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Measurement of oxytocin in the saliva of cattle

Diplomarbeit

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Table of contents

1. Introduction 1
2. Material and methods 4
   1) Animals, housing and management 4
   2) Experimental design 4
      a) “Control” condition 5
      b) “Familiar” condition 5
      c) “Unfamiliar” condition 5
   3) Behavioural observations 6
   4) Physiological analyses 8
      d) Analysis of oxytocin concentration 8
      e) Analysis of cortisol concentration 10
   5) Data preparation and statistical analysis 11
3. Results 13
4. Discussion 20
5. Summary in German and in English language 25
   6) Summary in German language 25
   7) Summary in English language 26
6. Reference list 26
7. List of tables and figures 29
   8) Tables 29
   9) Figures 30

1. Introduction

Animal welfare is not only based on physical health, but also on the animals' feelings and experiences, positive and negative (Hewson 2003; Duncan 2005; Mellor 2012; Rault et al. 2020a). The components of positive animal welfare depend on the ethical views of the scientist or the observer (Rault et al. 2020a). Some researchers do not only talk about welfare in general but focus on positive animal welfare. The World Organization for Animal Health (2019) defined good welfare as a state in which "the animal is healthy, comfortable, well-nourished, safe, is not suffering from unpleasant states such as pain, fear and distress and is able to express behaviours that are important for its physical and mental state". "Positive welfare" Rault et al. (2020a) defined as "what the animal likes (positive affective state) and what the animal wants (positive motivation to obtain a resource)" and "mental and physical states that exceed what is necessary for immediate survival", emphasizing the "welfare benefits of providing animals with greater opportunities for positive experiences, in addition to minimizing negative experiences".

One of the factors that can promote good or positive animal welfare is a positive human-animal-relationship, which has additional positive effects; for example dairy cattle give milk faster and kick less if they have a better relationship with their caretaker (Bertenshaw et al. 2008; Rault et al. 2020b). So familiar caretakers can have a positive influence on animals' welfare and production if they include routines like stroking cattle in their daily work and avoid negative experiences for the animals to improve their relationship to humans. Stroking is most effective at the ventral neck: it is a body region where cattle often lick each other in the context of positive social behaviour and being stroked by a human at the ventral neck causes a lower heart rate and more neck stretching, which is a behaviour connected with comfort, than being stroked at other body regions (Schmied et al. 2008).

Affective states may influence physiological processes, such as changes in hormone concentrations, in human and animals. Oxytocin is a mammalian peptide hormone that is released during parturition and lactation but also during social contact and it affects social behaviour. Oxytocin is such a hormone which is influenced by affective states and it is important in social adaptation of children (Feldman 2012). Social attachment may also be promoted by oxytocin (Mellor 2012; Crockford et al. 2013). Furthermore oxytocin has, with its multiple effects on the animals, an important influence on animal welfare (Rault et al. 2017).

Oxytocin can be released during different situations with social and physical contact, like breast feeding in human (White-Traut et al. 2009) or animals which played with human and were nuzzled by human (MacLean et al. 2017). Positive social behaviour was promoted by intranasal oxytocin administration in dogs towards their owners and familiar conspecifics (Romero et al. 2014). In another study, with a stranger in the room, dogs' reaction towards their owner was less friendly than without an approaching stranger after the application of oxytocin (Hernádi et al. 2015).Oxytocin can be measured in animals' blood (Bienboire-Frosini et al. 2017), cerebrospinal fluid (Rault 2016), urine (Mitsui et al. 2011) and in saliva (MacLean et al. 2018; Wang et al. 2019). In humans, there are already several studies about the reliable measurement of salivary oxytocin concentrations and their change in context with social contact, for example in anticipation of breast feeding: oxytocin concentrations were highest before breast feeding, decreased during breast feeding and increased again after breast feeding, but not as high as before breast feeding (White-Traut et al. 2009). In pigs, Rault (2016) investigated oxytocin concentrations in the cerebrospinal fluid during and after human contact. After positive human-animal interactions the oxytocin concentration was higher than after negative human-animal interactions (Rault 2016). In dogs it is also known that positive human-animal interactions increase oxytocin concentration in saliva and blood (MacLean et al. 2017). In cattle there is a lack of knowledge about salivary oxytocin concentration and its changes. There is only one study where salivary oxytocin was used to investigate cow-calf bonding in different breeds but without validation (Geburt et al. 2015). Salivary oxytocin could be a good alternative to taking blood samples, which has been done most often up to now, because saliva sampling is non-invasive.

The frequency and duration of behaviours can be an indicator of a positive affective state and may relate to oxytocin concentrations (Rault et al. 2020a). Thus, it is important not only to investigate the oxytocin concentration but also the behaviour and the connection between them. The linking between oxytocin and familiarity is often discussed and there are no final results until now (Marshall-Pescini et al. 2019). In wild chimpanzees Crockford et al. (2013) investigated urine oxytocin concentrations after grooming with kin bond conspecifics, non-kin bond conspecifics and bond partners. They found out that neither only grooming nor only the presence of a bonded partner raised the urinary oxytocin level (Crockford et al. 2013). Hence, both physical and psychological factors are crucial, as oxytocin only increase after grooming

with a bonded partner. However further research is still needed to get more clarity about this connection between oxytocin and familiarity. We hypothesized that oxytocin increases after positive human-animal interactions, but more after the interaction with a familiar human oxytocin than after the interaction with an unfamiliar human. Being stroked by a familiar person induces oxytocin release in dogs (Uvnäs-Moberg et al. 2014). We assume that cattle also release oxytocin while being stroked. Cattle express their enjoyment while being stroked by stretching their necks (Schmied et al. 2008). It can thus be assumed that cattle that enjoy stroking more than others will also be stroked for a longer time, show more neck stretching and have a higher increase in oxytocin concentration.

Therefore, we hypothesized that oxytocin is detectable, and that the oxytocin concentration can be reliably measured in saliva samples from cattle. Finally, we hypothesized that salivary oxytocin concentration and the shown behaviours, especially acceptance of stroking and neck stretching, are positively associated with each other.

2. Material and methods

1) Animals, housing and management

Eighteen Austrian Simmental were part of the study. They were between 14 and 26 months old and primiparous. Twelve of them were pregnant for between one and 183 days. The cattle lived in groups in a pen with a barn and a paddock. They were housed in three different groups. For the first batch all of them were in the same group and in the second batch they were in two different groups. The heifers on the farm are mainly used as replacement animals for the Vetfarm Kremesberg, or used for research. All of the animals in the study were used to human contact.

This project was reviewed and approved by the institutional ethics and animal welfare committee in accordance with GSP guidelines and national legislation (ETK 06/11/2017).

