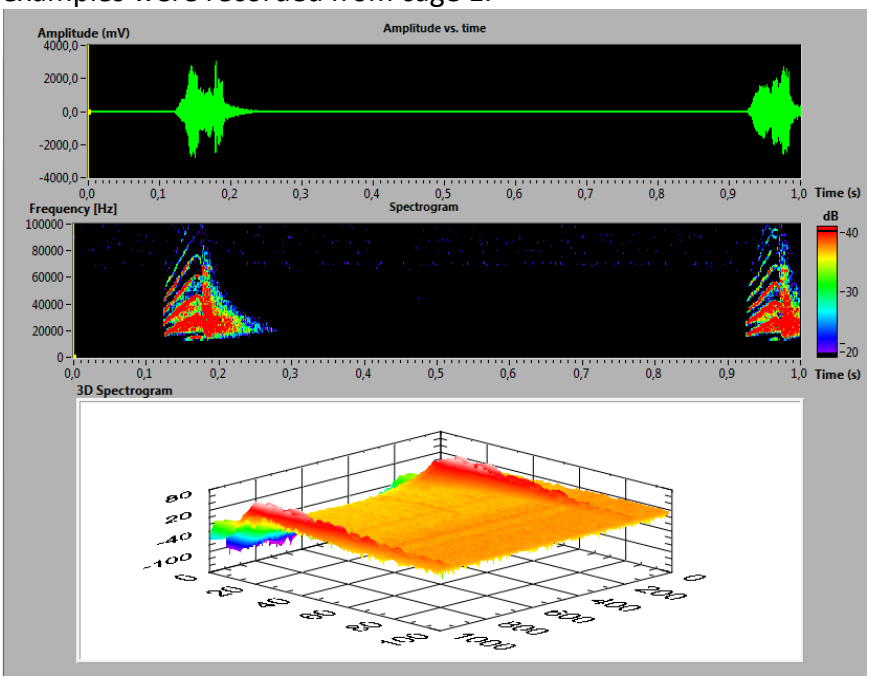

Figure 28: USV recorded during snake in the cage in experiment 3, trial 2a.

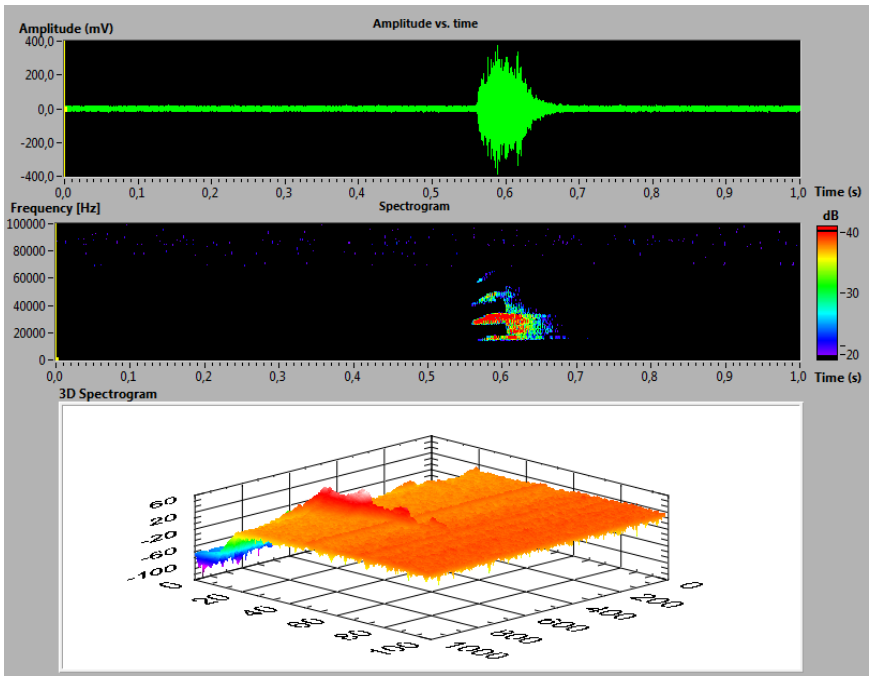

Figure 29: USV recorded during cow in the cage in experiment 3, trial 2b.

