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# The influence of previous stroking and talking on heart rate, heart rate variability and salivary cortisol of heifers during an isolation test with and without the presence of a human

Master's thesis

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submitted by

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**List of abbreviations**

AD ..... avoidance distance
ADT ..... avoidance distance test
AHR ..... animal-human relationship
HF ..... high frequency
HPA ..... hypothalamic-pituitary-adrenal
HR ..... heart rate
HRV ..... heart rate variability
IBI ..... inter-beat-intervals
LF ..... low frequency
RMSSD ..... root mean square of successive differences
SDNN ..... standard deviation of all inter-beat-interval

## 1. Introduction

Taurine cattle were domesticated some 10,500 years ago (Bollongino *et al.* 2012) and used by humans for diverse purposes (Isaac 1962). Nowadays a large number of animals kept on farms is often a reason for a decreased contact between animals and stockpersons (Boivin and Braastad 1996). From a formerly close relationship between stockpersons and their farm animals (Lensink 2002) the animal-human relationship (AHR) nowadays may be affected adversely by the modern farming and management methods (Waiblinger *et al.* 2006). Among farm practices, some unpleasant or aversive ones exist, e.g. veterinary treatments, restraining, dehorning (Waiblinger *et al.* 2006). The AHR is affected by the previous experiences, depending on their intensity and quality, humans and animals had with each other, with handling by the stockpersons as main variable influencing the quality of the AHR, i.e. whether animals are fearful or confident towards humans (Waiblinger *et al.* 2006). These interactions can vary from farm to farm and can be physical or non-physical, ranging from positive to negative (Waiblinger *et al.* 2006). The human-animal relationship can be evaluated by observing the stockpersons' behaviour towards the animals and assessing their attitudes towards the animals (Breuer *et al.* 2000, Waiblinger *et al.* 2006). The concept of AHR considers the relationship from the animal's perspective. Measuring the animals' behaviour during interactions with humans is one possibility to assess the AHR (Waiblinger *et al.* 2006).

A negative perception of interactions with humans, which characterizes a poor quality of the AHR, implies that negative emotions like fear and anxiety are elicited in animals resulting in fear-related behaviour, e.g. flight reactions (Munksgaard *et al.* 2001). This can reduce the stockpersons' time efficiency and may decrease both human and animal safety (Waiblinger *et al.* 2006). A negative AHR can impair growth and reproduction and thus run counter to the economic purposes of the farm and wellbeing of the animals (Boissy 1995, Coleman *et al.* 1998, Hemsworth 2003). The presence of a handler that treated dairy cows negatively, i.e. by shouting, striking and using a cattle prod, caused an elevated heart rate and increase in residual milk (Rushen *et al.* 1999b). Reproductive performance in pigs decreased due to unpleasant handling (Hemsworth *et al.* 1986).

A neutral or positive AHR, which means that the animals have at least a low level of fear of humans (Rault *et al.* 2020), can facilitate handling of animals during management practices and may improve the productivity of farm animals (Waiblinger *et al.* 2006). For example, laying hens that received additional contact with humans showed a lower level of fear and a higher

number of eggs than hens without additional human contact (Barnett *et al.* 1994); milk yield and milk fat in dairy cows was positively correlated with the time that a cow spent nearby the experimenter (Breuer *et al.* 2000), and avoiding fear based behaviour can reduce the risk of injury for animals and humans (Rushen *et al.* 1999b).

Additional physical contacts classified as "positive" or rewarding, e.g. gentle touching, stroking or allowing to suck on the fingers, reduced calves' withdrawal and latency to interact with a familiar and unfamiliar human (Lensink *et al.* 2000). Calves interacted more frequently and for a longer time with humans compared to calves with no additional contact (Lensink *et al.* 2000). Gentle handling by humans evokes positive reactions, i.e. stretching the neck, leaning against the person, similar to those reactions cows show in social licking interactions (Waiblinger *et al.* 2004). Hand feeding and gentle handling influenced attachment responses of early-weaned lambs to their stockperson (Boivin *et al.* 2000). The presence of a familiar human who had provided gentle handling in the past could reduce stress in cows during aversive procedures, e.g. veterinary treatments (Waiblinger *et al.* 2004). Dairy cows that experienced stroking on the ventral neck for 3 consecutive weeks prior had a significant lower increase in heart rate and less stepping during rectal palpation than the cows that were not stroked and experienced only the presence of a human (Schmied *et al.* 2010). Stroking the ventral neck of dairy calves with additional talking to the animals in a soft and soothing way led to a decrease in the avoidance distance and an increased average daily gain in body weight (Lürzel *et al.* 2015a). The average daily gain in body weight in dairy calves is connected to an enhanced first lactation milk yield (Soberon and van Amburgh 2013). More important, the reduction of avoidance distances can be an indicator of an improved AHR (Rault *et al.* 2020). It is concluded that gentle interactions may be effective to decrease calves' fear of humans in the short-term and could be applied under commercial farm conditions (Lürzel *et al.* 2015a).

How an animal perceives humans and reacts to them is modulated by different emotions and motivations (Waiblinger *et al.* 2006). The dimension of emotions, i.e. positive/pleasant or negative/unpleasant, constitute the quality of the AHR (Waiblinger *et al.* 2006). The way an animal perceives humans is not easy to assess in practice, but a positive AHR is expressed in the animal's behaviour and some signs can be observed directly, e.g. the animal approaches the humans voluntarily (Hemsworth *et al.* 1986, Rault *et al.* 2020). Stressful and/or adverse situations for animals can be perceived as less aversive if the AHR quality is high (Waiblinger *et al.* 2006). Cows brushed by a familiar person during isolation showed reduced signs of fear, i.e. defecation, urination and vocalization, compared to cows left alone (Rushen *et al.* 2001). The assessment of the AHR requires a holistic analysis and the consideration of many

indicators that are needed to obtain a full understanding (Rault *et al.* 2020). These can include several behavioural, e.g. avoidance of (Munksgaard *et al.* 2001) and approach towards humans (Hemsworth *et al.* 1986), and physiological reactions of animals (Waiblinger *et al.* 2006).

By nature, each species has its own repertoire of behaviours, which is species-specific (Kotrschal *et al.* 2007). The changes of behavioural patterns due to an environmental stimulus are the most visible ones in animals and can be a tool to assess the AHR (Deen 2010). A poor AHR can not only result in behaviours that are associated with fear, e.g. avoidance behaviour, but also elicit a physiological stress response (Lensink 2002), e.g. increases HR, decreased heart rate variability (HRV) or increased cortisol concentrations.

HRV is the oscillation in temporal distance between heart's consecutive beats (Task Force 1996), e.g. the constantly changing time of inter-beat-intervals (IBI) (Mohr et al. 2002). IBIs can be processed by using different statistical calculations and methods to analyse HRV in either in the time domain or in the frequency domain. The time domain indices provide information about the variability of the IBIs (Stein *et al.* 1994). The frequency domain components indicate the total variance of the HR, with high frequency (HF) power and low frequency (LF) power (Stein *et al.* 1994). A reduced HF and increased LF occur during psychological stress (Delaney and Brodie 2000). From the frequency domain the HF power is parasympathetically mediated (Stein et al. 1994) and reduced in individuals in stressful situations (Heathers 2014). The ratio between LF and HF can be calculated which is suggested to reflect the balance between sympathetic and vagal activities (Task Force 1996). An increase in this ratio can be interpreted as a domination of the sympathetic branch (Borell *et al.* 2007), as HF is reduced under stress from baseline, and LF is increased (Delaney and Brodie 2000). A decrease of the HRV and HF and an increased LF/HF ratio can occur during short-term psychological stress (Delaney and Brodie 2000).

The simplest parameter of the time domain is the standard deviation of all IBI (SDNN), which reflects both parasympathetic as well as sympathetic influences on HR (Mohr *et al.* 2002).The root mean square of successive differences of IBI (RMSSD) is a time domain parameter deriving from IBI differences (Malik 1996) and reflects alterations in the autonomic nervous system that are predominantly mediated by parasympathetic activity (Stubsjøen *et al.* 2015).

It is generally accepted that the increase of HRV indicates an increased adaptability in stress situations (Geitel 2016). As a simple statistical index, which can be used as a substitute for the ratio LF/HF, the ratio RMSSD/SDNN can be evaluated (Langbein *et al.* 2004; Borell *et al.* 2007) especially for short-time recordings (Task Force 1996). The IBI and so the HRV parameters

vary as a result of physical and mental stress (Stein *et al.* 1994). In recent years HRV has been used as a non-invasive technique to assess stress in animals (Borell *et al.* 2007). During a stressful situation the time domain parameters RMSSD and SDNN of HRV were reduced (Delaney and Brodie 2000). The measurement of HRV in cattle can be used to assess stress from physical, pathological and emotional origins (Borell *et al.* 2007). Calves that were exposed to internal and external stress load (diarrhea and high temperature plus insect harassment, respectively) showed a decline in HRV (Mohr *et al*. 2002).

Due to stress load and aversive, but also rewarding stimuli the hypothalamic-pituitary-adrenal (HPA) axis can be activated (Koolhaas *et al.* 2011) and then secrets steroid hormones like cortisol (Matteri *et al.* 2000).The increase in plasma cortisol concentrations is considered an indicator of stressful conditions (Minton 1994). Cortisol can be measured in blood; however, to avoid additional stress in animals, saliva samples can be used alternatively. Sampling is easy and non-invasive (Peeters *et al.* 2011) and the cortisol levels in plasma and saliva correlate with each other (Beerda *et al.* 1996; Möstl and Palme 2002). It is known that management routines in livestock can activate the HPA axis (Minton 1994) and cattle are still sensitive to stressors they experience in their everyday farm life (Rushen *et al.* 1999c). Dairy cows showed increased plasma cortisol concentrations and HR during social isolation (Rushen *et al.* 1999a). Dairy calves that were stroked and talked to during their first four weeks of life had lower concentrations of salivary cortisol before and after an arena test which included two phases of isolation and in between one phase with human presence (Lürzel *et al.* 2015b). During the test phase with the presence of a human tail-flicking occurred less often in the calves that were previously stroked and talked to compared to control calves that did not receive such a treatment (Lürzel *et al.* 2015b).

As the quality of the AHR is based on previous experiences and animals' behavioural and physiological reactions to humans depend on how they perceive humans, this can be used to investigate AHR (Waiblinger *et al.* 2006), e. g. latency of approaching a human and duration of voluntary interactions with a human (Rault *et al.* 2020). The three main categories of tests to measure the animals' reactions to humans are (1) reactions to a stationary human, (2) reactions to a moving human and (3) responses to actual handling (for detailed review see (Waiblinger *et al.* 2006). The test location can be familiar or if not, it has to be considered that an unfamiliar environment can evoke fear in animals (Waiblinger *et al.* 2006). Farm animals as social species can be influenced to a high degree by isolation (Waiblinger *et al.* 2006) and also exposure to a novel situation can lead to an emotional response in animals (Boissy 1995). Studies that include novelty and/ or isolation as central features can be used to test a human's

capacity to provide social support to animals (Waiblinger *et al.* 2006).

Tactile interactions were combined in many studies with vocal interactions (dairy cows: Schütz *et al.* 2012; Waiblinger *et al.* 2004; sheep: Hild *et al.* 2011; calves: Lensink *et al.* 2000; Lürzel *et al.* 2015a; goat kids: Boivin and Braastad 1996). The kind of vocal interactions during milking alone or summed with tactile interactions – talking in a quiet and soft or in a harsh and loud way – was associated with dairy cow behaviour, i.e. frequency of flinching and kicking during milking (Breuer *et al.* 2000; Schütz *et al.* 2012; Waiblinger *et al.* 2004) approach behaviour in a test arena (Breuer *et al.* 2000) and avoidance distance in the barn (Waiblinger *et al.* 2002) . Studies that included talking in the gentle interactions did not yet differentiate between the effects of stroking and talking alone to the animals. In the present study we aimed to investigate the effects of stroking and talking alone and the combination of the both. There is evidence that cattle respond to humans addressing them verbally (Albright *et al.* 1966). Just one former study questioned if cows have a preference for people speaking in a gentle voice, but it did not find that cows choose the human talking in a gentle voice significantly more often than the mere presence of a human (Pajor *et al.* 2003). Using the human voice to address animals could be a mean to impact on cattle when they are held in big herds or extensively kept. It could also be a mean to improve the AHR in animals that cannot be touched because of their fearfulness of humans.

To this purpose, we assigned heifers to one of five treatments: stroking with talking (ST), talking (T), stroking (S), human presence (P) and control (C). To investigate the capacity of a familiar human to provide social support to heifers an isolation test with the temporary presence of a familiar handler in an unknown arena was conducted after the treatment phase. The main hypothesis was that stroking, talking in a gentle voice and the combination of stroking and talking during 3 weeks prior to the isolation test improve the capacity of heifers to receive social support by a familiar handler to different degrees. The first prediction was that heifers that had experienced stroking, talking oder both with the handler during a previous 3-week period will perceive the human as more reassuring when present in an isolation test and therefore will show fewer signs of behavioural (analyzed in the context of another master's thesis, see Cords 2020) and physiological stress (lower heart rate, higher HRV, lower salivary cortisol concentrations after the test) than heifers that did not experience stroking or talking at all, i.e. treatment groups P and C. The second prediction was that heifers that had been solely stroked during a previous 3-week period will show fewer signs of stress compared to heifers that had experienced only vocal interactions. The third prediction was that heifers that had experienced both vocal and tactile interactions during a previous 3-week period will show fewer signs of

stress compared to heifers that had experienced only one type of interaction alone.