2) Experimental design

In the morning the test area was built in the pen which had a size of 4 x 5.50 m for the first batch and a size of about 4.70 x 5.10 m for the second batch. For the second batch the testing pen was an irregular rectangle. The testing pen was divided in thirds with adhesive tape as marker to see how close to the human the animal was located. The test took place consistently between 11 am and 2 pm to minimise the influence of a potential circadian rhythm on the measures being collected. A video recorder (HD-CX900 E, Sony, Germany) was set up above the pen to record the animal's behaviour during the test. The first seven animals were drifted into the feeding rack, which was locked after the cattle were inside. From the second day we locked the first five animals in the feeding rack because the animals waited too long without food on the first day. The remaining cattle of the group were drifted into the barn. After at first locked animals finished their test, they were drifted back into the barn and the missing animals were drifted into the feeding rack. The animals in the feeding rack did not have hay while waiting to avoid food residue to interfere with saliva measurements. After 30 minutes without food, the video recorder was started and the first saliva sample was taken. Sampling meant that saliva was collected with a swab held by the experimenter with a dressing forceps and left in the mouth angle for at least 45 s and a maximum of two minutes. The experimenter was unknown to the animals.

For saliva sampling, SalivaBio Children's Swabs (Salimetrics, USA) were used, which contain a cotton swab to collect saliva samples from humans or non-human animals. The duration of 45 s until two minutes has been selected to exclude that the concentration of oxytocin in the saliva changes because of the handling procedure. After sampling, the swab was put back in the plastic tube and immediately stored in a Styrofoam box on dry ice. Back at the institute the samples were stored at -20 °C until analysis. Altogether, six samples were collected from each animal, one before and one after each test condition.

Just after the sample prior to the test the animal was drifted to testing pen. The animal to be tested was separated physically from its group within a familiar pen just before testing to avoid interference from other animals during the test. It was still able to see and smell its conspecifics to avoid separation distress. It stayed inside for ten minutes and was presented with one of the three conditions: control condition, familiar treatment or unfamiliar treatment.

a) "Control" condition

The cattle stayed alone for ten minutes in the testing pen, no human was in sight.

b) "Familiar" condition

The cattle stayed with a familiar person standing in one corner in the testing pen. The test area was entered by the familiar person while the animal was sampled. The human did not move and tried to attract the animal by talking and doing friendly gestures. The human ceased trying to attract the animal after five minutes. When the animal came close enough the person stroked it. In case that the animal did not approach within the first two minutes the human tried to approach to the animal. If the animal stayed in the same place, the person started stroking it, if not the person remained standing where she was after the approach.

c) "Unfamiliar" condition

The sampling and test was executed the same way as it was in the condition "familiar" treatment. The only difference was that the human was unknown to the animal.

All animals experienced all conditions once, serving as their own controls in a within-subject design. Every animal was subjected to the three different conditions on three different days over three following weeks with one week in between tests. The order was randomized and counterbalanced across subjects to avoid potential order effects.

After the test the second saliva sample was taken. The animals were distracted with stroking by two people from both sides to make them standing still. Ten times the cattle had to be locked in the feeding rack for the second sample because it was impossible to take the sample while the animal was free to move. On four times it took longer than two minutes for the second sample. After sampling the animal was drifted back to the feeding rack. The procedure was the same for the following animals.

3) Behavioural observations

The animals' behaviour was recorded during the tests. The videos were analysed with the software BORIS, using an ethogram for the observation (Table 1). An inter- and intra-observer reliability was done. Every test was watched twice. Once for the behaviour and once for the localisation in the testing pen. As every test had a duration of ten minutes, 600 s were analysed. An aberration of one second was accepted. Two tests could not be analysed because of technical problems the tests were not recorded completely. One was "familiar" condition and one was "control" condition. The videos were from two different animals.

Table 1: Ethogram of behaviours analysed in cattle.

Categories and behaviours	**Definition**
Position	
Square (1-9; numbering starts with square containing human -> square 1)	The animal stands in square with the major part of the foremost forefoot; as soon as the major part of the foremost forefoot is set into another square, that square is recorded. Square number is modified with "h" if the human is in the same square and with "n" if not. If the animal moves backwards the square with the major part of the foremost forefoot is recorded.
Square 0	With the head in feeding rack or outside of testing pen , no modifier if human is in the same square or not

Behaviour	
Being touched	Human actively touches the animal with hand or finger movements such as petting, scratching, stroking. Behaviour ends if interrupted for > 2 s.
Alert	Both ears forward, head up, immobile > 2 s.
Alert towards human/human corner	The animal is immobile for > 2 s and has its head oriented toward the human or (in control condition) square 1 at an angle < 45° ; ears erected and toward the human or square 1
Locomotion	The animal moves its feet. Behaviour ends if interrupted for > 2 s. Behaviour is not scored while the animal is being touched or showing exploration.
Exploration	The animal's snout/muzzle is less than 10 cm from the ground or an object. Behaviour ends if interrupted for > 2 s. Behaviour is not scored while the animal is being touched.
Inactive	The animal is standing still or lying down and does not show any other of the defined behaviours.
Self-directed behaviour	The animal scratches or licks itself, using the tongue, teeth, feet or an object.
Defecation	The animal releases manure.
Urination	The animal releases urine.
Vocalisation	The animal emits a sound.
Feeding	The animal takes a food item in its mouth and chews; includes licking/nibbling on food items or salt stone and head through the feeding rack in a position to

	allow feeding. Behaviour is not scored while the animal is being touched.
Other behaviour	Behaviour not listed above (comment about which behaviour seen)
Not visible	Not sufficient visual information on what the animal is doing
Neck stretching	The animal positions neck and head actively in an outstretched line, either up, down, or forward, for at least 2 s. Behaviour ends if interrupted for > 2 s., modified with "h" if the animal is in contact with the human and with "n" if not.

4) Physiological analyses

d) Analysis of oxytocin concentration

Oxytocin concentration was analysed from both samples, and cortisol concentration from the post-test sample. Before analysing the samples, the validation was done by the following steps. The samples were used neat and an extraction was done. For the extraction, a modified Enzo oxytocin extraction protocol (Enzo Life Sciences (ELS) AG, Switzerland) was used, according to Bienboire-Frosini et al. (2017). An equal volume of 0.1 % trifluoroacetic acid (TFA) in ultrapure water (TFA-H2O) was added to the sample. It was centrifuged at 1700 g for 15 minutes at 4 °C to clarify and save the supernatant. After that 200 mg C18 Sep-Pak column with 1 ml acetonitrile was equilibrated, followed by 10 ml of 0.1 % TFA-H2O. The supernatant was applied to the Sep-Pak column and washed with 10 ml of 0.1 % TFA-H2O. The wash was discarded. The centrifuged sample was added at the column, without possible sediment. The sample was slowly eluted by applying 3 ml of a solution comprised of 60 % acetonitrile and 40 % of 0.1 % TFA-H2O. The eluate was collected in a glass tube. It was evaporated to dryness using a centrifugal concentrator under vacuum and kept at cold temperature (Speedvac system ISS100 SAVANT with cooling system). Extracted samples were then stored at -20 °C overnight and reconstituted with Assay Buffer immediately before the assaying. At least 220 µl volume

was needed per sample in order to have enough material to run duplicates. After that the intra- and inter-assay coefficients of variability was done.