The USV in figure 28 shows a tendecy to go up in frequency, while the USV in figure 29 seems to stay equal in frequency. Both USV consist again of multiple frequency levels.

General results

In all experiments there where situations which are believed to be stressful for the animals. To see if there is a correlation between a stressful situation and the USV per minute found, all possible stressful situations are compared in figure 30. From experiment 1 this is the unsedated blood sampling, from experiment 2 the new arrivals in the room, separation from a cage mate, the showing of the blue soft leather catching glove and showing of the bottom of a boot; from experiment 3 were included new born animals in the family group with their different ages of the new born animals, unstable family group, one animal apart due to fighting in the group, a 'snake' in the cage and a stuffed animal in the cage. Since there were more trials per stressful situation, the average was taken into account for creating figure 21.

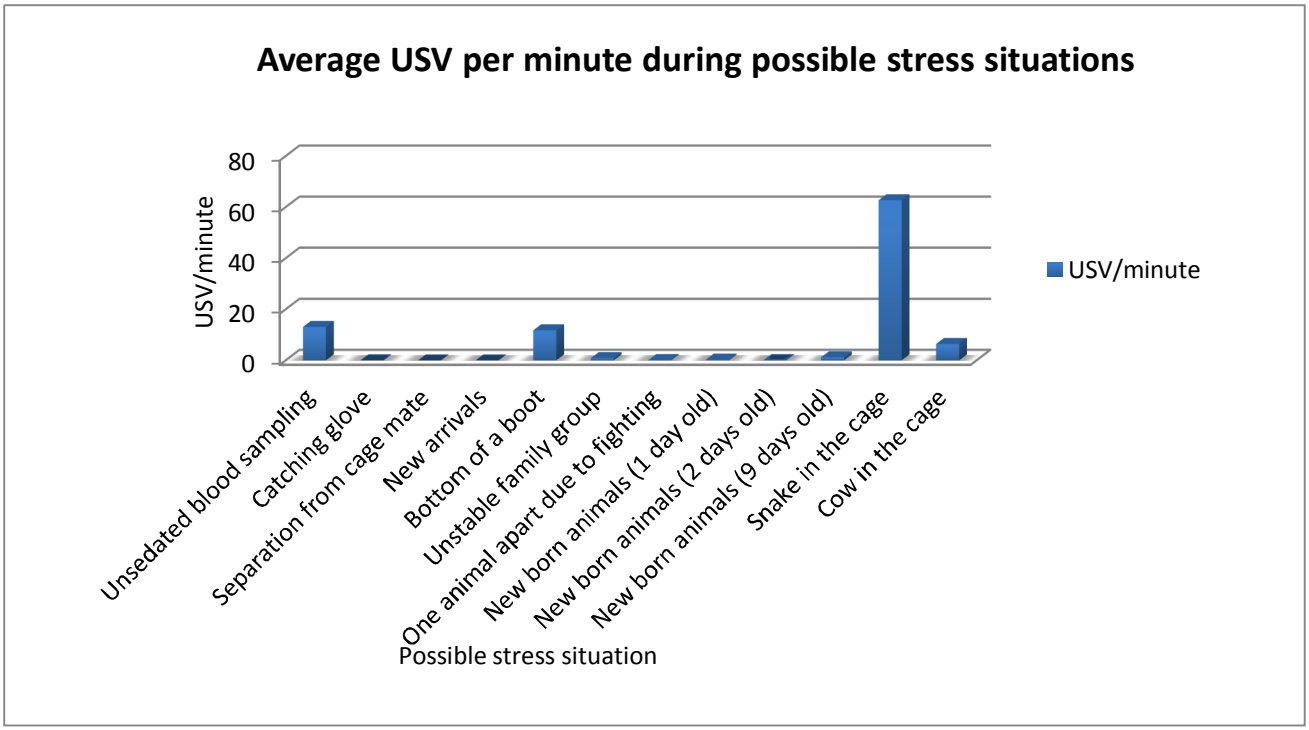

Figure 30: USV per minute recorded during possible stress situations.