## 2. Methods

### 2.1 Animals, housing and management

The experiment was carried out at the youngstock rearing unit of the 'VetFarm', i.e. the farm of the University of Veterinary Medicine, Vienna, in Rehgras (Furth an der Triesting, Niederösterreich) from May to June 2019. The farm unit Rehgras was leased and managed by a tenant. The sixty heifers involved in the study were housed in two herds. Herd 1 consisted of 30 Austrian Simmental heifers aged between 25 and 15 months (mean ± SD: 18.4 ± 2.9 months), with 19 animals born at the dairy cow unit of the 'VetFarm' in Kremesberg and 11 animals born at the tenant's dairy farm (see Annex 1: Table A 1). Herd 2 consisted of 30 heifers (28 Austrian Simmental, two Belgian Blue x Austrian Simmental crosses) aged between 15 and 7 months (mean ± SD: 10 ± 2.1 months), with eight animals born at Kremesberg and 22 born at the tenant's farm (see Annex 1: Table A 2).

At the tenant's farm in winter calves were born in a deep-litter group-calving pen, where animals had visual and auditory contact to the herd. In summer calves were born on pasture. Calves born during daytime were separated from their dams within one hour. If the calves were born at night separation took place within six hours. From day 1 to day 14 the calves were kept in calf igloos (area: 2.21 m²) with an outdoor enclosure (area: 2 m²) with straw bedding. They were usually kept in pairs. Between 2 and 10–12 weeks of age the calves were housed in groups in a separate non-insulated barn. It consisted of two pens with an area of 4.92 × 5.22 m², each containing a group of eight calves. This was a two-area system with deep litter plus elevated concrete feeding area. After weaning, at an age of 12 up to 14 weeks the calves were brought to a barn nearby the farm and kept together in one group. The first dose of colostrum was given immediately after birth with a teat bottle. The calf was assisted until it drank independently, which could take up to 2 days. The tenant's calves were fed warm milk from day 1 to day 7 via teat bucket twice a day. From day 8 to day 14, 10 litres milk replacer (Sprayfro Royal Kälbermilch, Trouw Nutrition Deutschland GmbH, Germany) was given via teat bucket twice a day. From day 5 on, calf muesli was additionally provided *ad libitum* (mixed by the tenant himself from straw, molasses, corn grits, plant oil). Feed was provided either by hand or tractor. From day 14 on until weaning the calves were fed milk replacer by an automatic milk feeder ("Urban Kälbermama Paula", Urban GmbH & Co.KG, 27798 Wüsting, Deutschland), that was accessible from both pens. Calves wore sensor collars so weaning could take place automatically, depending on growth and feed intake. Weaning was finished

at the latest with 14 weeks of age. The tenant himself had approximately 0.5 h per day contact to the calves. Additionally, customers had access to the calves until weaning, whenever they came to buy milk, mainly on weekends.

At Kremesberg calves were always born in a separate deep-litter pen in the loose-housing system. Auditory and visual contact to the herd was possible. Calves were also separated from their dams immediately after birth, i.e. within 1 to 6 h. Right after birth until the age of 14 days the calves were kept singly in calf igloos (area: 2 m²) with an outdoor enclosure (area: 2 m²) and straw bedding. Visual contact to the neighbour calves was possible. With the age of 14 days the caretakers used a tractor to bring the calves to a separate calf barn. There the calves were housed in groups of eight in an open-front barn including a deep-litter area, size 4.8 x 4.9 m², and an elevated feeding area with individual feeding stalls (each measuring 0.45 x 1.3 m²). For feeding the calves were restrained in the feeding stalls and teat buckets were placed in front of the calves. At Kremesberg colostrum was fed right after birth via teat bottle. Two litres of colostrum were provided from day 1 to day 6 three times a day in teat buckets. The calves were assisted until they drank independently. Following to that three litres of pasteurised milk was provided from day 7 on twice a day until weaning. Additionally, concentrates ("Kälberstart vital", Garant Tiernahrung GmbH, Raiffeisenstr. 3, 3380 Pöchlarn, Austria) and hay were provided *ad libitum* from day 1 onwards. Weaning was completed with 14 weeks of age latest. Subsequent to that hay, silage and concentrate were fed. As Kremesberg is one place of the teaching and research facilities of the University of Veterinary Medicine, Vienna, some calves had already been used in studies and experienced contact to other humans in addition to the permanent caretakers.

With approximately 4 months of age the calves of both origins were brought to Rehgras and kept together in groups (12–16 animals) in an open-front barn with a roofed deep-litter area and a concrete outdoor run and feeding area (pens 1 and 2 in Figure 1). They were fed hay, corn silage, straw, and corn grit. The Rehgras barn additionally included two deep-litter pens (A and B) and two cubicle housings (C and D) all with access to outdoor runs, respectively (Figure 1). A small, roofed pen E was an extension of the passage that connected the outdoor runs D and C. All outdoor runs had concrete floor (Figure 1). With approximately 6 months of age the animals were kept on pasture, except if weather or ground conditions did not allow for it (e.g. in winter). Then the heifers stayed in the cubicle barn C and D with access to outdoor runs. When staying in the barn, they were fed hay, corn silage and corn grit. In the barn and on pasture, water and mineral salt were provided *ad libitum*. At Rehgras the tenant did the daily routine work, sometimes with the help of his father or his wife. Heifers were familiar with

the usual barn management methods. For charging the feeding alley with feed and for mucking out the barn a tractor was used, but feed distribution by hand happened as well. On day 9, i.e. the first day of treatment, one heifer of herd 2 needed treatment by a veterinarian (0.5 h) because of a sole ulcer. This animal plus a companion stayed in the barn for 5 nights and were not turned out to pasture like the rest of the herd. After an additional control of the hoof on day 14, these two heifers were reintroduced to their herd. Because of lameness another animal's claw was controlled by the tenant on day 14 but no injury was detectable.

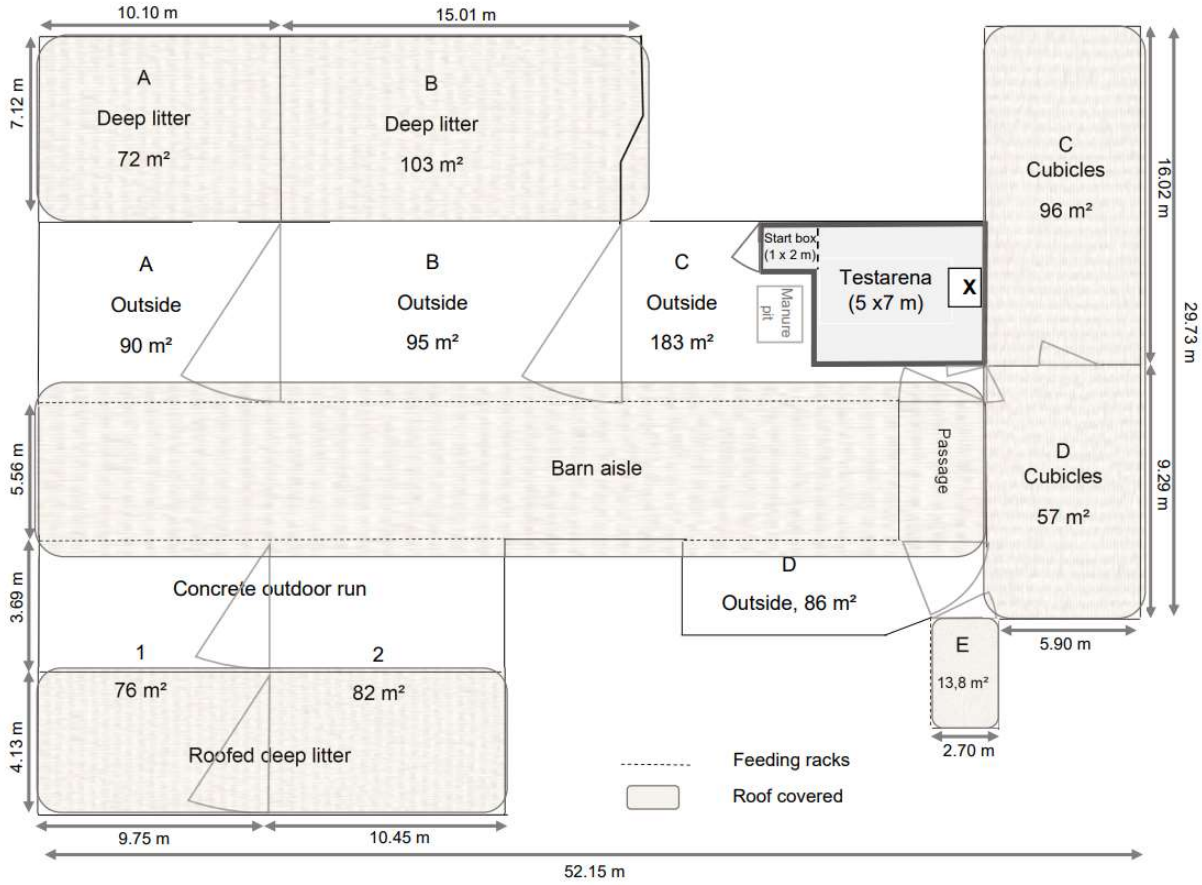


Figure 1: Schematic drawing of the barn (adapted from © Regien van Hasselt). "A outside" was the run to gather the herd after it had been brought in from the pasture. The animals were sorted in A according to treatments and brought to outdoor pens C, D or E, where feeding racks had been prepared for the animals to lock themselves in. Animals that already received treatment were moved to cubicles C and D until every treatment was finished. After the treatment, all animals were again moved to run A before bringing the herd back to pasture.

## 2.2 Experimental design and treatment

All procedures applied during this study were discussed and approved by the institutional ethics and animal welfare committee in accordance with guidelines for Good Scientific Practice and with national legislation (ETK 56/03/2019).

Each heifer was randomly assigned to one of five treatments balanced for avoidance distance (AD, see 2.4). The different treatments were as follows:

- Stroking with talking (ST): The experimenter approached and then stroked the animal, while talking in a calm, soothing way.
- Talking (T): The experimenter approached and then talked to the animal without stroking.
- Stroking (S): The experimenter approached and then stroked the animal without talking.
- Group P (human presence): The experimenter approached but neither stroked nor talked to the animal.
- Group C (control group): The animals experienced no additional human contact.

Each heifer was treated 5 min/d on 5 d/week for 3 weeks (2.2.1, Figure 2) similar to the duration in a previous study (Schmied *et al.* 2008a) while all heifers of the corresponding treatment were restrained in the feeding rack. Thus, heifers remained at least 30 min per day (6 × 5 min) in the feeding rack. For treatment ST, S, T, P the experimenter approached the heifer to be treated from the left while addressing her in a standardized manner, talking in a calm and soothing way. Then the experimenter walked slowly to the treatment position, which was always on the left side of the animal close enough so that the animal's neck could be reached comfortably with both hands. Additionally, only for the treatments ST and S, the experimenter wore rough latex-coated gloves (PowerGrab Katana® 310, Towa Corporation, Japan) for stroking. Speed of stroking was between 40 and 60 strokes/min, imitating the speed of social licking (Schmied *et al.* 2005). For the talking treatments ST and T, the experimenters spoke with long vowels and a lowered pitch at the end of the sentence, in a calm and soothing way. To apply the stroking and talking treatment in a similar way, the two experimenters calibrated and harmonized pressure and voice before the beginning of the treatment phase, so that both experimenters used a similar medium pressure for stroking.

To assess the potential treatment effects on the AHR two different kinds of tests were conducted. Three AD tests (ADT) were performed, with a first ADT before the treatment phase

(see 2.2.1, Figure 2) to assign the heifers to the different treatments and to have baseline values for comparing them with the values of AD collected in a second and a third test after the treatment phase. Every heifer was also tested once in an isolation test with the temporary presence of the experimenter (see 2.5) to investigate the capacity of a familiar human to provide social support. This thesis focuses on the physiological signs of stress caused by the isolation to study the capability of the heifers to receive social support, while another master's thesis used the results of behavioural observations of the heifers in the isolation test and ADTs (see Cords 2020).

### 2.2.1 Schedule

On day 1 heifers were divided into herd 1 and herd 2 (see 2.1; Annex 1: Table A 1, Table A 2). All heifers were habituated to the daily routines necessary for treatment on six days (between day 1 and day 12, 35.5 h in total, Figure 2). Herd 2 was additionally habituated to use the feeding racks on three days (6.5 h in total). A first ADT was conducted on day 6 to assign the heifers to the different treatments balanced for their AD. The treatment phase took 3 weeks (day 14 to 34). A second ADT was conducted on day 35. Building up the arena for the isolation test took 3 days, so an isolation test was then conducted on days 38 till 42. Finally, a last ADT took place after the isolation test, on day 49.