For the oxytocin assay itself, samples were spun in an Eppendorf cool centrifuge at 1500 g for 20 minutes with 4 °C. The aliquots were taken in ice after spinning. For the analysis the Oxytocin Elisa Kit (Cayman Chemical, USA) was used. It was stored at -20 °C. The extract was resuspended in 220 µl of ELISA Buffer and was vortexed after. The next step was to prepare the Assay-Specific Reagents. The contents of the Oxytocin ELISA Standard were reconstituted with 1 ml ultrapure water. 7.5 nm/ml was the concentration of the solution which is storable for one week with a temperature of 4 °C or -20 °C. For the standard eight test tubes were numbered from one to eight. Into tube number one were 900 µl Elisa Buffer and into tube number two till 500 µl Elisa Buffer pipetted. 100 µl of 7.5 ng/ml solution was transferred into tube number one and was mixed afterwards. 500 µl were removed from tube one and pipetted to tube number two. Then it was mixed. The next 500 µl were taken from tube two to tube three. This procedure was repeated until tube number eight. Within one hour the diluted standards must be used. For the Oxytocin AChE Tracer were reconstituted with 6 ml ELISA Buffer. The same was done with 100 dtn Oxitocin Polyclonal Antiserum for the Assay. A 96-well plate was used which was included in the kit. Every plate had to contain two blank wells, two NSB wells, two B0 wells and eight standards run in duplicate. Now everything was prepared for the Assay. First 150 µl ELISA Buffer were added to the NSB wells and 100 µl to the B0 wells. 100 µl from the number eight tube of Oxytocin ELISA Standard were pipetted into the standard wells with the number eight. The Oxytocin ELISA Standard from tube number seven were added to the standard wells with the number seven. This was repeated to all the other Oxytocin ELISA Standards. 100 µl sample were added to each well, with each sample ran in duplicates. Except of the Total activity well and the blank wells in every well 50 µl of Oxytocin AChE Tracer were added. The same amount of Oxytocin Polyclonal Antiserum was pipetted into every well except of the Total Activity, the Blank and the NSB wells. The plate was covered with a plastic film and was incubated overnight which means 18 hours at a temperature of 4 °C. Ellman's Reagent was reconstituted with 100 dtn vial Ellman's Reagent and 20 ml of ultrapure water. It is important to prepare this right before use because it is unstable. Wells were emptied and washed five times with Wash Buffer. 200 µl Ellman's Reagent were added to each well. 5 µl of tracer were pipetted to the TA wells. The plate was covered with plastic film and mixed on

an orbital shaker. It took 90 minutes to develop. Before reading the plate, it was cleaned so the bottom was wiped with a clean tissue. The cover was removed carefully. The plate was read at between 405 and 420 nm. The B0 wells had to be in the range of 0.3-1.0 A.U.. Absorbance reading of the blank wells was subtracted from the absorbance readings from the rest of the plate. The absorbance readings from the NSB and the B0 wells were averaged. The average NSB was subtracted from the B0 average. This was done to get the corrected maximum binding. For the remaining wells B/B0 was calculated. This was done by subtracting the average NSB absorbance from the S1 absorbance. Afterwards it was divided by the corrected B0. This was repeated for the sample wells and S2-S8. The Standard Curve was plotted by the formula: logit (B/B0) = In [B/B0/(1-B/B0)]. A linear regression fit was performed. For the sample concentration B/B0 value was calculated for each sample. The concentration of the samples was determined by using the equation obtained from the standard curve plot. The unity of the result was pg of oxytocin per ml of saliva.

e) Analysis of cortisol concentration

For cortisol the enzyme immunoassay kit (Salimetrics, USA) was used. It was stored at 4 °C. All reagents were mixed and brought to room temperature and the microtiter plate as well. A wash buffer was prepared. The plate layout was determined with standards, controls and saliva samples as duplicates. The strips were put in the strip holder. Non-specific binding wells were placed and the strips one and two were removed from the strip holder and the bottom wells were broken off. The strips were placed back in the strip holder and H-1, 2 with the NSB wells were left blank. Two were broken off from the strip of NSB included in the foil pouch. They were placed in H-1, 2. The foil pouch with unused wells and desiccant was released. It was stored in 4 °C. 24 ml of Assay Diluent were pipetted into the disposal tube. Afterwards it was set aside. 25 µl of standards, controls and saliva samples were pipetted into appropriated wells. The same amount of Assay Diluent was pipetted into two wells which were served as zero. 25 µl of Assay Diluent were pipetted into each NSB well. The Enzyme Conjugate was diluted 1:1600 by adding 15 µl of the conjugate to the 24 ml tube of Assay Diluent. The conjugate tube was centrifuged for a few seconds to bring the liquid down to the tube bottom. Immediately the diluted conjugate solution was mixed and 200 µl were added to each well with a multichannel pipette. On a plate rotator the plate was mixed for five minutes at 400 rounds per minute and incubated at room temperature for one hour. With 1X wash buffer the plate was washed four

times by gently squirting wash buffer into each well with a squirt bottle and discarding the liquid over a sink. After each wash the plate was thoroughly blotted on paper towels before turning upright. After washing 200 µl of TMB Substrate Solution were added to each well with a multichannel pipette. For five minutes it was mixed again on a plate rotator at 400 rounds per minute and afterwards incubated in the dark at room temperature for additional 25 minutes. With a multichannel pipette 50 µl of Stop Solution were added. The plate has to be mixed again on the plate rotator at 400 rounds per minute for three minutes. It had to stay on the plate until every well turned to yellow. The bottom of the plate had to be wiped off with a cloth which was water-moistened and lint-free scarf. After that it had to be wiped dry. The plate has to be read within ten minutes after adding the Stop solution. It should be read at 450 nm in a plate reader. For the quality control with every assay High and Low Controls had to be done.

For the calculation the average optical density had to be computed for the duplicate wells. The average optical density of the NSB wells had to be subtracted from the optical density of the standards, zero, controls and saliva samples. The percent bound is calculated by dividing the optical density of each well by the average optical density for the zero. It must be done for every control, standard and saliva sample. The control and saliva samples concentrations were determined by interpolation with a data reduction software. It is recommended to use a 4 parameter non-linear regression curve fit. If the Cortisol concentration was higher than 3 µg/dl it was repeated with an Assay Diluent in order to fall within the detectable range of the assay.