The only significant difference here is the difference in USV per minute during unsedated blood sampling and presenting of the catching glove (P = 0.0048). Lack of significance between the other stress situations may be due to the small sampling size.

One goal of this study was to establish whether there is a difference in USV production between animals in the experimental housing and the animals in the breeding facility. Therefore the average USV per minute recorded are listed in figure 31. For experiment 2, the average USV per minute from trial A was used since this was considered to be a neutral situation. And for experiment 3, the average USV per minute from trial 1 was used since again this was the neutral situation. The cages with newborn animals, unstable family group and one animal apart from the rest of the family due to fighting were also included, since this was something that happened without influence of the experimenter. To rule out possible differences due to group size, the cages in experiment 3 were divided into three different groups: groups with two animals, groups with three animals and groups with five or more animals. In experiment 2, the recordings while arrival of new animals in the room were also included. It can be seen that the animals in the breeding facility produce more USV per minute then the animals in the experimental facility, regardless of the group size. There was no significance difference between any of the groups in figure 31.

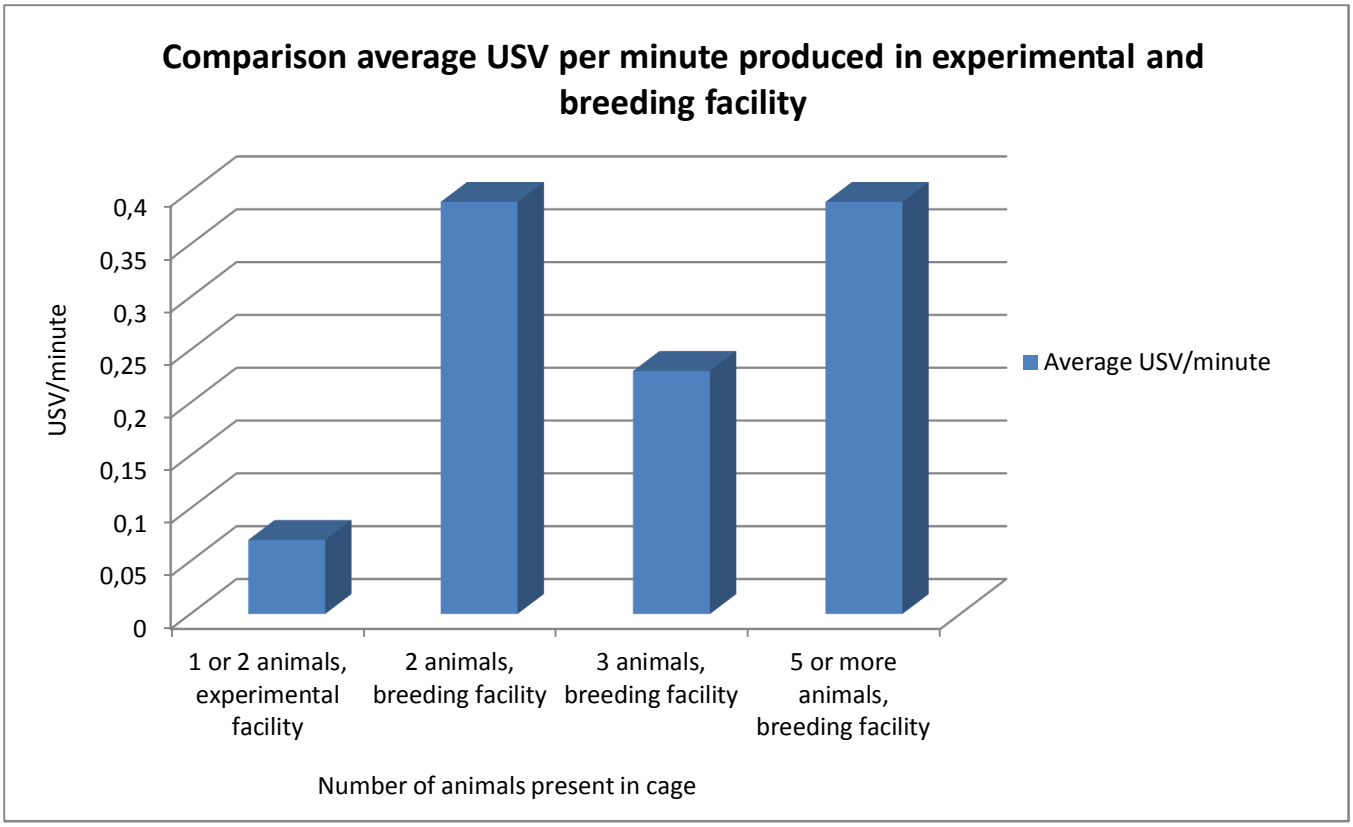

Figure 31: Comparison of the average USV per minute in neutral situation in the experimental facility (experiment 2) and the breeding facility (experiment 3).

DISCUSSION

Regarding the results of experiment 1, it seems to be that USV are possibly more related to stress then they are to (acute) pain, which is also the case in mice (Williams et al., 2008). This conclusion can readily be made because the number of USV per minute produced by the marmoset, did not increase when the needle had to be inserted multiple times. Since inserting of the needle would be expected to be painful, one would expect that, if USV correlate with (acute) pain, more USV would be recorded from animals where the needle was multiple times inserted. This was however not the case, even the opposite was true; marmosets were the needle was only inserted once, produced more USV. This difference was not significant and this may be due to the small sampling size or this difference is just coincidence. It is more likely however, that the number of USV recorded per minute has more relation with the amount of stress a marmoset experience from restrained and that the difference is due to individual variation and previous experiences during restrained. However, it may also mean that the insertion of a needle is not painful at all and that therefore there is no apparent correlation between (acute) pain and the production of USV in this experiment. To be sure that USV have indeed no correlation with (acute) pain, other painful stimuli should be used during the recording of USV.