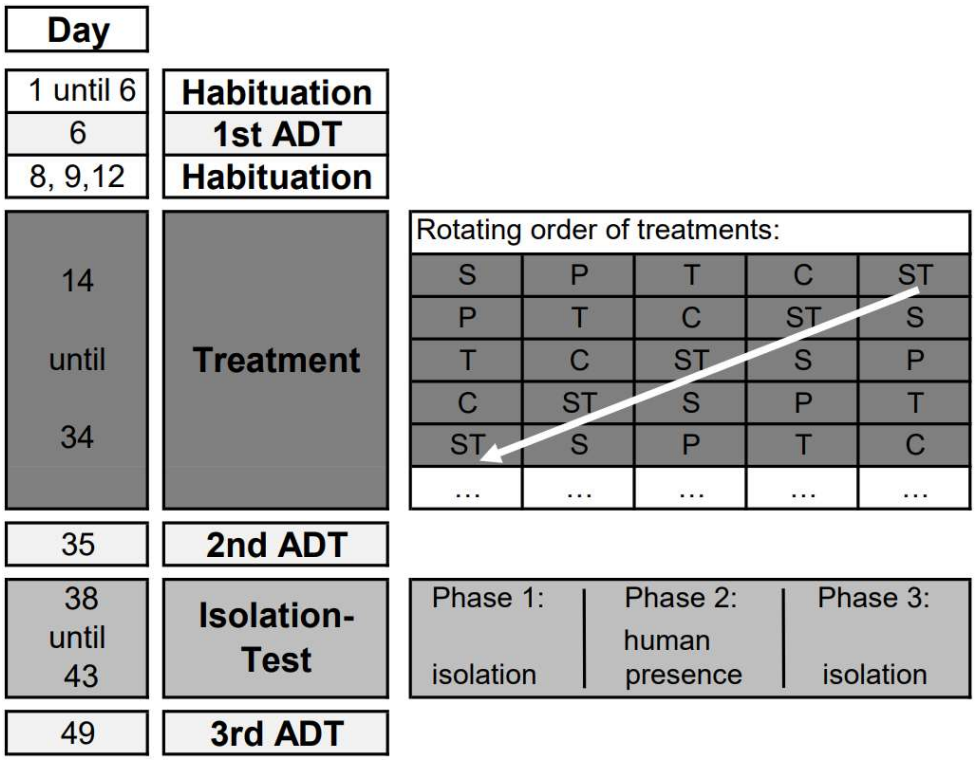


Figure 2: Overview over the schedule of the experiment. Habituation: Before the start of the treatment, the heifers were habituated to the experimental procedures on several days. Treatment: Each heifer was treated in total 3 weeks, on 5 days per week, for 5 minutes every day. In total three avoidance distance tests (ADT) were conducted. Three days after treatment has ended an isolation test was conducted.

### 2.3 Experimenters and procedures during treatments

All procedures of the study were performed by two female students, further called experimenters. Experimenter A had a height of 176 cm and long, blond hair. Experimenter B had a height of 172 cm and short, blond hair. Both experimenters were always dressed in green overalls and wore black boots. Experimenter A performed the treatment of herd 1, Experimenter B conducted the treatment to the animals of herd 2. Each experimenter was blinded to the treatment allocation of the other experimenter's herd and thus was absent during the treatment sessions of the other experimenter. Both experimenters prepared the daily treatment setup for both herds together, i.e. arranging the treatment pens, moving the herds from pasture to the barn, sorting the heifers into groups for the treatments and supplying them with water and hay between testing single animals.

The treatment of herd 1 was performed between 8:15 and 13:15 and the treatment of herd 2 between 12:30 and 20:30. On the first day the order of the different groups to be treated was pseudo-randomized. Every day the group subsequent to the first group of the day before was treated first, so the order rotated daily (Figure 2). Before the beginning of the treatment phase both herds were brought to the barn and groups of six animals were driven to all different pens A, B, C, D and E to habituate them to all parts of the barn that were used during the treatment phase. As herd 2 consisted of younger animals not used to being restrained in the feeding rack, they also needed to be habituated to this management method: corn grit was scattered on the feeding alley in front of opened feeding racks to encourage naïve heifers to use them and to get locked.

During the experimental period, the heifers were kept on pasture, but were brought to the barn for experimental procedures, i.e. treatment and tests (ADT 1, 2 and 3; isolation test). Run “A outside” (Figure 1) and the adjacent deep-litter area was the location to gather the herd when it was brought in from pasture and before it was brought back; it was also the pen where heifers waited to be treated. The outdoor run C was used as treatment pen for herd 1. Due to the smaller body and head size of the heifers of herd 2, the outdoor runs D and E, equipped with smaller feeding racks, were used for the treatment of herd 2.

For treating the heifers, the feeding racks were used to restrain the animals with minimum two empty feeding places between two animals. To encourage the heifers to enter the feeding racks and restrain themselves corn grit and a handful of hay were placed on the feeding alley in front of the accessible feeding places. The experimenters prepared the places, i.e. run “C outside” for herd 1 and runs D and E for herd 2, before the heifers entered the treatment pen to avoid any association with humans. In some cases, it was necessary to lure single animals into the feeding rack by presenting the corn grit directly on the shovel. The heifers that were already treated were moved to “C cubicles” and “D cubicles” (Figure 1), so there were up to 15 animals in pen C and up to 10 animals in pen D. As soon as the last treatment was finished all heifers were released and gathered in run A again. While restrained, hay was submitted to the heifers that were not treated and they were regularly supplied with water. As run B was located between run A, where animals waited to be treated, and treatment run C, it was supposed that the heifers of different treatments would not pay attention to the treatment procedures of the other groups.

### 2.4 Avoidance distance test (ADT)

The AD is the distance to which the experimenter can approach a heifer until it shows withdrawal. The AD assessed in a loose housing system validly reflects the quality of AHR (Waiblinger *et al.* 2003). The AD of heifers towards the two experimenters were recorded in three ADTs (according to Waiblinger *et al.* 2002, Windschnurer *et al.* 2009). Each experimenter tested herd 1 in the morning and herd 2 in the afternoon, with a minimum of 30 min between the testing, respectively. The first AD measurement was the basis value and also served for allocation of animals to treatments, the second AD was to assess if the treatments caused different improvements of the AHR in the short-term and the third ADT was to determine if there were longer-term effects on the quality of AHR. The first and second ADT was performed in the outdoor run of C, the third was performed in the outdoor run A plus B, due to management reasons, while heifers were allowed to move freely in the outdoor run. The experimenter started approximately 3 m away from each heifer. Start position was in front of the animal or from a slight angle of maximum 45°, one arm raised at 45° angle with the back of the hand towards the animal. The heifer was able to move freely and was aware of the experimenter approaching. The experimenter wore an earphone and heard the sound of a metronome to standardize her speed of approaching, i.e. 1 step/s. The distance between the hand and the muzzle in the moment when the heifer was withdrawing, i.e. stepping away (at least one step) or turning the head in response of the approaching human was estimated in steps of 10 cm (Lürzel *et al.* 2015a, Waiblinger *et al.* 2002). If the heifer was not taking a step away and turning the head was not followed by a step away, the experimenter touched the heifer's nose, slid the hand further to the cheek to stroke it for a maximum of 5 s or as long the heifer allowed stroking; the seconds the heifer accepted stroking was noted down (0S1-0S5). If withdrawal happened at the moment of touching "0A" was recorded. If the muzzle could be touched but cheek could not be stroked "0B" was recorded.

### 2.5 Isolation test with the temporary presence of a human

The isolation test with the temporary presence of the familiar experimenter (adapted from (Boivin *et al.* 2000), suggested by (Waiblinger *et al.* 2006) to assess the AHR, was conducted on days 38 to 43. One animal of each treatment per herd was tested per day; therefore, it took six days in total to test all animals (59 in total). The tests for herd 1 were conducted between 07:30 and 14:00 and the tests for herd 2 between 12:45 and 20:30. The order for testing the

heifers of the different treatments was pseudo-randomized. Each experimenter performed the procedure of test phase 2 (see below) for the heifers she treated on days 14 until 34, i.e. experimenter A for heifers of herd 1, experimenter B for heifers of herd 2.

The test consisted of three phases: The first 5 min each heifer was alone in the test arena (phase 1), then the experimenter entered the arena through a side door, placed herself at the middle of the wall (at position “X”, Figure 1) and talked during the 5 min to the heifer in the same calm, soothing way as she did during the treatments ST and T (phase 2). If the animal walked towards the experimenter and stayed within arm’s reach, the experimenter attempted to stroke the animal at the head or neck. She tried this maximally three times; if the animal avoided the stroking attempts repeatedly, she only kept talking soothingly to the animal. After 5 min, the experimenter left, and the heifer spent again 5 min alone in the arena (phase 3).

The arena was constructed in outdoor run “C” (Figure 1) and was an unfamiliar object for the heifers. As the experimenters had to drive the single animals into the arena the heifers had to pass the arena on the two days before the test started (2 h each herd) to habituate them. The arena walls were constructed of 1 m x 2 m plywood boards, enclosing an area of 5 x 7 $m^2$, preventing heifer’s visual, but not audible contact with conspecifics. To move the heifers into the test arena it had a start box, which was a corridor (1 m x 2 m surface area). An additional door enabled the experimenter to enter the arena from the opposite side.

Before testing, the five heifers were separated from the herd, restrained in the feeding rack in pen D and equipped with a chest girth including electrodes and transmitters for measuring HR (see 2.5.1). The visual and spatial isolation of a single animal from conspecifics usually causes physiological and behavioural signs of stress. These signs were recorded during the whole test to control if the presence of the experimenter could provide social support. One by one, the animals were brought into the arena. As soon as the heifers had entered the arena, a sliding door was closed from the outside by an experimenter and the test started. After phase 3, the sliding door of the start box was opened. In most cases, the heifers walked out by themselves. Some heifers did not make any attempt to walk out, so the other experimenter entered through the side door and moved the heifer to the exit.

### 2.5.1 Heart rate (HR) monitoring

During the isolation test, IBIs were recorded in order to calculate HR and HRV using a commercial HR monitoring system (horse trainer transmitters and S810 monitors, Polar Elektro Oy, Helsinki, Finland). The equipment was attached to elastic chest girths and fitted to the

heifers. On the left body side, the transmitter was placed at the lateral thorax and the (-) electrode was placed in the area of cardiac dullness (Figure 3). The (+) electrode was placed dorsally on the right lateral thorax, behind the right scapula. Ample ultrasound gel was applied to ensure contact between the electrodes and the skin, and an additional elastic girth kept the transmitter in place and included a pocket for the HR monitor. On the test days, the heifers were equipped at least 32 min (mean ± SD: 2:05 h ± 00:58 h, maximum 4:05 h) before the start of the isolation test. The HR monitor was started immediately before the heifer was released from the feeding rack and driven into the test arena. The monitor was stopped immediately and equipment was removed after the heifer was restrained in the feeding rack after the test and a cortisol sample was collected (2.5.2).

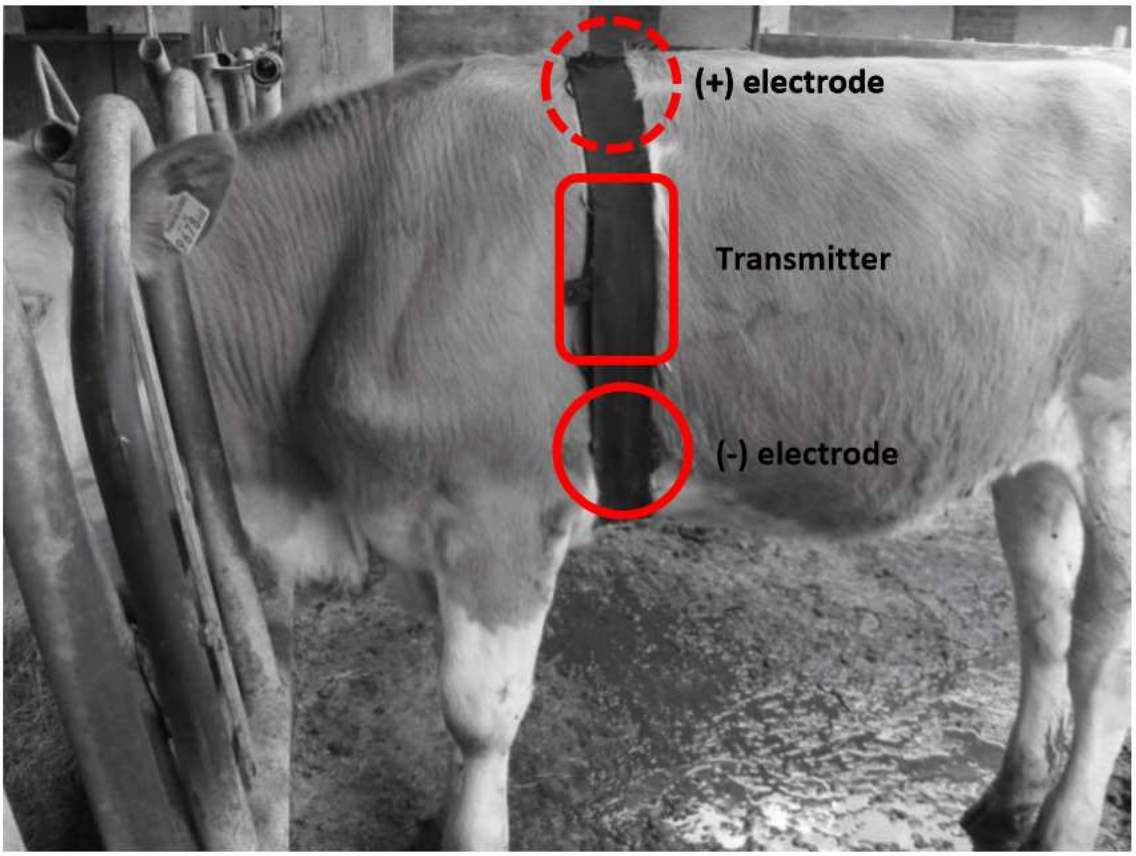


Figure 3: Heifer equipped with heart rate (HR) monitoring equipment. On the left body side, the transmitter was placed at the lateral thorax and the (-) electrode was placed in the area of cardiac dullness. On the right body side, the (+) electrode was placed dorsally on the lateral thorax, behind the right scapula. The Equipment was attached to an elastic chest girth and an additional elastic girth kept the transmitter in place and included a pocket for the HR monitor.