5) Data preparation and statistical analysis

The statistics were made with the program R. From two cattle on two different days, one in “control” condition and one in “familiar” condition, there were no data because of technical problems. The amount of oxytocin from pre to post was determined in for each animal for the different conditions as well as the amount and the percentage of the oxytocin change in the different conditions. The spearman rank correlation was determined from the pre-test Oxytocin concentration and the pre-test saliva quantity, the post-test Oxytocin concentration and the post-test saliva quantity, the pre-test oxytocin concentration and the start number. This was the statistics of the physiological variables. The next thing was to test if the saliva quantity was individual based which was done with a Spearman coefficient from the pre- and post-test saliva quantity. To control if the saliva quantity differs between the different conditions a linear mixed

model was used. One linear mixed model for the pre and one for the post saliva quantity was generated. Because there could be more factors which could have an influence on the post saliva quantity except of the condition, several other factors had to be proved. This was done with four Spearman's rank correlations. For them it was not possible to compute an exact p-value. Test order and the start (Spearman's rank correlation, $\rho = 0.96$, $p < 2.2e\text{-}16$), the human squared and the duration of being touched ($\rho = 0.95$, $p < 2.2e\text{-}16$), the numbers of the squares and the locomotion ($\rho = 0.81$, $p < 0.001$) were significantly. To avoid multicollinearity only one factor of each test was used for the following models. The next thing was the change by condition. A linear mixed model with random slopes was generated. Chi2 was charged for Start, saliva quantity and the interactions between time and condition and time and interval. As intercept pre-test time, condition "familiar", batch one was used. The estimate was determined for time post, the condition "unfamiliar", batch two, interval, start, saliva quantity, time post and "unfamiliar", time-post and "control", time-post and interval. Next it was counted how often which behaviour occurred. *Urination* occurred only three times and *defecation* seven times. These were considered too rare and therefore were not analysed. The cattle vocalised 13 times, which is quite a low number, but it was used in our statistics. Afterwards it was shown in graphs how often the behaviours occurred in different conditions. Interesting to know was if the oxytocin change was influenced by the animals' behaviour. Again, a linear mixed model was generated. Self-directed *behaviour* (-293.263) had the highest Log likelihood so it was chosen to keep the major random slope. First Chi2 was calculated for the linear mixed model *self-directed behaviour* and afterwards it was charged for the rest of the behaviours. For the Intercept which was batch one and post-test oxytocin was used the Estimate was calculated for the behaviours, batch two and pre-test oxytocin. The last to test was if the behaviour was associated with condition or pre-test oxytocin. With GLMMs it was analysed but only for neck stretching and being stroked. As condition only familiar and unfamiliar could be used because they could not appear in the control condition.

3. Results

Pre-test oxytocin and pre-test saliva quantity (Spearman's rank correlation, $\rho = -0.37$, $p = 0.006$) were significantly, but weakly correlated. Post-test oxytocin and post-test saliva quantity ($\rho = -0.28$, $p = 0.04$) were also significantly but weakly correlated. Pre-test oxytocin and test order tended to be correlated ($\rho = -0.23$, $p = 0.09$).

The linear mixed-effects models did not reveal a significant effect of the condition on the saliva quantity (LMM, pre-test saliva quantity $\chi^2 = 0.154$, $df = 2$, $p = 0.926$, post-test saliva quantity $\chi^2 = 0.317$, $df = 2$, $p = 0.853$).

The comparison of the full and null model did not reveal a significant effect of the test condition on oxytocin concentration ($\chi^2 = 2.1$, $p = 0.35$) (Fig. 1). Regarding the confounding variables, saliva quantity ($\chi^2 = 12.28$, $p < 0.001$) and start time ($\chi^2 = 8.57$, $p=0.003$) showed a negative association with the oxytocin concentration. Batch ($\chi^2 = 5.54$, $p = 0.02$) showed a positive association with the oxytocin concentration (Tab. 2).

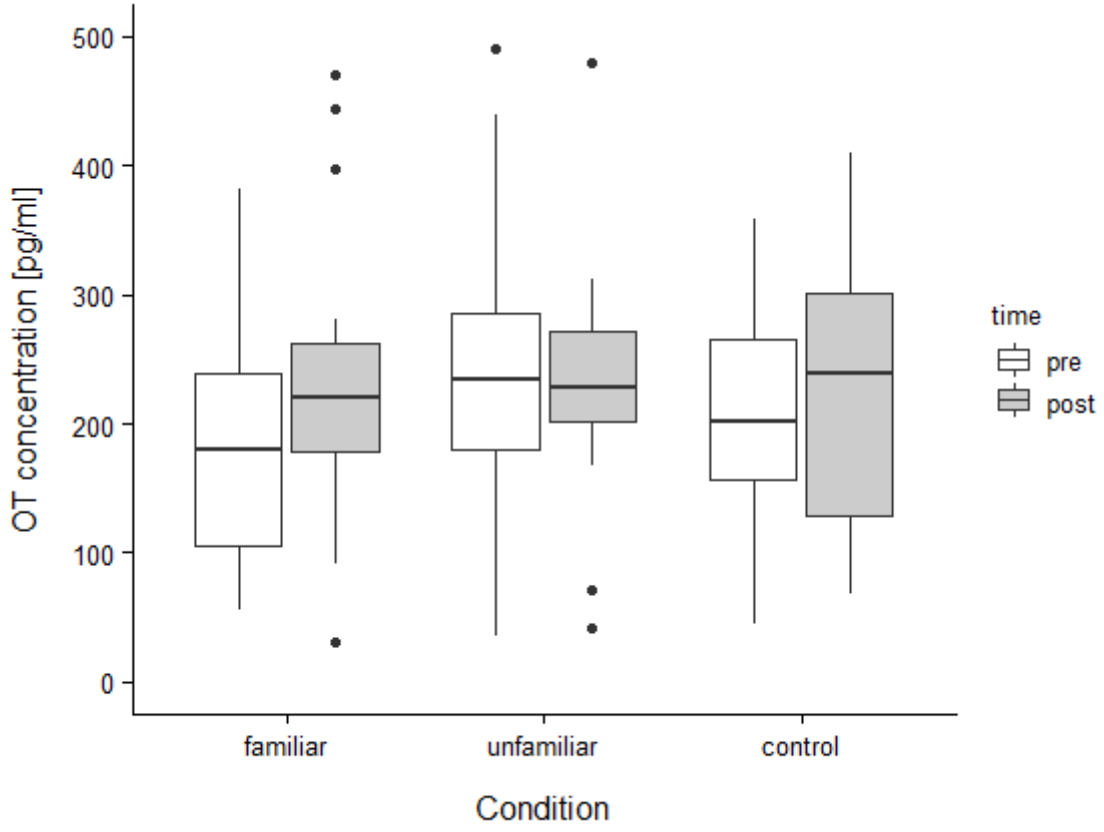

Figure 1: Pre- and post-test oxytocin concentration from 18 Simmental heifers in the experimental conditions familiar, unfamiliar and control. The tested main effect was the interaction between time and condition (p = 0.35) where no significance was revealed.