Males tended to produce more USV per minute then females, but not significantly more. The lack of significance may be due to the small sample size or it may just be that again this difference is due to individual variation and previous experiences during restrained influences the amount of stress a marmoset experiences and so alters his USV production. The marmosets produced dominantly chatter vocalizations during restrained, these vocalizations seem to be a sign of intra- and intergroup aggression (Bezerra and Souto, 2008; www.marmosetcare.com). However, they are now not produced within a group or between two groups of marmoset, but from a single marmoset that is restrained. It may however be, that this is also an aggressive vocalization in this context, since they obviously do not like being restrained and fight against it by trying to bite the catching glove. Notable however, most marmosets stopped vocalizations and fighting against the restrained when the animal caretaker had restrained the animal completely.

In the positive trial of experiment 2, there were no USV recorded during the presentation of actual positive stimulus, the marshmallow. In only one instance did the marmosets react on the marshmallow with an audible vocalization. Since this was the only positive stimulus in this study, there were no USV found in a definite positive situation. One may conclude that marmosets may not produce USV in positive situations. However, in unpublished data from Herma van der Wiel, it was suggested that during play, which is regarded as positive, there were USV produced by marmosets. There are different possible explanations for this. One is that marmosets do produce USV in positive situations, but not with a marshmallow as a stimulus, this may well be the case. Another explanation may be that they do not see a marshmallow as a positive stimulus. However, since they are trained with marshmallows as a reward and training with this reward goes very well, this is not that plausible. A third explanation may be that, during what was regarded as play in the experiment of Herma van der Wiel, this was not all positive and therefore the USV recorded there may be due to negative interactions between the marmosets during the play. To be sure that marmosets indeed do or do not produce USV during positive situations, more positive stimuli should be explored.