### 2.5.2 Cortisol samples

Saliva samples were taken directly before and after the isolation test to determine cortisol concentrations. It was not possible to take saliva samples from free moving heifers, so they had to be locked in the feeding racks. Before the test, any feed was removed between 3 and 27 min (mean ± SD: 00:14 h ± 00:05 h) before taking the sample. The time from removing the feed before taking the sample A was shorter than 10 min for 11 heifers (n = 1, 3 min; n = 1, 5 min; n = 1, 7 min; n = 3, 8 min; n = 5, 9 min). For taking the B sample feeding racks were opened in the run of C. As soon as the heifer has left the arena an experimenter was standing on the feeding alley presenting corn grit (inaccessible) to encourage the heifer to restrain itself. The time from test end until taking the saliva sample depended on the time it took for restraining. The second cortisol sample was taken within the first five minutes for 41 heifers. Sixteen heifers took longer than 5 min to restrain themselves (n = 6, 6 min; n = 2, 7 min; n = 3, 8 min; n = 1, 11 min; n = 1, 12 min; n = 1, 13 min; n = 1, 16 min; n = 1, 23 min; in total: mean± SD: 4.25 min ± 4.18 min). Saliva was collected using absorbent cotton (Salivette®, Sarstedt AG, Nürnbrecht). A rubber teat was put over an arterial forceps and then the cotton was clamped by the forceps. The device was inserted into the heifer's mouth, allowing the heifer to chew on the cotton for 30–120 s. All samples were stored within 60 s in the freezer (-20 °C) until further analysis. To analyze the saliva cortisol samples, they were defrosted at room temperature and then centrifuged (20 min at 1500 *x g*) to separate the saliva from the cotton. Then the samples were analysed using a cortisol enzyme immunoassay as previously described (Palme and Möstl 1997; Wagner *et al.* 2013). The sensitivity of the assay was 0.5 ng/ml.

## 2.6 Data preparation, data description

One animal was excluded from the treatment right after its selection according to the first ADT because it could be stroked for more than 5 seconds, implying that any further improvement of the AHR would not be measurable with our method and the effect of treatment would have been impossible to determine. In total, cortisol data were obtained for 57 animals. One animal was excluded from the isolation test because it was not possible to move it into the test arena and another animal was excluded due to its missing B saliva sample, because it could not be restrained to take the B sample. Overall, 31,6 % (in total 18 samples) of the saliva samples

had cortisol concentrations below the assay's detection limit of 0.05 ng/ml. For analysis the minimum detection limit of 0.05 ng/ml was used for calculation.

In total HR data were obtained from 58 animals. The HR data were processed using the software Polar Precision Performance SW, version 4.01.029 (Polar Electro Oy 2004, Kempele, Finland). First, the 5-min measurements of phases 1, 2 and 3 were identified and cut separately as it is recommended (Malik *et al.* 1996). Afterwards, each 1-min sequence of the recordings was inspected for artefacts. If the error rate was larger than 5 % (Hagen *et al.* 2005) according to the software's standard settings, the 1-min sequence was discarded. If the error rate was below 5 % the software's correction procedure was applied. Sequences with 0 % error rate remained unchanged. Additionally, the suggestions for the corrections were inspected visually for plausibility of the interpolations.

Because the resulting number of 5-min sequences was not sufficient for analysis, it was decided to divide the sequences further into 1-min sequences and use their average. Discarded 1-min sequences were error corrected if they were situated before or after a 1-min window to be analyzed. For some animals all minutes of a single phase (1, 2 or 3) were missing, and remaining data of these animals were discarded completely. In total the HR data of 44 heifers could be used, 26 animals from herd 1 and 18 animals of herd 2. HR measurements were then processed using Kubios, version 2.1.0.0 (Biosignal Analysis and Medical Imaging Group, Department of Applied Physics, University of Eastern Finland, Kuopio, Finland). To account for the respiratory rate, frequency bands were set to 0.04–0.2 Hz for the low frequency (LF) band and 0.2–0.58 Hz for the high frequency (HF) band (Borell *et al.* 2007). The ages of herd 1 and herd 2 ranged from 25 to 7 month so it was decided that the frequency bands for adult cattle fit the best. The following parameters were obtained and further analyzed: mean HR, SDNN, RMSSD, the ratio of RMSSD and SDNN and the power of the HF obtained via fast Fourier transform. For the HF components of HRV, a recording of about 1 minute is sufficient to evaluate them, while to account for the LF component, about 2 minutes are required. Therefore, it was not possible to analyze the LF power and the ratio LF/HF. As measures from the time domain SDNN, RMSSD and the ration RMSSD/SDNN were analyzed. As measures of the frequency domain HF power was analyzed. The mean value of HR, SDNN, RMSSD and HF per animal and phase was calculated.

### 2.7 Statistical analyses

The statistical unit was the individual animal. Data were analyzed with the software package R version 3.5.2 (R Core Team, 2020). HR, HRV data and cortisol concentrations were analyzed using linear mixed models (LMMs) with the "lme4" package (Bates *et al.* 2015). Interactions with $p \leq 0.05$ are referred to as significant, and with $0.05 < p \leq 0.1$ as a trend. To avoid cryptic multiple testing (Forstmeier and Schielzeth 2011), for each dependent variable a full model was compared with a null model that lacked the variables of interest but was otherwise identical.

The full and null models comprised treatment (ST, S, T, P and C), herd (1 or 2), origin (tenant or Kremesberg) and the confounding variables changes of squares, duration of rumination and age as fixed effects. The confounding variables changes of squares and duration of rumination were included to control for effects of physical activity, the age of the heifers was included to control for an effect of age; these variables were centered.

The full model of HR, SDNN, RMSSD and HF additionally included phase (1, 2 and 3) and the interaction between treatment, phase and herd and all lower-order interactions as fixed effects. The models of SDNN, RMSSD and HF additionally included HR as a fixed effect to consider the effect of the sometimes strong correlation of HR and HRV parameters. So, the models considered the effects of treatment, origin, changes of squares, duration of rumination and age on the HRV parameters while controlling for differences in HR (Lange *et al.* 2020). To control for an effect of herd this was included in the interaction, too.

For the HR and the HRV parameters the interaction between treatment and phase was the effect of main interest, as we wanted to investigate if heifers of different treatment groups differ in their HR and HRV parameters during the phases of the isolation test. The interaction was thus excluded from the null model, leading to the exclusion of the higher-level interactions. Therefore, the null model contained the two-way interactions treatment * herd and phase * herd as well as the confounding variables.

For SDNN the full/null model comparison revealed a trend for the interaction of treatment and phase, but the three-way interaction was not significant, therefore a reduced model without the three-way interaction was calculated additionally. It included the two-way interactions treatment * phase, treatment * herd and phase * herd (see 3.3).

In the full model of salivary cortisol concentration, the time point (A and B) and the interaction between treatment, time point and herd and all lower-order interactions were additionally included as fixed effects.

In the full model of cortisol the interaction of treatment and timepoint was the effect of main interest, as we wanted to investigate if the progress in heifers' cortisol concentrations before the isolation test (timepoint A) and after the isolation test (timepoint B) differs between treatment groups. The null model thus lacked the interaction of treatment and timepoint but was otherwise identical.

Likelihood ratio tests using the ANOVA function were used to compare the full model with null model. To check if the residuals of the cortisol, HR, SDNN, RMSSD and HF met the assumption of homoscedasticity and were normally distributed, the data were visually inspected for these preconditions. Only the residuals of HR were normally distributed. Residuals of cortisol, SDNN, RMSSD and HF revealed a positive skew in the distribution, therefore, data were normalized using a log10 transformation.

For graphical representation, the R packages "ggplot2" (Wickham 2016) and "cowplot" (Wilke 2019) were used. Data depicted as Tukey-style boxplots have the median marked as the bold line. The lower line of the box corresponds to the first quartile, the upper line to the third quartile. The lowest and highest values that are still within 1.5 x interquartile range are depicted by the whiskers. All values outside of 1.5 x interquartile range are depicted as points.

## 3. Results

### 3.1 Heart rate (HR)

The comparison of the full and the null model of HR revealed a strong trend (LMM: $\chi^2 = 25.93$, df = 16, p = 0.055) towards a difference between the models. The three-way interaction between treatment, phase and herd was significant (p = 0.050) (Annex 2: Table A 3; Annex 3: Table A 7). The estimated means of HR decreased from phase 1 to phase 2 in all treatment groups of herd 2 and in all treatment groups of herd 1, except in group C (Annex 3: Table A 8), although the decrease was partly less than 1 bpm (in C and ST of herd 1). In treatment T of herd 1 the decrease was strongest with more than 16 %, though treatment T had the highest baseline value in phase 1, and in treatment S of herd 2 the decrease was second strongest with nearly 13 %. From phase 2 to phase 3 the estimated means of P and ST increased again in both herds less than 1 bpm. In herd 1 the means increased also again in treatment T to an intermediate extend (6 %), but decreased in groups C and S; in herd 2, this pattern was reversed (see also Figure 4; Annex 9: Figure A 1).

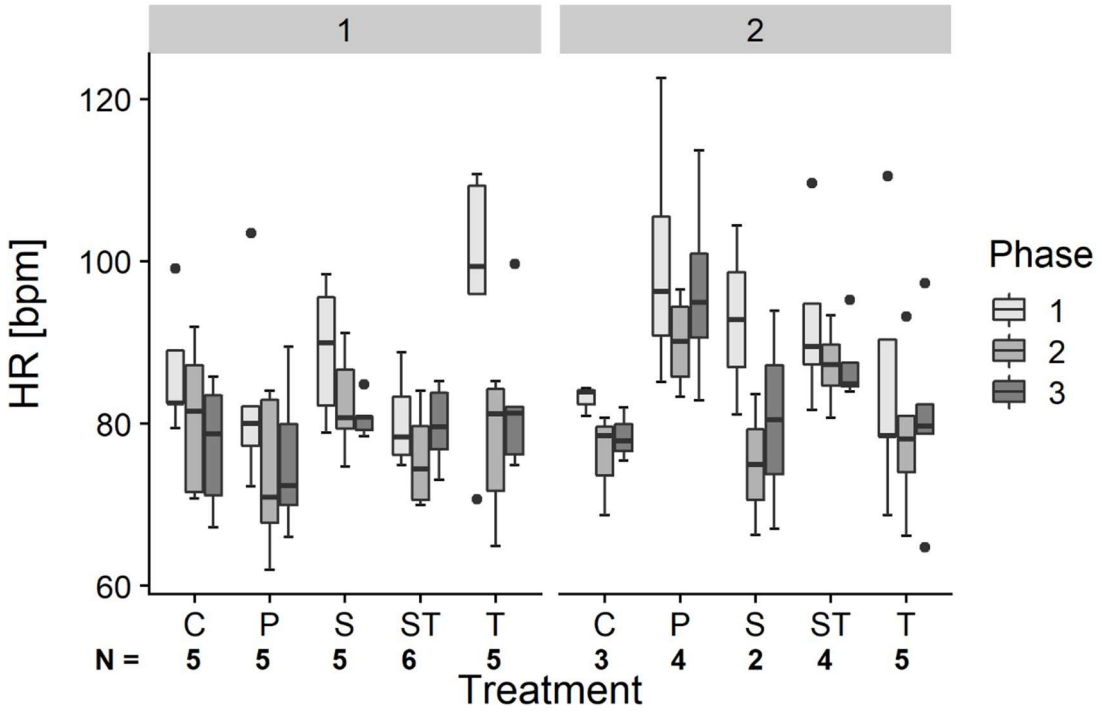


Figure 4: Mean heart rate [bpm] of heifers in herd 1 (n = 26) and herd 2 (n = 18 ) belonging to five different treatment groups: C – control, P – human presence, S – stroking, ST – stroking and talking in a gentle voice, T – talking in a gentle voice. Comparison of the mean HF between the phases 1, 2 and 3 in an isolation test (phase 1 and 3: 5 min alone in isolation, phase 2: 5 min with familiar human present). Data were averaged across 1-min segments within phase and animal. Statistics: Linear mixed model: interaction treatment * phase * herd, p = 0.05.