Table 2: Full model of the oxytocin concentration

Effects	**Coefficients**	**SE**	**χ^2**	**Df**	**P**
(Intercept)	158.336	23.326			
Time			-		
Post	36.05	26.59			
Condition					
Unfamiliar	51.849	26.545			

Control	26.054	26.747			
Batch			5.544	1	0.019
Batch 2	58.081	22.99			
Interval	-2.192	12.272			
Start	-25.803	8.087	8.568	1	0.003
Saliva quantity	-40.752	9.054	12.284	1	<0.001
Time : condition			2.097	2	0.350
Time post : unfamiliar	-53.415	37.484			
Time post : control	-17.034	37.686			
Time : interval			0.611	1	0.434
Time post : interval	-12.827	15.499			

Regarding the association of post-oxytocin concentration with the different behaviours, the full and the null model differed significantly ($\chi^2 = 18.76$, df = 8, p = 0.02). *Vocalisations* (LMM, $\chi^2 = 6.804$, p = 0.009), *self-directed behaviour* ($\chi^2 = 6.516$, p = 0.01) and *neck stretching* ($\chi^2 = 3.831$, p = 0.05) showed significant positive associations with post-test oxytocin concentration (Fig. 2). Pre-test oxytocin ($\chi^2 = 6.354$, p = 0.012) and batch ($\chi^2 = 7.39$, p=0.007) as co-variables showed a positive association with the post-test oxytocin concentration. (Tab. 3).

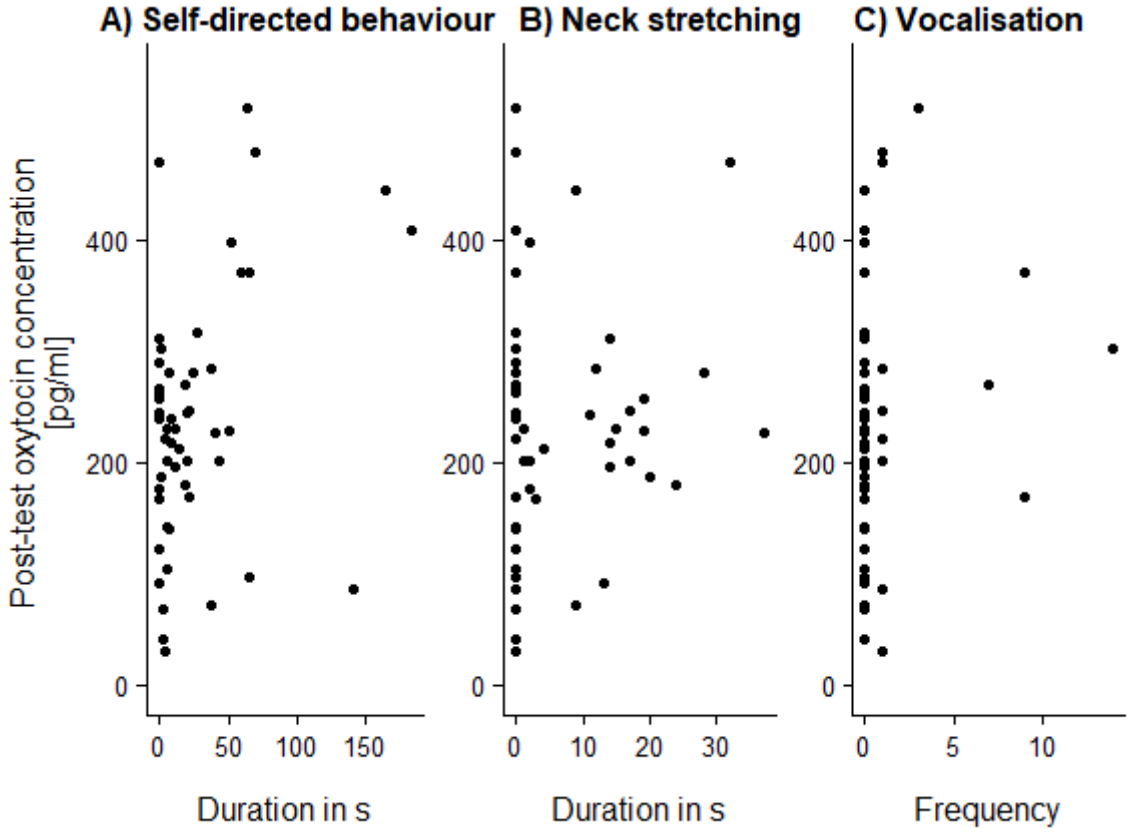

Figure 2: Post-test oxytocin concentration for the durations (of the total time observed) of self-directed behaviour (A), neck stretching (B) (only in the conditions familiar and unfamiliar) and the frequency of vocalisation (C) (in the total time observed) from Simmental heifers (n = 18) on the three experimental days. Statistics LMM: the three behaviours self-directed behaviour (p = 0.01), neck stretching (p = 0.05) and vocalisation (p = 0.009) showed a significant positive association with post-test oxytocin concentration.

Table 3: Full model for the associations of behaviours with post-test oxytocin concentrations. Statistically significant results appear in bold if the effects were taken out of the null model.

Effects	**Coefficients**	**SE**	**χ^2**	**df**	**p**
(Intercept)	202.105	14.447			
Alert	8.584	15.415	0.309	1	0.578
Alert to human	13.801	10.597	1.636	1	0.200

Touched	-56.652	67.380	0.696	1	0.404
Expolaration	-71.014	46.009	2.300	1	0.129
Inactive	-0.837	27.605	0.001	1	0.976
Locomotion	6.005	20.346	0.085	1	0.770
Neck stretching	**22.787**	**11.425**	**3.831**	**1**	**0.050**
Self-directed behaviour	**59.897**	**22.704**	**6.516**	**1**	**0.011**
Vocalisation	**28.940**	**10.500**	**6.804**	**1**	**0.009**
Pre-test oxytocin	29.811	10.882	6.354	1	0.012
Batch			7.390	1	0.007
Batch 2	68.724	23.037			

Null and full model for *being touched* differed significantly (GLMM, $\chi^2 = 9.543$, $p = 0.02$). Pre-test oxytocin ($\chi^2 = 2.633$, $p = 0.008$) and the condition ($\chi^2 = 3.832$, $p = 0.050$) have both a significant effect on the behaviour *being touched* (Fig. 3). Pre-test oxytocin has this effect because of an association and the condition has a causal effect. In both conditions every animal was stroked. In the "familiar" condition the animals were stroked in average for 255.1 s and for 210 s in the "unfamiliar" condition.

For the behaviour *neck stretching* the null and full model also differed significantly (GLMM, $\chi^2 = 9.210$, $p = 0.027$). For the behaviour *neck-stretching* the condition had a significant effect ($\chi^2 = 8.206$, $p = 0.004$) (Fig. 3) while there was no significant effect for the pre-test oxytocin ($\chi^2 = 0.684$, $p = 0.408$) which was an association (Tab. 4). In the "familiar" condition 13 animals showed neck stretching while being stroked and in the "unfamiliar" condition eleven animals showed neck stretching while being stroked. The cattle showed in average *neck stretching* for 12.6 s in the "familiar" and for 2 s in the "unfamiliar" condition.