During the different negative stimuli in experiment 2, there were virtually no USV recorded, with the exception of one USV recorded during showing of the catching glove. A possible explanation for the low number of USV recorded on showing the catching glove may be that the catching glove is not that stressful for the animals as thought. Another explanation may be that the catching glove is stressful, but that the animals react on this with staying still and silent instead of vocalizing and trying to flee from the object. It may also be that just holding the glove in front of the cage is not that stressful at all and that bringing the glove into the cage would be more stressful for the animals, which might provoke USV production. In experiment 1 it was seen that especially restrained in the catching glove was stressful what resulted in many audible vocalizations accompanied by USV. It may therefore be that not the catching glove itself is a negative stimulus for the marmosets, but that restrained is experienced as stressful. The single ek (or egg: Bezerra and Souta, 2008) call during presenting of the catching glove, is a vocalization believed to be made in situations of some alarm and vigilance behaviour, so therefore it is likely that presenting of the catching glove did alarm the marmosets in cage 2 to some extend. Whether this is because of the catching glove and the association with being caught or restrained, or whether this is because of the experimenter standing directly in front of the cage with an object, is not known.

Since the marmoset is a highly social primate, it was expected that the separation from their cage mate would be very stressful to the animals, but no USV were found here. A possible explanation for this may be that the separation was not that stressful for the animals since they were kept at the same, familiar cage during the trial and could still smell and hear their cage mate and so lost only sight of their cage mate. It was notable that only the marmoset that was kept at the lower part of the cage produced audible vocalizations. These vocalizations however have not been specified so it is hard to say if indeed the marmoset kept in the lower part of the cage, experienced stress from the separation. It can also be that these marmosets vocalized because at general, the marmosets stay more in the upper part of the cage then they do in the lower part of the cage, so the audible vocalizations may be evoked more because of confinement in the lower part of the cage then to be evoked by separation from their cage mate. To see if marmosets indeed do not communicate with vocalizations that extend in to the ultrasonic range during separation, a different kind of separation trial should be conducted. In this trial the animals should not be able to see or smell each other, but still be able to hear each other. It may also be that placing one marmoset in an unfamiliar cage, in sight and smell distance from his cage mate, is more stressful for the animal and would evoke USV production.

It was surprising to find that the sight of the bottom of a boot of the experimenter provoked a reaction with tsik-ek vocalizations, which were accompanied by USV. The reason for this is not known. It is thought that tsik-ek vocalizations are made in situations of some alarm (marmosetcare.com). According to Bezerra and Souta, who call this vocalization a tsik-egg call, it is associated with vigilance behaviour (Bezerra and Souta, 2008). Whether the reaction on the bottom of a boot is about curiosity or fear, remains uncertain. It is notable that in cage 2, not every tsik-ek vocalizations was accompanied by an USV. The reason for this is not known, it might be that the USV part of the vocalization is only present in certain situations.

The highest number of USV per minute in experiment 2 was recorded in the neutral trials. This was, however, all due to one animal; the single housed marmoset in cage 1. This marmoset produced multiple loud shrills during all trials, but especially during the neutral

trials. The loud shrill is regarded to be an isolation call (www.marmosetcare.com), so the fact that this animal was producing many loud shrills may be due to the fact that it was single housed. However, the marmoset in cage 3 did not produce loud shrills and was also single housed. Others describe this type of vocalization as a (long) phee call and state that these mostly are produced during isolation (Bezerra and Souta, 2008; Pistorio et al.; 2006). It is therefore most plausible that this marmoset indeed produced loud shrills/long phee calls due to the fact that it was single housed. An explanation for the fact that the other single housed marmoset in cage 3 did not produce loud shrills/long phee calls may be that this marmoset was moved in this housing room just recently and was therefore not that long isolated as the marmoset in cage 1.

During unrest in two different cages in experiment 3, there were USV found that pretty much resemble each other (see figures 25 and 26), the types of audible vocalizations during the unrest were not specified, so it is difficult to understand what these USV might mean, since the unrest might came from negative interactions between the animals (e.g. mild type of fighting, establishing the pecking order) or from a startle reflex from anything in their surrounding (e.g. sudden movement from the experimenter, noise in other cages). The USV in figure 23 however, was made during actual fighting in the group, so it is plausible to say that these types of USV can be found during at least fighting between family members. Whether these types of USV also occur during other events (e.g. fighting between different family groups, other negative events) remains unknown, however, they were not found during any other recording, so it may well be that these types of USV are indeed only occur during fighting. Since the second type of USV in figure 23 seems to resemble the ones in figures 25 and 26, it may be that the unrest in both groups was because of some mild type of fighting to establish the pecking order. However, since behaviour was not scored during the recording, this remains uncertain.