### 3.2 Square root of the mean squared differences of successive inter-beat intervals (RMSSD)

The comparison of the full and null model was significant (LMM: $\chi^2 = 27.97$, df = 16, p = 0.032). The three-way interaction between treatment, phase and herd was significant (p = 0.01) (Annex 2: Table A 3; Annex 4: Table A 9). From phase 1 to phase 2 the estimated means of RMSSD of herd 1 and herd 2 increased in most treatments except in treatment ST of herd 1 and S of herd 2 (Annex 4: Table A 10). Estimated means of treatment C from herd 1 increased the highest by 58 %. The estimated means of treatment T for RMSSD in herd 1 increased the second most and in herd 2 the most. Treatment ST of herd 2 had the second strongest increase with about 35 %. (Annex 4: Table A 10). From phase 2 to phase 3, the estimated means increased in both herds in

treatment P and decreased in both herds in groups C and T. In herd 1 the means increased also in group ST but decreased in group S; in herd 2, this pattern was reversed (see also Figure 5; Annex 9: Figure A 2).

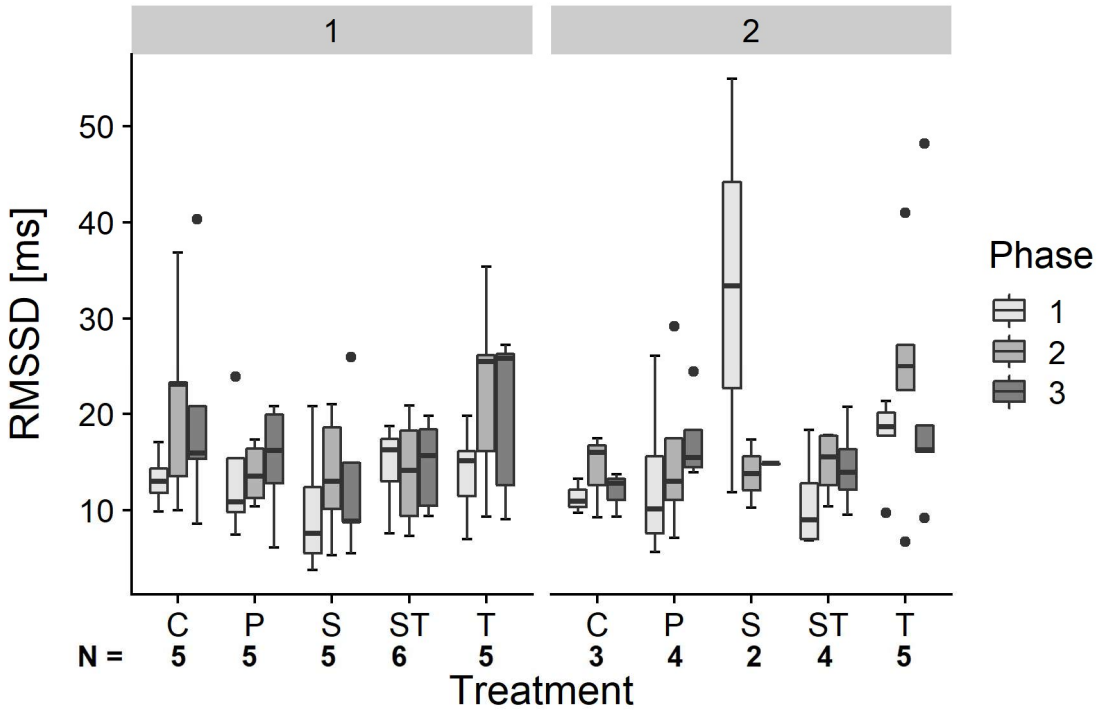


Figure 5: Mean square root of the mean squared differences of successive inter-beat intervals (RMSSD) [ms] of heifers in herd 1 (n = 26) and herd 2 (n = 18 ) belonging to five different treatment groups: C – control, P – human presence, S – stroking, ST – stroking and talking in a gentle voice, T – talking in a gentle voice. Comparison of the mean RMSSD between the phases 1,2 and 3 in an isolation test (phase 1 and 3: 5 min alone in isolation, phase 2: 5 min with familiar human present. Data were averaged across 1-min segments within phase and animal. Statistics: Linear mixed model: interaction treatment * phase * herd, p = 0.01.

### 3.3 Standard deviation of all inter-beat-intervals (SDNN)

The comparison of the full and null model of SDNN revealed a trend (LMM: $\chi^2 = 24.04$, df = 16, p = 0.089) towards a difference between the models (Annex 2: Table A 3; Annex 5: Table A 11), but the three-way interaction of treatment, phase and herd was not significant ($\chi^2 = 9.88$, df = 8, p = 0.27) (Annex 2: Table A 3; Annex 5: Table A 11; for estimated means see Annex 5: Table A 12). Therefore a reduced model without the three-way interaction was

calculated. The two-way interaction treatment * phase revealed a trend (LMM: $\chi^2 = 14.16$, df = 8, p = 0.08), the two-way interaction treatment * herd was significant (LMM: $\chi^2 = 11.1$, df = 4, p = 0.03) (Annex 2: Table A 4; Annex 5: Table A 13). In general, the estimated means increased from phase 1 to phase 2 in all treatments except treatment S (Annex 5: Table A14). For both herds the strongest increase was in treatment P, the second strongest increase was in treatment ST and the third most was in treatment C based from the basic values. From phase 2 to phase 3 the estimated means increased again in both herds except for treatment C. (see also Figure 6, Figure 7; Annex 9: Figure A 3, Figure A 4).

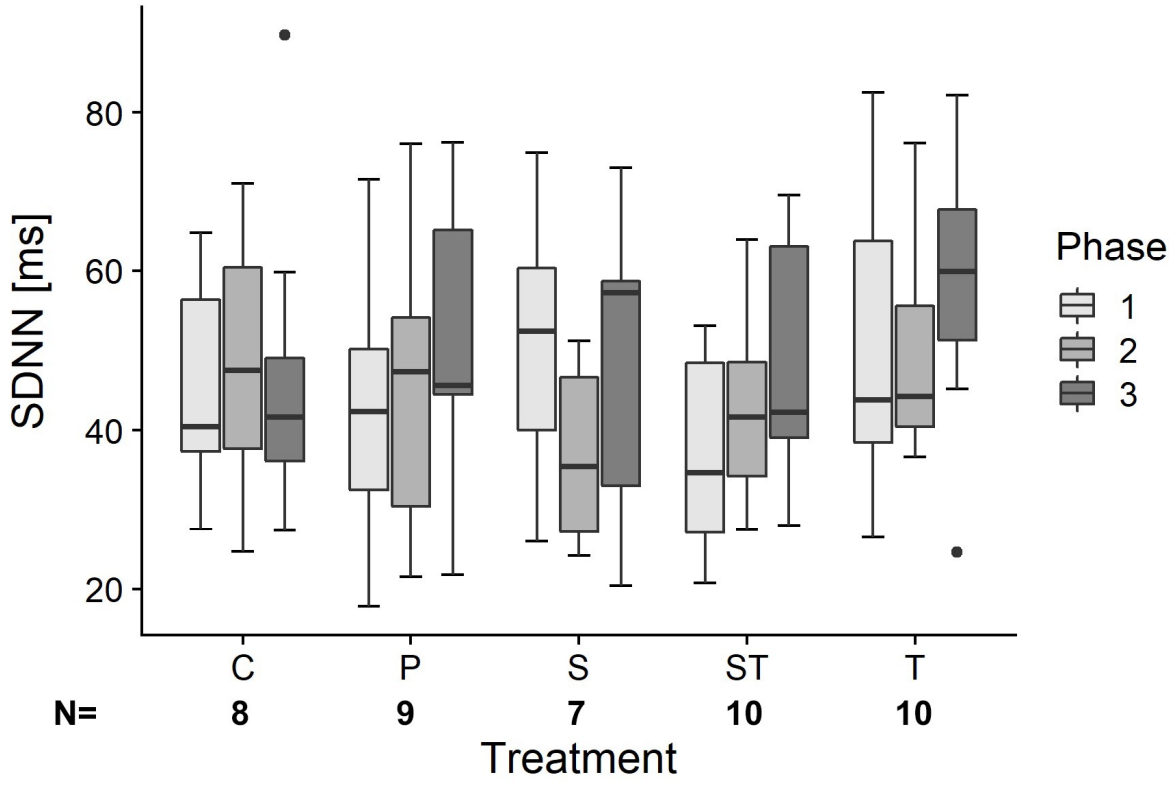


Figure 6: Mean standard deviation of the inter-beat intervals (SDNN) [ms] of all heifers (n = 44) belonging to five different treatment groups: C – control, P – human presence, S – stroking, ST – stroking and talking in a gentle voice, T – talking in a gentle voice. Comparison of the mean SDNN between the phases 1, 2 and 3 in an isolation test (phase 1 and 3: 5 min alone in isolation, 2: 5 min with familiar human present). Data were averaged across 1-min segments within phase and animal. Statistics: Linear mixed model: interaction treatment * phase, p = 0.08.

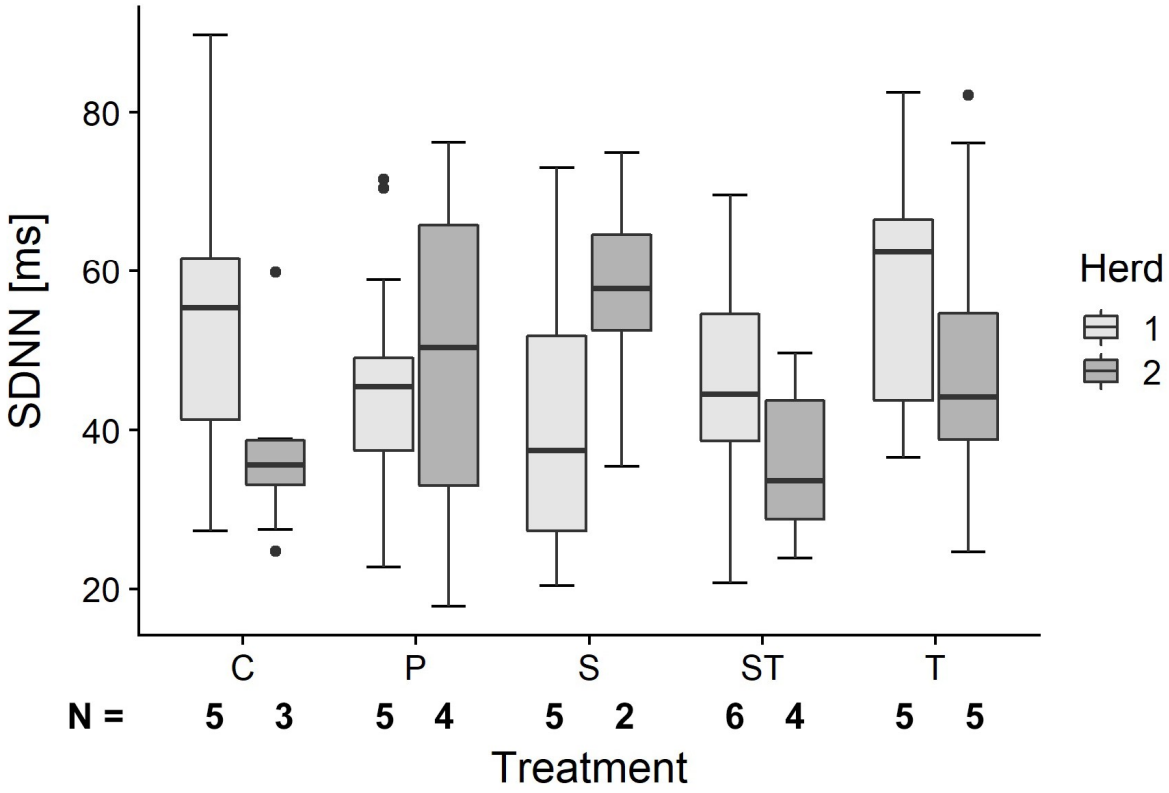


Figure 7: Mean standard deviation of the inter-beat intervals (SDNN) [ms] of heifers in herd 1 (n = 26) and herd 2 (n 18) belonging to five different treatment groups: C – control, P – human presence, S – stroking, ST – stroking and talking in a gentle voice, T – talking in a gentle voice. Comparison of the mean SDNN between the herds.. Data were averaged across 1-min segments within animals. Statistics: Linear mixed model: interaction treatment * herd, p = 0.03.

### 3.4 High frequency (HF)

The comparison of the full and null model of HF revealed no effect (LMM: $\chi^2 = 14.99$, df = 16, p = 0.53) towards a difference between the models (Annex 2: Table A 3; Annex 6: Table A 16). The three-way interaction between treatment, phase and herd was not significant (p = 0.67) (Annex 6: Table A 16; for estimated means see Annex 6: Table A 17).