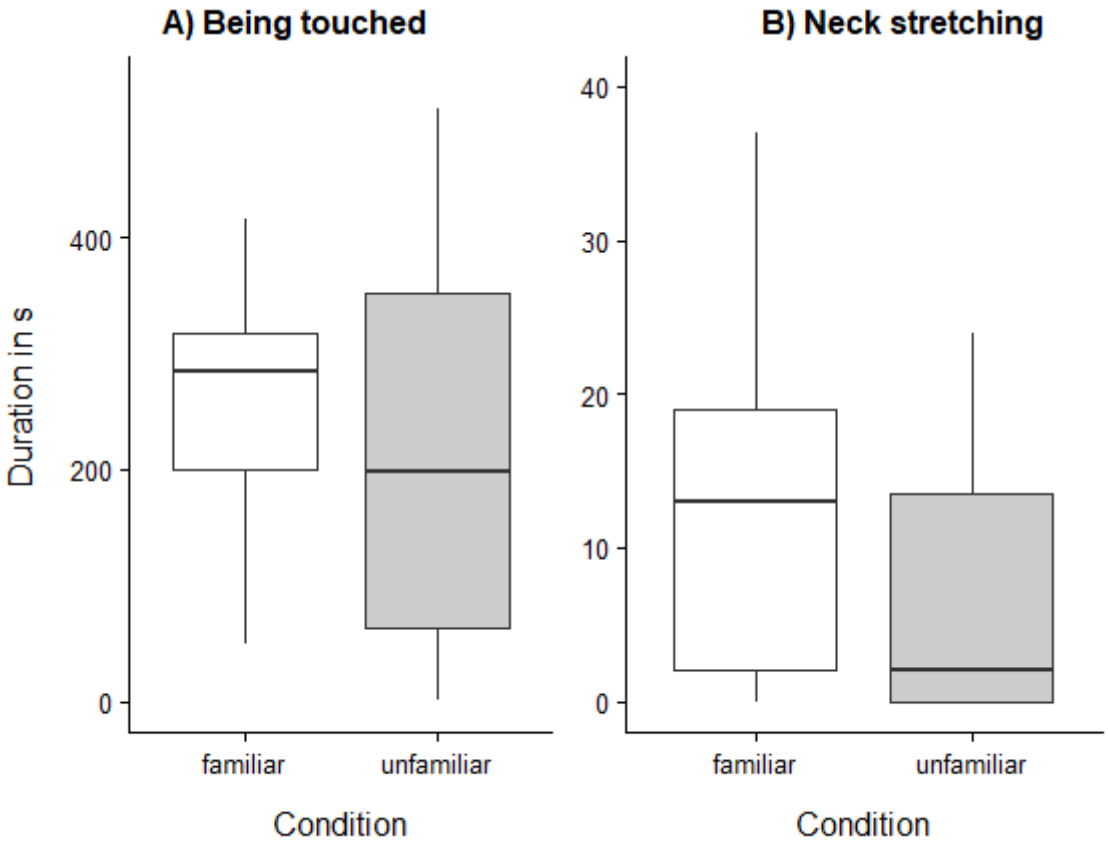

Figure 3: Mean durations (as a proportion of the total time observed) for being touched (A) and neck stretching (B) of Simmental heifers (n = 18) in the two conditions familiar and unfamiliar. Statistics for GLMMs: the condition had a significant effect for the two behaviours being touched (p = 0.05) and neck stretching (p = 0.004).

Note that the y-axis scale varies to allow for sufficient resolution for the neck stretching.

Table 4: Full models for being touched and neck stretching. Statistically significant results appear in bold if the effects were taken out of the null model. Statistics: GLMMs

Being touched					
Effects	**Coefficients**	**SE**	**χ^2**	**df**	**p**
(Intercept)	-0.166	0.286			
Pre-test oxytocin	0.377	0.143	6.309	1	0.012

Condition			**3.832**	**1**	**0.050**
Unfamiliar	-0.581	0.292			
Batch			0.079	1	0.778
Batch 2	-0.093	0.329			

Neck stretching					
Effects	**Coefficients**	**SE**	**χ^2**	**df**	**p**
(Intercept)	-3.393	0.173			
Pre-test oxytocin	0.056	0.067	0.684	1	0.408
Condition			**8.206**	**1**	**0.004**
Unfamiliar	-0.314	0.097			
Batch			0.071	1	0.790
Batch 2	-0.062	0.232			

4. Discussion

The results from this study confirmed that oxytocin could be reliably detectable in the saliva of cattle, although it requires extraction of the sample prior to analysis in order to yield reliable measurement. We found high oxytocin concentrations in the saliva of cattle. We had a mean pre-test oxytocin concentration of 213.55 pg/ml and a mean post-test oxytocin concentration of 236.23 pg/ml. Regarding other species, dogs also have high oxytocin concentrations (MacLean et al. 2018; Wang et al. 2019) in the saliva while human have a much lower salivary oxytocin concentration (Carter et al. 2007; White-Traut et al. 2009; Javor et al. 2014). MacLean et al. (2018) and Wang et al. (2019) used different kits for their oxytocin measurements. They measured unextracted samples with the Arbor Assay kit (mean 258 pg/ml), the Cayman Chemical kit (mean 679 pg/ml) and the Enzo Life Science kit (mean 690 pg/ml) (MacLean et al. 2018; Wang et al. 2019). The extracted samples were measured with the Arbor Assay kit (mean 41 pg/ml) and the Cayman Chemical kit (mean 260 pg/ml) (MacLean et al. 2018; Wang et al. 2019). White-Traut et al. (2009) had oxytocin concentrations in humans in their study between 6.44 pg/ml and 61.05 pg/ml. Javor et al. (2014) had median concentrations of 18.1 pg/ml, 16.19 pg/ml and 16.42 pg/ml. In humans, a concentration of the sample is necessary to make the concentration of oxytocin reliably measurable (Carter et al. 2007), and in cattle an extraction of the sample before the analysis is needed because it is not possible to achieve reliable results without an extraction. If the samples were not extracted before doing the enzyme immunoassay the spiked oxytocin was over-recovered, which indicated that sample matrix effects had an influence on the measurement (Szeto et al. 2011).So the extraction was important "to eliminate the effect of potentially interfering molecules or reduce sample matrix effects" (Szeto et al. 2011).

The only study that quantified oxytocin in cattle' saliva found lower oxytocin concentrations than ours (Geburt et al. 2015). However, this study is not comparable with our study as the authors did not describe any validation for the oxytocin measurement. Furthermore, there was a difference in the storage of the samples. Geburt et al. (2015) stored the samples for one day at 4 °C before further processing. As oxytocin is known to be very sensitive to heat and a protease enzymatic breakdown is thus possible (Wang et al. 2019), even if the 4° C are still in the recommended storage temperature (Nguyen et al. 2019; Vries et al. 2021), we decided to

stay on the safe side by storing the samples on dry ice and -20 °C afterwards, as other researchers recommended (MacLean et al. 2017; MacLean et al. 2018; Wang et al. 2019).