The USV found during a loud shrill (see figure 24) seems to resemble the USV found during a loud shrill in experiment 2 (see figure 15). In experiment 2 it was thought that this loud shrill or long phee call was uttered because of isolation. However, the animal that uttered the loud shrills in experiment 3, was part of a family group, what makes an isolation call less plausible. According to Bezerra and Souta, long phee calls are also uttered to make contact with conspecifics when in the presence of another group of common marmosets, this may then be an aggressive or territorial call (www.marmosetcare.com). This seems to be the case in experiment 3 since the marmoset uttering these calls, was making them towards other cages with marmosets. Whether the loud shrills of the animal in experiment 2 were isolation calls or were produced to make contact with other marmosets, is therefore uncertain.

The animals in cage 5 made a lot of tsik vocalizations and were very skittish about the Sonotrack™ and the experimenter. They were uttered in single series and therefore would be a call made during distress of animals (Bezerra and Souto, 2008; www.marmosetcare.com). Therefore it is plausible that this type of USV is uttered in alarming situations for the marmoset.

The USV recorded during the snake and cow present in the cage do not seem to resemble each other (see figures 28 and 29). The USV found with the cow in the cage tend to stay equal in frequency while the one found with the snake in the cage, tend to go up in frequency. During the snake present in the cage, the marmosets produced tsik vocalizations that rapidly followed each other, also known as a mobbing call which is known to be uttered in the presence of a predator (Bezerra and Souto, 2008; Clara et al., 2008;

www.marmosetcare.com), and this reaction was not seen during the cow in the cage. In all cages the animals reacted most on the snake. Cage 8 received the cow stimulus at first, so it is not plausible that the reaction on the cow was less intense because of habituation to a stuffed animal in the cage. Most research on the recognition of a snake model by marmosets has been done with animals that were not entirely snake naïve since most of this research have been done with animals that possible could have seen a snake in their direct environment (Cagni et al., 2011; Emile and Barros, 2009; Clara et al., 2008). It was therefore not known whether this mobbing reaction on a snake was a learned reaction or not. These animals could not have seen a snake before since they are born at the BPRC and no snakes live in the surrounding environment. The results of this experiment suggest that at least a part of the predator response is native to the animals and not entirely a learning progress. However, they did not flee from the snake, so it may well be that some parts of the predator response are indeed learned.

Comparing the different possible stress situations carried out during this study, there are four possible stress situations that by far cause the most USV per minute to be produced by the marmosets. These (see figure 30) are the unsedated blood sampling, showing the bottom of a boot, a snake in the cage and a cow in the cage. One explanation for this may be that these situations are the most stressful for the marmosets and therefore are accompanied by the most USV per minute. However, why the showing of the bottom of a boot is such a stressful situation, is not clear. This does not seem to resemble a possible predator and in contrast with the cow, it was not placed inside of the cage. However, the cow raises a similar question, since this also does not seem to resemble a predator. So in both cases the explanation may be that the animals react this way on something new in, or close by, their environment. For establishing this, more novel objects should be presented to the marmosets during recording of the USV they produce to see if they give a similar response to all novel objects that do not resemble a predator. Regarding the USV production, it seems to be that the moving in of new animals in the housing room in experiment 2, was not that stressful for the animals. However, the animal in cage 1 increased his scent marking behaviour, which is regarded to be stress behaviour in the common marmoset. It may therefore be that marmosets do not produce USV in every stressful situation or it may be that the production of USV has no correlation at all with stressful situations.