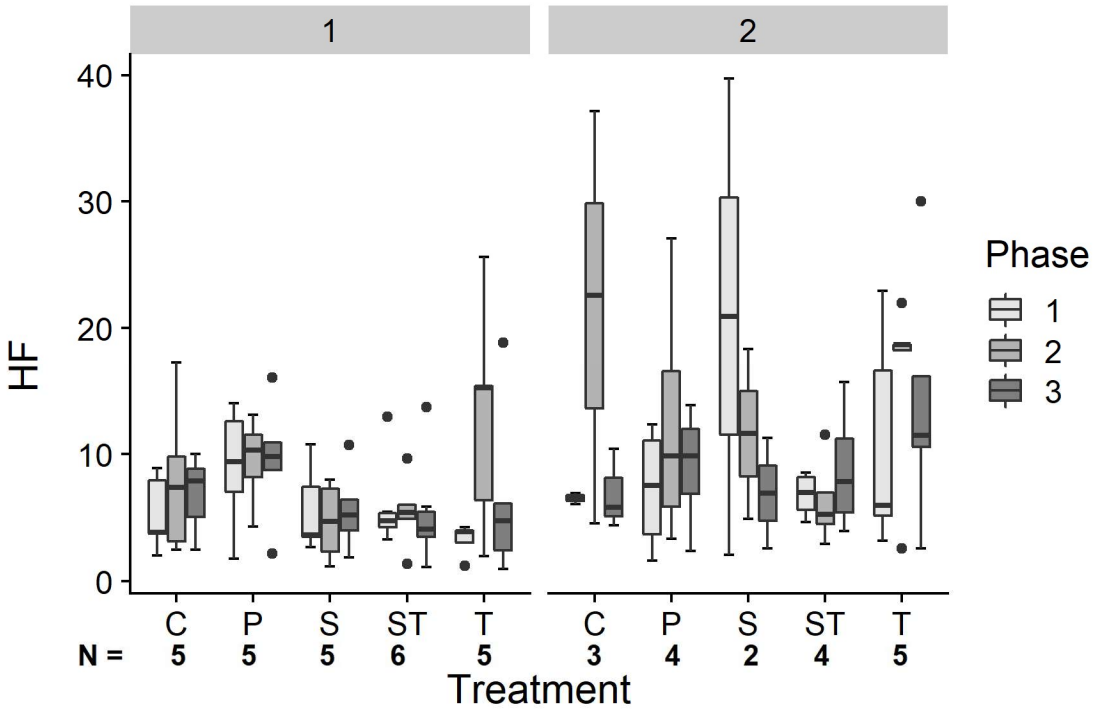


Figure 8: Mean normalized high frequency power (HF) of herd 1 (n = 26) and herd 2 (n = 19) for heifers in herd 1 (n = 26) and herd 2 (n = 18 ) belonging to five different treatment groups: C – control, P – human presence, S – stroking, ST – stroking and talking in a gentle voice, T – talking in a gentle voice. Comparison of the mean HF between the phases 1, 2 and 3 in an isolation test (phase 1 and 3: 5 min alone in isolation, phase 2: 5 min with familiar human present). Data were averaged across 1-min segments within phase and animal. Statistics: Linear mixed model: interaction treatment * phase * herd, p = 0.67.

### 3.5 Ratio RMSSD/SDNN

The comparison of the full and the null model of the ratio revealed no significant effect (LMM: $\chi^2 = 20.07$, df = 16, p = 0.22) towards a difference between the models (Annex 2: Table A 3; Annex 7: Table A 18). The three-way interaction between treatment, phase and herd was not significant (p = 0.37) (Annex 2:Table A 3; for estimated means see: Annex 7: Table A 19).

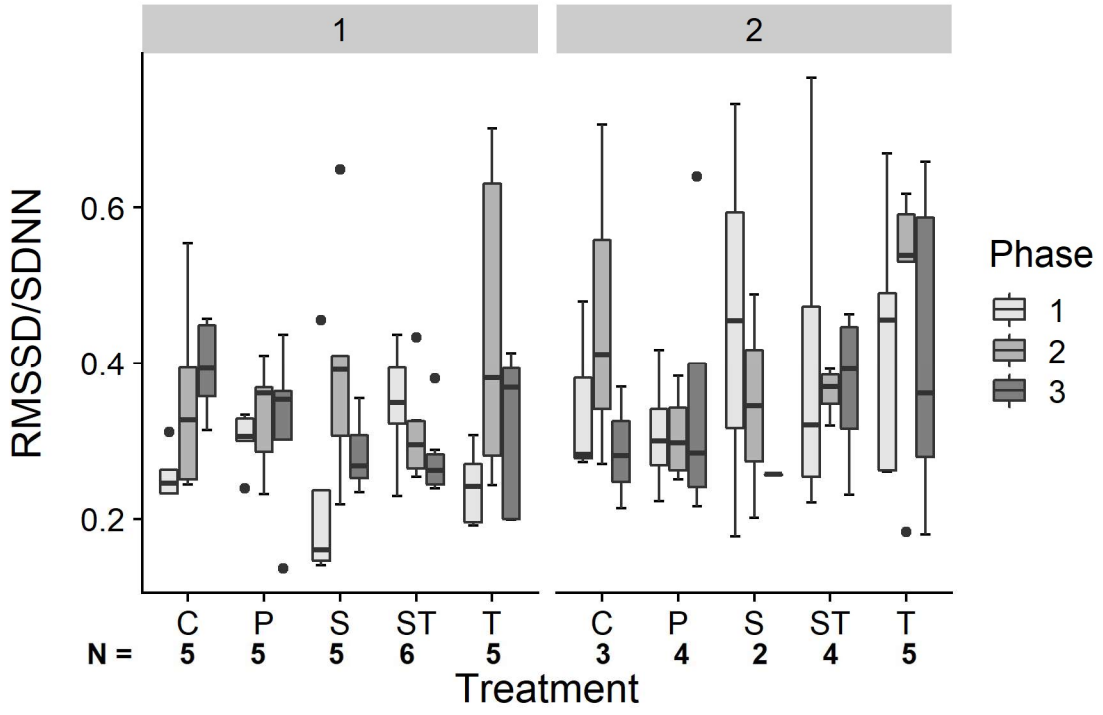


Figure 9: Mean RMSSD/SDNN of heifers in herd 1 (n = 26) and herd 2 (n = 18) belonging to five different treatment groups: C – control, P – human presence, S – stroking, ST – stroking and talking in a gentle voice, T – talking in a gentle voice. Comparison of the ratio between the phases 1, 2 and 3 in an isolation test (phase 1 and 3: 5 min alone in isolation, phase 2: 5 min with familiar human present). Data of RMSSD and SDNN were averaged across 1-min segments within phase and animal. Statistics: Linear mixed model: interaction treatment * phase * herd, p = 0.37.

### 3.6 Cortisol

The comparison of the full and null model of salivary cortisol revealed no significant effect (LMM: $\chi^2 = 0.09$, df = 8, p = 0.43) towards a difference between the models (Annex 2: Table A 5; Annex 8: Table A 20). The three-way interaction between treatment, time point and herd was not significant (p = 0.75).

The time from removing the feed before taking the sample A was shorter than 10 min for 11 heifers (n = 1, 3 min; n = 1, 5 min; n = 1, 7 min; n = 3, 8 min; n = 5, 9 min) and the time from leaving the arena until sample B was taken was longer than 5 min in 16 heifers (n = 6, 6 min; n = 2, 7 min; n = 3, 8 min; n = 1, 11 min; n = 1, 12 min; n = 1, 13 min; n = 1, 16 min; n = 1, 23 min). As this could influence the cortisol concentrations, two additional models without these A and B samples were calculated (Annex 2: Table A 6). The comparison of the full and null model without the mentioned A samples was not significant (LMM: $\chi^2 = 10.47$, df = 8, p = 0.23). The comparison of the full and null model without the mentioned B samples pointed towards a trend (LMM: $\chi^2 = 2.65$, df = 8, p = 0.12). The estimated means of the cortisol concentrations increased in all treatments from timepoint A (before the isolation test) to timepoint B (after the isolation test) in herd 1 and herd 2 (Annex 8: Table A 21; see also Figure 10; Annex 9: Figure A 7).

The mean cortisol concentration at timepoint B was 4.69 times higher than at timepoint A. Mean concentrations increased in treatment C by a factor of 5.31, in treatment P by 6.11, in treatment S by 3.92, in treatment ST by 4.09 and in treatment T by a factor of 5.50.Seventeen samples of the 18 samples below the assay's detection limit were of time point A, with two heifers belonged to treatment group C and S, respectively, four belonged to P and T, respectively, and five belonged to group ST. One sample was from time point B; this heifer belonged to treatment group C. Cortisol concentrations were higher after the test (time point B) in 53 heifers (Annex 1: Table A 1, Table A 2). Two of the four heifers with lower cortisol concentrations of sample B compared with sample A ruminated at the time of sampling and the time it took from test end till taking their samples was 13 min and 8 min, respectively. One heifer could only be lured in the feeding rack with a bucket of water and therefore drank before the experimenter could take sample B, this heifer was the above mentioned animal with 23 min from test end till taking sample B (treatment C) and had also a lower concentration in the B sample than in the A sample. The fourth of the lower B sample was taken within 3 min from test end till B sample for one heifer (treatment T). In total, three heifers ruminated at the time

of taking the B sample: the two above mentioned heifers and one heifer with B sample taken within 6 min after test end.

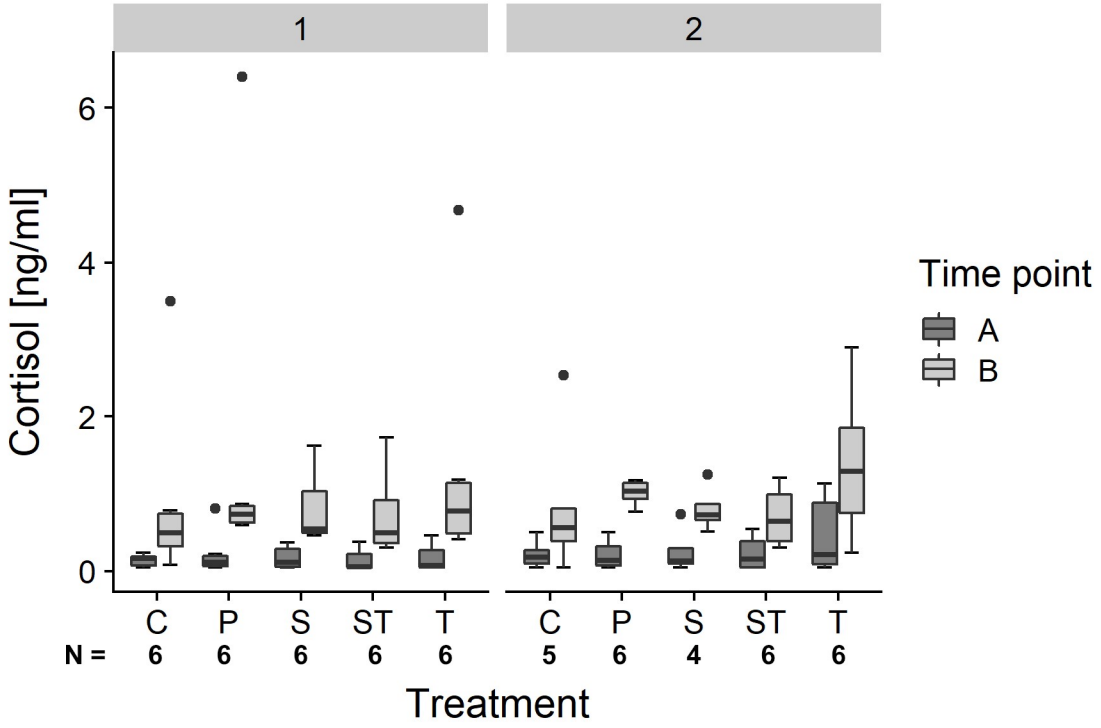


Figure 10: Mean cortisol concentrations [ng/ml] of heifers in herd 1 (n = 30) and herd 2 (n = 27) belonging to five different treatment groups: C – control, P – human presence, S – stroking, ST – stroking and talking in a gentle voice, T – talking in a gentle voice. Comparison of the mean cortisol concentrations between the timepoints A and B for sampling (A – before isolation test, B – after isolation test). Statistics: Linear mixed model: interaction treatment * timepoint * herd, p = 0.75.

## 4. Discussion

Predictions were that heifers of treatments ST, S and T had a stronger decrease of HR and salivary cortisol concentrations and stronger increase of RMSSD, SDNN, RMSSD/SDNN and HF from phase 1 (5 min isolation) to phase 2 (human present) than heifers that only experienced P or no treatment at all. From phase 2 to phase 3 (5 min isolation) the predictions were that heifers of treatment ST, S and T had a stronger increase of HR and salivary cortisol concentration and stronger decrease of RMSSD, SDNN, RMSSD/SDNN and HF in phase 3 compared to phase 2. The results do not support these predictions.

### 4.1 HR and HRV parameters

The decrease of HR from phase 1 to phase 2 in nearly all treatment groups (except treatment C of herd 1) could be due to the presence of the experimenter. A cofounding factor could also be the heifers' habituation to the novel environment. For example, a study found that HR returned to reference values after 5 min in a novel arena, with reference value of HR measured while the cow was standing in the feeding rack before this arena test (Hopster 1998). The increase of HR from phase 2 to phase 3, also in treatments without gentle interactions (again except for treatment C in herd 1) could support a general calming effect of the human presence, with increasing arousal of heifers when they were alone in the arena again, however it did not occur in all treatments that had experienced gentle interactions. In contrast, a study with calves that experienced 40 min of gentle interactions (stroking and talking) during their first four weeks of life found that HR differed from the control group in a similar arena test (isolation: increase of HR, human presence: decrease of HR, isolation: again increase) in *post hoc* comparisons (Lürzel *et al.* 2015b). Previously stroked (3 weeks, 5 min/s) dairy cows differed from animals that experienced mere presence of a human with respect to HR during a veterinary procedure in a familiar barn (Schmied *et al.* 2010). These studies did not differentiate between stroking and talking alone.