As oxytocin was reliably measurable with the methods used in this study, it is a good alternative to using other samples like blood or cerebrospinal fluid, especially from the ethical point of view, because it offers a non-invasive sampling approach. Furthermore, from a practical point of view it is easier to collect a saliva sample than handling the animal for a blood sample or having to anaesthetise it to place a catheter such as for collecting cerebrospinal fluid. The relationship between oxytocin concentrations measured in different matrices, for example in saliva and blood or saliva and urine, needs further investigation in this species. Some findings regarding this questions already exist in other species, for example in humans (Feldman et al. 2011; Javor et al. 2014; Martin et al. 2018) and in dogs (Mitsui et al. 2011; MacLean et al. 2017). While Javor et al. (2014) did not find a correlation between oxytocin in plasma and in saliva ($\rho = 0.252$, $p = 0.18$; $\rho = -0.005$, $p = 0.98$; $\rho = 0.043$, $p = 0.821$) , Feldman et al. (2011) found a significant correlation between plasma oxytocin and salivary oxytocin ($\rho = 0.41$, $p < 0.001$). Feldman et al. (2011) did not find a significant correlation between plasma oxytocin and urinary oxytocin ($\rho = -0.06$, ns) and also not between salivary oxytocin and urinary oxytocin ($\rho = -0.03$, ns). Martin et al. (2018) found a significant correlation between salivary oxytocin and oxytocin in cerebrospinal fluid ($\rho = 0.657$, $p < 0.001$), between cerebrospinal fluid oxytocin and plasma oxytocin ($\rho = 0.412$, $p = 0.003$) and between salivary oxytocin and plasma oxytocin ($\rho = 0.361$, $p = 0.01$). Mitsui et al. (2011) measured plasma oxytocin and urinary oxytocin. He revealed that the urinary oxytocin concentration peak is one hour later than the salivary oxytocin concentration peak. He did not calculate a correlation between these two matrices. MacLean et al. (2017) also did not calculate a correlation between salivary oxytocin and plasma oxytocin which he used in his study. He only compared pre-test salivary oxytocin with post-test salivary oxytocin and pre-test plasma oxytocin with post-test plasma oxytocin. These divergent findings in humans and the non-existent research in this area in dogs reveal that there is still a big lack of knowledge.

To validate that cattle familiarity to the human matters, we had three different conditions. As we expected the condition had a significant effect on the behaviours *being stroked* and *neck*

stretching. The behaviours *being stroked* and *neck stretching* had a longer duration in the "familiar" condition than in the "unfamiliar" condition; *neck stretching* also had a higher frequency in the "familiar" condition. This shows that the cattle differentiated between the familiar and the unfamiliar human. Another explanation could be a difference in stroking: possibly, the cattle enjoyed being stroked by the familiar person to a higher degree. This could be because of the better relationship to the familiar person, which might have led to a more pronounced motivation for more proximity. Pérez Fraga et al. (2020) already investigated that dogs are seeking for proximity with their caretaker in a room with their caretaker and a stranger. In addition, the familiar person knew the animals better and probably knew what the cattle enjoyed most leading to a stroking style perceived more positively by the animals. We tried to exclude a difference in stroking style with a training before the study. The familiar person explained and showed the unfamiliar person the technique, which means the used pressure while stroking and the localisation, how the cattle enjoyed stroking the most.

We tested not only if the oxytocin is reliably measurable but also whether the oxytocin concentration is linked with different behaviours or if the oxytocin concentration is connected to familiarity. To test if oxytocin release is specific to familiarity, we had three different conditions. We did not find a significant influence of the interactions with an experimenter on post-test oxytocin concentrations. This could be because the cattle did not feel comfortable or did not enjoy the interaction with the caretaker. However, this seems to be very unlikely as the cattle were on average stroked for 39 % of the test duration, which shows that the animals spent a lot of time voluntarily in interaction with human. This leads to the assumption that the cattle enjoyed the stroking and the proximity of a human being. The relationship between positive human-animal interactions and oxytocin concentration is very complex. Also, in human there are unclear results; for example an oxytocin increase after breast-feeding was found by White-Traut et al. (2009) but not by Jong et al. (2015). Several studies revealed an oxytocin increase in dogs' blood or saliva after positive human-animal interaction with a familiar caretaker (Handlin et al. 2011; Rehn et al. 2014; MacLean et al. 2017). Others did not find a relationship between positive human-animal interactions with a familiar person and the oxytocin concentration (Marshall-Pescini et al. 2019). The study by Marshall-Pescini et al. (2019) using

dogs is similar to our study. They also collected samples to analyse oxytocin before and after treatment from the dogs. One difference was that they used urine as sample. Their treatments were "owner cuddle", which is comparable to our "familiar" condition, and "familiar cuddle", which did a person, which was known by the dog but did not live in the same household. We chose a completely unknown person in our "unfamiliar" condition. Another difference was that Marshall-Pescini et al. (2019) had no "control" condition that would have been comparable to ours in their study. As they also had two persons with different relationships towards the tested animal, it is very interesting that their results were comparable in that they did not find a significantly higher oxytocin concentration comparing pre-test and post-test samples in their "owner cuddle" treatment vs. the "familiar cuddle" treatment.

However, some behaviours were associated with oxytocin concentration. While *being stroked* by a human had a positive association with pre-test oxytocin, *neck stretching* had a positive association with post-test oxytocin. Hence, the cattle liked to be stroked longer if they had higher pre-test oxytocin concentration, suggesting that oxytocin concentration predicted greater solicitation, and possibly enjoyment, of stroking by the human subsequently during the test. The association between *neck stretching* and greater post-test oxytocin is interesting as it corroborates behavioural findings suggesting that *neck stretching* is a sign of enjoyment in cattle (Schmied et al. 2008). Both behaviours had a longer duration in the "familiar" condition than in the "unfamiliar" condition.

The other behaviours that were positively associated with post-test oxytocin were *self-grooming* and *vocalisation*. Both behaviours were most common in the "control" condition. *Vocalisation* in the "control" condition could be a sign of missing direct social contact as conspecifics were visible but not approachable during the test. *Self-grooming* could be a sign of comfort as they are relaxed in their familiar environment, but also it is possible they did most *self-grooming behaviour* in the "control" condition as displacement behaviour after exploring the testing area as there was neither food nor conspecifics or any other distraction.

There are still a lot of unknown details about oxytocin and its release, the best sampling method, why there are so different concentrations in different matrices and how are they related to each other, and oxytocin's effects on social behaviour. It is still unknown how behaviour and oxytocin influence each other. This lack of knowledge is underlined by the contrasting results

in various studies in different species. For example, MacLean et al. (2017) investigated oxytocin concentrations in blood and saliva before, in the middle and after a ten minutes of positive human-animal interactions and found an increase in oxytocin concentrations. There were no treatments comparable to our “familiar” and “control” condition. Their experimenter, because he or she is not described otherwise, seemed to be unknown to the dogs, which would be comparable to our “unfamiliar” condition. If the results from our study and the two similar studies by MacLean et al. (2017) and Marshall-Pescini et al. (2019) are compared to each other the question remains if oxytocin is associated with physical contact but independent on the identity of the person. We could not find a general effect of our testing condition on oxytocin, suggesting that the presence of a human, or its familiarity, on oxytocin concentrations. Nevertheless, we found significant associations between specific behaviours such as stroking or neck stretching that relates to the experience by the animal of this interaction. This overall suggests a fine relationship between human-animal interaction and oxytocin, which is possibly linked to the perception by the animal of the situation, rather than the situation that we exposed to the animal to per se.