Regarding the results of this study, it seems to be that animals in the breeding facility, produce more USV then do the animals in the experimental facility. One explanation could be that with bigger groups, more vocalizations are necessary to keep the group together and to keep the social relations stable. However, in the breeding facility there are several cages containing only two or three relatives and they do not significantly produce less USV then to the bigger family groups and the still produce more USV on average then the animals in the experimental facility (see figure 31). Another explanation may be that, since the difference between the breeding facility and the experimental housing is not significant, this is just coincidence, since the sampling size is too small to be sure about this, this remains uncertain. A third possible explanation may be that the production of USV correlate with the housing conditions. The cages in the breeding facility are more spacious, posses an inside and outside enclosure, have more enrichment and the animals are less handled by the animal caretakers. This may well contribute to the production of the USV. However, again more research should be conducted to establish whether this is the case or if the difference found is due to coincidence.

There are some limitations on this study, the behaviour of the animals was not scored during recording, this makes it difficult to link types of USV to an animals' state of mind. Only vocalizations in the ultrasonic range of sound were recorded, since many audible vocalizations of the marmoset are described in the literature, simultaneously recording of both the audible vocalizations and USV could see more about the meanings of different USV. Not every audible vocalization was specified during this experiment what makes it more difficult to link types of USV to an animals' state of mind.

During every recording, the experimenter was present in front of the cage what may have altered the marmosets behaviour and vocalizations, since they were not familiar with the experimenter and the Sonotrack™ standing in front of the cage. This was not seen as a major barrier in this study since most attention went to the question if the common marmoset indeed produces USV and if so, if these have a possible correlation with positive and negative stimuli. However, if one would like to know how the production of USV is in marmosets that are not disturbed at all, the animals should get accustomed to the Sonotrack™ and then be recorded without the presence of a person. Ideally, behaviour would be recorded on camera simultaneously to establish possible correlations between different types of USV and behaviour. Moreover, it seems that it may also be interesting to record not only the USV but also the audible vocalizations. There might be a difference in calls that are now classified as the same, since there were some instances where a loud shrill was not accompanied by an USV and cases where a loud shrill did extend into the ultrasonic range. Therefore it might be that these calls serve a different purpose, which can give possible new insight in the behaviour and meaning of different types of vocalizations of the common marmoset.

In all three experiments, there were USV recorded from marmosets. It seems however, that USV do not come solely as is the case in rats and mice, but are accompanied by audible vocalizations. It seems to be that certain audible vocalizations of the common marmoset extend in the ultrasonic area. In this study this was the case for tsik, chatter, mobbing calls, loud shrills, screams and tsik-ek vocalizations. All these vocalizations are, accordingly to the marmoset care website, negative welfare indicators. Since not all audible vocalizations were specified due to more attention towards the USV, this may mean that possible more audible vocalizations extend into the ultrasonic range. There were no USV recorded without a simultaneously audible vocalization in experiment 2. Only in experiment 2 there was special attention to the audible vocalizations, so it is not plausible that USV are produced solely by the common marmoset. This rises the question whether USV alone are a good indicator for welfare in the marmoset. However, not with every tsik and loud shrill vocalizations there were USV recorded, so in some cases these vocalizations extend into the ultrasonic range, but in other cases they do not. It may therefore be that vocalizations that extend into the ultrasonic range have another meaning then the same vocalizations that do not extend into the ultrasonic range. To establish whether this is true, a playback experiment should be conducted to test if the USV serve a function in intraspecific communication in the common marmoset.

CONCLUSION

In conclusion; the common marmoset indeed produces USV but it seems to be that audible vocalizations extend into the ultrasonic range rather then there are solely USV produced by the common marmoset. Most USV recorded in this experiment were during stressful situations, with the highest USV per minute during a stuffed snake model present in a cage. However, not in every stressful situation there were USV recorded, so it may be that the production of USV has no correlation with stressful situations. More research is necessary to establish this. The meanings of the USV are not clear, to gain more knowledge about the meanings of the USV, more research is necessary. This would preferably be a study where vocalizations, both audible and ultrasonic, are combined with behaviour scoring. This may also give new insights in the meanings of the behaviour of the common marmoset.
To establish whether USV have a function in marmosets, a playback experiment would be necessary. With this, it may also be necessary to adjust the auditory threshold found by Osmanski and Wang (2011). As far as USV regarding as an objective non-invasive and non-intrusive measurement for the assessment of pain and distress by common marmosets, it seems to be more promising to use vocalizations in total, rather then using the USV alone.

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