The increase of RMSSD from phase 1 to phase 2 in treatment C and the decrease in treatment S in herd 2 and in treatment ST in herd 1 are not in line with the prediction. A small increase in RMSSD from phase 1 to phase 2 for herd 1 and herd 2, regardless of treatment, could be due to the presence of the human. From phase 2 to phase 3 the RMSSD changed only slightly; but the decrease even for treatment C in herd 1 and herd 2 could indicate a calming effect of the human presence in phase 2. The decline of RMSSD was more

pronounced than SDNN, but both parameters are congruent in their ability to reflect changes of the HRV parameters due to stress (Mohr *et al.* 2002).

The strong decrease of SDNN from phase 1 to phase 2 in treatment S and the increase in C does not correspond to the prediction. All other treatments showed an increase in SDNN, pointing again to a potential effect of the experimenter as social support independent of the treatment, even for heifers of treatment C. From phase 2 to phase 3 SDNN only decreased for treatment C, probably indicating that the heifers of treatment C perceived being alone in this phase most stressful. It is not in line with the predictions that for all other treatments SDNN further increased even though in some treatment groups just to a low extent. The changes of SDNN may be less clear than the ones of RMSSD because this parameter reflects both parasympathetic as well as sympathetic influences on HR (Mohr *et al.* 2002). The further increase of SDNN from phase 2 to 3 for the groups that experienced gentle interactions and the mere presence of a human may indicate that the presence of a human resulted in support going beyond phase 2.

The results for HF showed no difference, and findings are not in line with the predictions, although another study found that RMSSD and HF were correlated – both reflecting vagal activity (Bigger *et al.* 1989) and therefore results similar to the ones of RMSSD would have been expected. Although the prerequisite to assess HF components was fulfilled, i.e. analysed sequences had a minimum length of 1-min, errors may have been amplified by the analysis of very short segments, because the sequences did not consist of consecutive time segments (Task Force 1996). Further, HF power is influenced by respiration rate, this must be considered in the analysis by adjusting the limits of the HF band, depending on the age of the animals (Hagen *et al.* 2005; Mohr *et al.* 2002). The age of heifers in this study was between 7 and 25 months, but we used the HF band for adult cows for all animals. Adjusting the bands for the age of individual animals might have been a better choice to account for the respiratory rate and might then have allowed for similar results as for RMSSD, since both parameters are predominantly mediated by the parasympathetic branch.

The ratio RMSSD/SDNN was expected to increase most for treatments with gentle interactions from phase 1 to phase 2, but no such pattern was detectable. Another study that used RMSSD/SDNN to assess the balance between the sympathetic and vagal activities obtained clearer results. Zebunke *et al.* (2011) found in pigs that RMSSD/SDNN was significantly affected due to a cognitive challenge and decreased suddenly while HR increased.

### 4.2 Cortisol

Cortisol concentrations increased in all groups, there was no difference between treatment or herd; the results do not support our hypotheses. In another study, cortisol concentrations were also not found to be significantly higher between cows left alone in an isolation chamber and cows in the isolation chamber accompanied by a familiar human brushing them (Rushen *et al.* 2001). The missing findings of a significant difference of cortisol concentrations between treatments and phases may result from various factors: Individuals perceive stress to different degrees and this may have different impacts on the increase of salivary cortisol concentrations (Schwinn *et al.* 2016), also salivary cortisol concentrations are substantially lower than in plasma and may stay below the detection limit of the kit (Negrão *et al.* 2004). This was the case for 17 samples of time point A and for one of time point B. The used minimal values (0.05 ng/ml) entered the model for these samples reflect not the true concentrations, but this fact was probably not problematic as a main effect of treatment was not expected.

Another reason might be the time differences of taking the food away before taking sample A and the time differences of test end until taking sample B. The time before feed was removed for sample A was shorter than 10 min for more than 19 % (n = 11) of animals and 27 animals (47.4 %) ruminated at that time. Only one study has examined the effects of feeding actions on salivary cortisol concentration and found no effect of feeding, drinking or rumination on the salivary cortisol concentration (Schwinn *et al.* 2016). In the present study, the time from the end of the test, i.e. when the heifers were released from the arena, was longer than 5 min in 28 % of the heifers and taken later than 10 min after the end of the test in more than 8 % of the B samples. It was necessary to get the animal restrained into the feeding rack to take a sample and the 8 % taking more than 10 min were all heifers from herd 2 that were habituated to the feeding racks only shortly before the start of the treatment. A study found that salivary cortisol concentrations still reflects the concentration in blood within a time lag of 10 min (Hernandez *et al.* 2015). Samples that have been taken within 5 min after the test end, i.e. 10 min after the end of phase 2, may still reflect the cortisol concentration in phase 2 (human present). However, studies disagree on whether there is a time lag between plasma and salivary cortisol concentrations (Hernandez *et al.* 2015; Schwinn *et al.* 2016). Samples from time point B taken later than 5 min after the end of the test are not expected to reflect the cortisol concentration in plasma of phase 2.

### 4.3 General Discussion

The main hypothesis was that treatments ST, T and S during the three weeks prior to the isolation test improve the capacity of heifers to receive social support by a familiar handler to different degrees. Further, it was expected that their capacity would be better than in the heifers which experienced only human presence or no interaction. Only the statistical analyses of HR and RMSSD showed significant results in the three-way interactions and their comparison of the full and null model. The trends of the estimated means (EMMs) pointing to a stress reduction when the human was present in phase 2, regardless of the type of treatment.

The results do not support the hypothesis. Looking at the performance of HRV data from phase 1 to phase 2 of treatments P and C, it seems that heifers of these treatments have a capacity to receive social support, as do heifers of treatments with gentle interaction. Furthermore, individual differences were seen, some of which had a large impact on the results due to less data. In addition, for the analysis of HRV parameters for herd 2 only two heifers of treatment S were left due to a lot of artefacts. The results must therefore be interpreted with caution because a single animal might have a huge impact on the data. Maybe a model excluding treatment S of both herds would show a clearer difference between the effects of the different treatments. For the test the heifers were separated not only from the herd, but even from the group within they were treated. The heifers were brought to the arena one by one. This pre-test condition could elicit stress in the animals to different extents (Waiblinger *et al.* 2006). Strong individual variation in HR reactions are possible, e.g. during a veterinary procedure, i.e. strong increase of HR to even decrease (Waiblinger *et al.* 2004). As short-term measures HRV parameters return to baseline fast (Task Force 1996), it is possible that they have already started to return to the baseline within the five minutes of phase 1.

Phase 2 was characterised not only by the presence of the experimenter, it also included talking. Experimenters did not talk to the heifers when they moved the heifers and sorted them for the treatment. Treatment P, C and S experienced talking for the first time in the arena and could also react differently to the human talking. Talking in a soothing way as how it was performed in this study (long vowels and a lowered pitch at the end of the sentence) were associated with a decrease in motor activity and might also have a calming effect (McConnell 1990). Talking in a calm and soothing way is also associated with positive cow behaviour (less kicking, stepping, Waiblinger *et al.* 2002) , but still the single effect of talking and stroking alone has to be further investigated.

The lack of significant effects for the cortisol concentration are probably due to experimental implementation, rather than the design of the isolation test. In a former study with a similar isolation test it was found that treatment had a significant main effect on salivary cortisol concentrations before and after the test (calves that were stroked and talked to vs control; Lürzel *et al.* 2015b).

In parallel to the physiological parameters evaluated in the present thesis, behavioural observations during the isolation test and an avoidance distance (AD) test at the feeding rack were performed within the framework of another thesis (Cords 2020). Cords (2020) found that the avoidance distance (AD) decreased across all treatments, but found no significant effect of the treatment on AD (Cords 2020). It is suggested that neutral handling can reduce fear of humans in farm animals (Waiblinger *et al.* 2002). The AHR might have been improved in this study by the general handling (moving, sorting heifers) that was performed in a calm, neutral way. Perhaps heifers became habituated to the experimenters in general through the preparations for the treatments, i.e. moving heifers from the pasture to the barn and back, sorting, moving the heifers between the pens etc.

## 5. Conclusion

The results of HRV parameter evaluation suggest that physiological signs of stress decreased from phase 1 to phase 2. Patterns in the parameter evaluations to demonstrate graded effects of different gentle treatments (tactile and/or auditory) were not seen clearly. It is possible that gentle interactions such as talking, stroking, or both affect heifers' capacity to receive social support and thus improve AHR to different degrees, but the expected differences could not be clearly proven in the present study.

## 6. Summary

A good animal-human relationship (AHR), based mainly on calm, positive handling of the animals, can improve animal welfare and is a prerequisite for animals being capable to receive social support by humans. In cattle, it is known that physiological signs of stress can decrease when they experience either stroking or stroking and talking in a soothing way simultaneously. This study investigated stroking and talking as separate interactions and their combined effect. It was evaluated how these gentle interactions applied over a period of time differ in their potential for stress reduction in a challenging situation, indicating an improvement of the AHR.

Sixty Austrian Simmental heifers were assigned to one of five treatments: stroking with talking (ST), talking (T), stroking (S), human presence (P) and control (C) and experienced the respective treatment for 5 min/day on 5 days/week for 3 weeks. Subsequently, an isolation test with the temporary presence of a human was performed in a test arena with opaque walls (phases 1 and 3: 5 min alone in the arena, phase 2: experimenter enters and talks soothingly to the heifer). During the test, heart rate (HR) was recorded for calculation of mean HR and HR variability (HRV) parameters. Before and after the test, saliva samples were taken for analysis of the cortisol concentration.

The three-way interaction of treatment, phase and herd was significant for mean HR and for the root mean square of the successive differences (RMSSD). As expected, mean HR decreased and RMSSD increased from phase 1 to phase 2 in almost all treatments. However, the exceptions from this rule were not consistently the groups that had not experienced gentle interactions. There were no significant effects on other HRV parameters, but the general pattern of the estimated means of the HRV data indicated a tendency for stress to decrease in phase 2 also for groups P and C. In some treatment groups within a herd, HRV results were not very meaningful because data from very few animals could be included in the analysis due to artefacts in the recordings. There was no significant difference in cortisol concentrations between the treatment groups. It is thus possible that the presence of the human exerted a calming effect in phase 2, but as there was no clear-cut difference between the groups that had experienced gentle interactions and those that had not, it cannot be excluded that the effects on HR and RMSSD were partially due to habituation to the test arena.

## 7. Zusammenfassung

Eine gute Tier-Mensch-Beziehung (TMB), die hauptsächlich auf einem ruhigen, positiven Umgang mit den Tieren beruht, kann das Tierwohl verbessern und ist eine Voraussetzung dafür, dass Tiere fähig sind soziale Unterstützung durch Menschen zu erhalten. Es ist bekannt, dass bei Rindern die physiologischen Anzeichen von Stress abnehmen können, wenn sie entweder gestreichelt werden oder gleichzeitig gestreichelt und beruhigend mit ihnen gesprochen wird. In dieser Studie wurde verglichen, in wie weit eine Phase mit regelmäßigem Streicheln oder beruhigendem Sprechen oder einer Kombination der beiden Interaktionen die TMB verbessert. Als Indikator für die TMB wurde die Fähigkeit des Menschen, die Reaktion auf eine Stress auslösende Situation zu vermindern, gewählt.

Sechzig österreichische Fleckvieh-Färsen wurden jeweils einer von fünf Behandlungsgruppen zugeteilt: Streicheln und beruhigendes Sprechen (ST), Streicheln (S), beruhigendes Sprechen (T), menschliche Anwesenheit (P), Kontrolle (C) und erfuhren die entsprechende Behandlung jeweils 5 Min/Tag an 5 Tagen/Woche drei Wochen lang. Anschließend wurde ein Isolationstest in einer Testarena mit temporärer Anwesenheit eines Menschen durchgeführt (Phasen 1 und 3: 5 Minuten allein in der Arena, Phase 2: Experimentatorin betritt Arena und spricht beruhigend mit der Färse). Während des Tests wurde die Herzfrequenz (HF) aufgezeichnet, um die durchschnittliche HF und verschiedene Parameter der HF-Variabilität (HRV) zu berechnen. Für die Analyse der Cortisol-Konzentration wurden vor und nach dem Test Speichelproben genommen.