Conclusion:

The results of this study demonstrates that oxytocin is detectable and reliably measurable after extraction in the saliva of cattle. There was no significant change in the oxytocin concentration under different testing conditions that varied inhuman presence and familiarity, however, the oxytocin concentration was associated with different behaviours. With our findings we significantly advance the field because there no comparable study has been done before and other researchers can do further research based on our findings about the oxytocin concentration in cattle and its connection with different behaviours. So, our results in the measurement of oxytocin and that oxytocin release seems not to be related with familiarity but is influenced by different behaviours gives more ideas for further research about oxytocin and its role in social behaviours.

5. Summary in German and in English language

6) Summary in German language

Studien zur Oxytocin-Konzentration im Speichel und möglichen Einflussfaktoren haben bislang widersprüchliche Ergebnisse geliefert. Beim Rind gab es bisher keine Validierung für die Messung von Oxytocin im Speichel und somit auch keine Studien. Wir haben untersucht, ob die Oxytocin-Konzentration im Speichel von Rindern zuverlässig messbar ist. Außerdem haben wir untersucht, ob positiver Kontakt mit Menschen in Form von freundlichem Sprechen sowie Streicheln, wenn die Tiere in Reichweite sind, einen Einfluss auf die Oxytocin-Konzentration hat. Zusätzlich haben wir getestet, ob der vermutete Anstieg der Oxytocin-Konzentration und das Verhalten der Tiere von der Vertrautheit der Person abhängen. Es wurden weibliche Jungrinder eingesetzt, die eine positive Beziehung zum Menschen hatten und im Rahmen von anderen Studien bereits positive Interaktionen mit der bekannten Testperson erfahren hatten. Das Verhalten der Tiere im Testbereich, entweder allein oder mit einer der Personen, wurde zehn Minuten lang aufgezeichnet und ausgewertet. Vor und nach dem Test wurde eine Speichelprobe genommen. Die Speichelproben wurden extrahiert; anschließend wiesen wir nach, dass Oxytocin via ELISA zuverlässig gemessen wurde. In unserer Studie gab es weder einen wesentlichen Anstieg der Oxytocin-Konzentration vor und nach positiven Mensch-Tier-Interaktionen noch einen signifikanten Unterschied zwischen den Testbedingungen. Die Tiere ließen sich jedoch von der ihnen bekannten Person länger streicheln und zeigten mehr Halsstrecken, einen Verhaltensindikator für positive Emotionen. Außerdem ließen sich Rinder mit einer höheren Oxytocin-Konzentration vor dem Test länger streicheln. Die Tiere, die mehr Halsstrecken zeigten, hatten eine höhere Oxytocin-Konzentration nach dem Test. Wir haben in dieser Studie eine zuverlässige nicht-invasive Methode zur Bestimmung von Oxytocin im Speichel von Rindern gefunden. Wir konnten nicht bestätigen, dass die Vertrautheit der mit dem Tier umgehenden Personen die Oxytocin-Konzentration beeinflusst. Weitere Forschung ist wichtig, besonders bezüglich des Zusammenhangs von Oxytocin-Konzentration im Speichel mit den Oxytocin-Konzentrationen im Blut oder in zerebrospinaler Flüssigkeit und bezüglich anderer Faktoren, die die Veränderung der Oxytocin-Konzentration beeinflussen können.

7) Summary in English language

Studies on oxytocin concentration in saliva and possible influencing factors reported conflicting results. In cattle, there has been no validation for the measurement of oxytocin in saliva and therefore no studies on this topic. We investigated whether the oxytocin concentration in the saliva of cattle can be reliably measured. We also examined whether positive contact with people in the form of talking in a gentle voice and stroking when the animals are within reach had an influence on salivary oxytocin concentration. In addition, we tested whether change in oxytocin concentration and the behaviour of the animals depend on the familiarity of the person. We examined heifers that had a positive relationship with humans and had already experienced positive interactions with the familiar test person in the context of other studies. The behaviour of the animals in the test area, either alone or with one of the test persons, was recorded for ten minutes and evaluated. A saliva sample was taken before and after the test. We demonstrated that oxytocin was reliably measurable using the enzyme immunoassay, but that it requires extraction prior to analysis. In our study, there was no significant change in oxytocin concentration between the test conditions. However, the animals accepted stroking longer by the known person and showed more neck stretching, a behavioural indicator of positive emotions. In addition, cattle with a higher pre-test oxytocin concentration accepted stroking longer. The animals that showed more neck stretching had a higher post-test oxytocin concentration. In this study, we found a reliable, non-invasive method to measure oxytocin in the saliva of cattle. We could not confirm that the presence of familiarity of the person affects the oxytocin concentration, but particular behaviours related to the human-animal interactions were associated with changes in salivary oxytocin concentration. Further research is important, particularly regarding the relationship between oxytocin levels in saliva and oxytocin levels in the blood or cerebrospinal fluid and other factors that may affect changes in oxytocin levels.

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7. List of tables and figures

8) Tables

Table 1: Ethogram of behaviours analysed in cattle. 6
Table 2: Full model of the oxytocin concentration 14
Table 3: Full model for the associations of behaviours with post-test oxytocin concentrations. Statistically significant results appear in bold if the effects were taken out of the null model. 16
Table 4: Full models for being touched and neck stretching. Statistically significant results appear in bold if the effects were taken out of the null model. Statistics: GLMMs 18

9) Figures

Figure 1: Pre- and post-test oxytocin concentration from 18 Simmental heifers in the experimental conditions familiar, unfamiliar and control. The tested main effect was the interaction between time and condition (p = 0.35) where no significance was revealed. 14
Figure 2: Post-test oxytocin concentration for the durations (of the total time observed) of self-directed behaviour (A), neck stretching (B) (only in the conditions familiar and unfamiliar) and the frequency of vocalisation (C) (in the total time observed) from Simmental heifers (n = 18) on the three experimental days. Statistics LMM: the three behaviours self-directed behaviour (p = 0.01), neck stretching (p = 0.05) and vocalisation (p = 0.009) showed a significant positive association with post-test oxytocin concentration..... 16
Figure 3: Mean durations (as a proportion of the total time observed) for being touched (A) and neck stretching (B) of Simmental heifers (n = 18) in the two conditions familiar and unfamiliar. Statistics for GLMMs: the condition had a significant effect for the two behaviours being touched (p = 0.05) and neck stretching (p = 0.004)..... 18