Die Dreifachinteraktion von Behandlungsgruppe, Phase und Herde war signifikant für die Parameter HF und RMSSD (root mean square of the successive differences). Die HF sank und der Wert der RMSSD stieg wie erwartet von Phase 1 zu Phase 2 in fast allen Behandlungsgruppen. Die Ausnahmen von dieser Regel waren jedoch nicht durchweg die Gruppen, die keine sanften Interaktionen erfahren hatten. Es gab keine signifikanten Effekte auf die übrigen HRV-Parameter, aber das allgemeine Muster der geschätzten Mittelwerte der HRV-Daten deutete auf eine Tendenz zur Stressreduktion auch für P und C in Phase 2 hin. In einigen Behandlungsgruppen innerhalb einer Herde waren die HRV-Parameter nicht sehr aussagekräftig, da aufgrund von Artefakten nur Daten von sehr wenigen Tieren in die Analyse einbezogen werden konnten. Bei den Cortisoldaten gab es keinen signifikanten Unterschied zwischen den Behandlungsgruppen. Es ist daher möglich, dass die Anwesenheit des Menschen in Phase 2 eine beruhigende Wirkung hatte. Da es jedoch keinen eindeutigen

Unterschied zwischen Behandlungsgruppen gab, die freundliche Interaktionen erfuhren oder nicht, kann nicht ausgeschlossen werden, dass die Auswirkungen auf HR und RMSSD unter anderem auf die Gewöhnung an die Testarena zurückzuführen sind.

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## Annex 1: Overview of heifers of herd 1 and herd 2

Table A 1: Heifers of herd 1: age [months], assigned to treatment, pen in which they were confined for treatment, origin (VF = VetFarm; T = Tenant), test-day. Salivary cortisol concentrations at timepoint A and B ("Cort A" [ng/ml], "Cort B" [ng/ml]), time feed was took away until taking sample A ("Time till A"), time from test end until taking sampling B ("Time till B"). Also, whether heifer was ruminating or not at the timepoint of sampling A and B ("Rum at A"; "Rum at B").

| Animal ID | Age | Treat-ment | Pen | Origin | Day | Time till A | Time till B | Rum at A | Rum at B | Cort A [ng/ml] | Cort B [ng/ml] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1330 | 25.5 | S | C | VF | 38 | 00:16 | 00:02 | yes | no | 0.05 | 0.46 |
| 1335 | 24.5 | T | C | VF | 39 | 00:07 | 00:00 | no | no | 0.10 | 0.52 |
| 1339 | 24.0 | C | C | VF | 41 | 00:09 | 00:08 | no | yes | 0.18 | 0.08 |
| 2467 | 17.3 | C | C | VF | 39 | 00:18 | 00:01 | yes | no | 0.05 | 0.62 |
| 2471 | 16.7 | ST | C | VF | 42 | 00:13 | 00:01 | no | no | 0.38 | 1.73 |
| 2476 | 16.4 | C | C | VF | 42 | 00:11 | 00:06 | no | no | 0.05 | 0.78 |
| 2478 | 16.3 | C | C | VF | 38 | 00:08 | 00:03 | no | no | 0.19 | 0.31 |
| 2481 | 15.9 | S | C | VF | 39 | 00:09 | 00:04 | no | no | 0.32 | 1.19 |
| 2482 | 15.9 | P | C | VF | 38 | 00:27 | 00:06 | yes | yes | 0.05 | 0.71 |
| 2486 | 22.9 | ST | C | VF | 39 | 00:27 | 00:03 | yes | no | 0.05 | 0.30 |
| 2490 | 22.2 | ST | C | VF | 41 | 00:21 | 00:08 | yes | no | 0.06 | 0.54 |
| 2494 | 20.8 | T | C | VF | 42 | 00:10 | 00:01 | no | no | 0.33 | 1.18 |
| 2497 | 20.4 | T | C | VF | 38 | 00:20 | 00:01 | yes | no | 0.05 | 0.47 |
| 2505 | 19.6 | P | C | VF | 40 | 00:19 | 00:02 | no | no | 0.05 | 0.60 |
| 2508 | 19.3 | P | C | VF | 42 | 00:12 | 00:01 | no | no | 0.81 | 6.40 |
| 2510 | 19.3 | S | C | VF | 40 | 00:08 | 00:01 | yes | no | 0.06 | 0.52 |
| 2511 | 19.2 | T | C | VF | 40 | 00:08 | 00:01 | no | no | 0.05 | 0.41 |
| 2515 | 18.6 | P | C | VF | 39 | 00:05 | 00:06 | no | no | 0.13 | 0.75 |
| 2521 | 18.1 | S | C | VF | 42 | 00:11 | 00:01 | no | no | 0.05 | 0.49 |
| 2592 | 21.9 | P | C | T | 41 | 00:17 | 00:04 | yes | no | 0.22 | 0.87 |
| 2597 | 21.5 | T | C | T | 41 | 00:13 | 00:07 | yes | no | 0.05 | 1.03 |
| 2634 | 18.8 | P | C | T | 43 | 00:10 | 00:04 | yes | no | 0.09 | 0.59 |
| 2636 | 18.2 | T | C | T | 43 | 00:11 | 00:04 | no | no | 0.46 | 4.68 |
| 2645 | 17.1 | ST | C | T | 38 | 00:22 | 00:02 | yes | no | 0.05 | 0.33 |
| 2646 | 17.0 | S | C | T | 41 | 00:16 | 00:00 | yes | no | 0.17 | 1.62 |
| 2652 | 16.3 | ST | C | T | 43 | 00:20 | 00:01 | no | no | 0.05 | 0.45 |
| 2655 | 16.2 | C | C | T | 43 | 00:09 | 00:04 | yes | no | 0.24 | 3.50 |
| 2658 | 16.0 | S | C | T | 43 | 00:14 | 00:01 | no | no | 0.37 | 0.57 |
| 2660 | 15.6 | ST | C | T | 40 | 00:15 | 00:01 | yes | no | 0.27 | 1.04 |
| 6268 | 22.7 | C | C | T | 40 | 00:19 | 00:07 | no | no | 0.14 | 0.36 |

Table A 2: Heifers of herd 2: age [months], assigned to treatment, pen in which they were confined for treatment, origin (VF = VetFarm; T = Tenant), test-day. Salivary cortisol concentrations at timepoint A and B ("Cort A" [ng/ml], "Cort B" [ng/ml]), time feed was took away until taking sample A ("Time till A"), time from test end until taking sampling B ("Time till B"). Also, whether heifer was ruminating or not at the timepoint of sampling A and B ("Rum at A"; "Rum at B").

| Animal ID | Age | Treat-ment | Pen | Origin | Day | Time till A | Time till B | Rum at A | Rum at B | Cort A [ng/ml] | Cort B [ng/ml] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2663 | 15.3 | ST | D | T | 42 | 00:10 | 00:02 | yes | no | 0.05 | 1.21 |
| 4920 | 9.9 | T | D | T | 39 | 00:18 | 00:03 | no | no | 0.32 | 0.88 |
| 4921 | 9.4 | S | E | T | 41 | 00:25 | NR[1] | NR[1] | NR[1] | 1.39 | NR[1] |
| 4922 | 9.4 | S | E | T | 38 | 00:12 | NR[2] | NR[2] | NR[2] | 0.07 | NR[2] |
| 4924 | 9.2 | P | D | T | 40 | 00:10 | 00:03 | no | no | 0.50 | 1.17 |
| 4925 | 8.9 | C | E | T | 40 | 00:09 | 00:02 | no | no | 0.10 | 0.81 |
| 4926 | 8.9 | ST | D | T | 43 | 00:13 | 00:03 | no | no | 0.43 | 1.10 |
| 4927 | 8.6 | T | D | T | 42 | 00:16 | 00:03 | no | no | 1.13 | 0.71 |
| 4929 | 8.2 | T | E | T | 40 | 00:13 | 00:16 | yes | no | 0.08 | 1.91 |
| 4931 | 7.5 | C | E | T | 42 | 00:21 | 00:06 | yes | no | 0.27 | 2.53 |
| 4935 | 7.5 | P | E | T | 38 | 00:03 | 00:12 | yes | no | 0.05 | 0.93 |
| 7571 | 14.7 | P | D | T | 42 | 00:10 | 00:01 | yes | no | 0.13 | 1.15 |
| 7572 | 14.2 | S | E | T | 42 | 00:27 | 00:06 | yes | no | 0.05 | 0.51 |
| 7578 | 13.2 | P | D | T | 41 | 00:25 | 00:01 | yes | no | 0.14 | 0.77 |
| 7579 | 13.0 | ST | D | T | 41 | 00:10 | 00:13 | no | no | 0.54 | 0.30 |
| 9536 | 9.9 | S | D | VF | 40 | 00:09 | 00:04 | yes | yes | 0.11 | 0.74 |
| 9537 | 9.9 | T | D | VF | 38 | 00:20 | 00:03 | yes | no | 0.05 | 2.90 |
| 9538 | 9.6 | ST | D | VF | 40 | 00:10 | 00:03 | no | no | 0.26 | 0.62 |
| 9539 | 9.5 | C | D | VF | 41 | 00:10 | 00:02 | yes | no | 0.18 | 0.56 |
| 9545 | 9.0 | P | E | VF | 39 | 00:11 | 00:08 | no | no | 0.05 | 1.12 |
| 9670 | 13.0 | C | D | T | 38 | 00:19 | 00:03 | yes | no | 0.05 | 0.05 |
| 9671 | 12.9 | C | E | T | 39 | 00:13 | 00:23 | no | no | 0.50 | 0.39 |
| 9674 | 12.1 | T | E | T | 41 | 00:12 | 00:06 | no | no | 1.07 | 1.69 |
| 9678 | 11.1 | S | D | T | 39 | 00:26 | 00:04 | no | no | 0.73 | 1.25 |
| 9681 | 10.8 | S | D | T | 43 | 00:14 | 00:11 | NR | no | 0.15 | 0.71 |
| 9684 | 10.4 | P | D | T | 43 | 00:13 | 00:01 | no | no | 0.38 | 0.94 |
| 9689 | 9.9 | ST | D | T | 38 | 00:19 | 00:05 | yes | no | 0.05 | 0.67 |
| 9773 | 12.6 | T | D | VF | 43 | 00:12 | 00:03 | no | no | 0.10 | 0.24 |
| 9777 | 12.1 | ST | D | VF | 39 | 00:23 | 00:04 | yes | no | 0.05 | 0.31 |

[1] Animal could not be moved into the arena and was excluded from test; data were not recordable

[2] Animal could not be locked in the feeding rack after the test, therefore B sample was not recordable

## Annex 2: Tables of P-values

Table A 3: Statistics of the full/null model comparison, the three-way interaction treatment * phase * herd. Significant results appear in bold; trends appear in bold and italics.

| **HR/ HRV parameter** | full/null model | | | treatment * phase * herd | | |
|---|---|---|---|---|---|---|
| | $\chi^2$ | df | p | $\chi^2$ | df | p |
| HR [bpm] | 25.93 | 16 | ***0.055*** | 15.73 | 8 | **0.050** |
| SDNN [ms] | 24.04 | 16 | ***0.089*** | 9.88 | 8 | 0.270 |
| RMSSD [ms] | 27.97 | 16 | **0.032** | 21.47 | 8 | **0.010** |
| RMSSD/SDNN | 20.07 | 16 | 0.22 | 8.71 | 8 | 0.37 |
| HF power | 14.99 | 16 | 0.53 | 9.88 | 8 | 0.27 |

Table A 4: P-values of the two-way interactions of the reduced SDNN model. Significant results appear bold; trends appear in bold and italics.

| Reduced model | treatment * phase | | | treatment * herd | | | phase * herd | | |
|---|---|---|---|---|---|---|---|---|---|
| | $\chi^2$ | df | p | $\chi^2$ | df | p | $\chi^2$ | df | p |
| SDNN [ms] | 14.16 | 8 | ***0.080*** | 11.1 | 8 | **0.030** | 2.22 | 2 | 0.33 |

Table A 5**:** P-values of the full/null model comparison of cortisol concentrations, the three-way interaction treatment * timepoint * herd. Significant results appear in bold; trends appear in bold and italics.

| **Cortisol concentrations** | full/null model | | | treatment * timepoint * herd | | |
|---|---|---|---|---|---|---|
| | $\chi^2$ | df | p | $\chi^2$ | df | p |
| Cortisol [ng/ml] | 8.09 | 8 | 0.43 | 1.94 | 4 | 0.75 |

Table A 6**:** P-values of the full/null model comparison of cortisol concentrations, the three-way interaction treatment * timepoint * herd; one model without A samples and one model without B samples. Significant results appear in bold; trends appear in bold and italics.

| **Cortisol concentrations** | full/null model | | | full/null model | | |
|---|---|---|---|---|---|---|
| | without A samples | | | without B samples | | |
| | $\chi^2$ | df | p | $\chi^2$ | df | p |
| Cortisol [ng/ml] | 10,47 | 8 | 0,23 | 12,65 | 8 | ***0,12*** |

**Annex 3: HR- statistics of the full model, estimated means (EMM), standard errors (SE) and the lower and upper confidence levels ($CL_{lower}$, $CL_{upper}$) of the full model**

Table A 7: Statistics of the full model for heart rate (HR) of heifers belonging to five different treatment groups in an isolation test with three phases (phase 1, 5 min in isolation; phase 2, 5 min with familiar human present; phase 3,5 min in isolation). C – control (n = 8), P – human presence (n = 9), S – stroking (n = 7), ST – stroking and talking in a gentle voice (n = 10), T – talking in a gentle voice (n = 10). Data were averaged across 1-min segments. Statistics: Linear mixed model. Significant results appear bold; trends appear in bold and italics.

| **Full model HR** | | | | | |
|---|---|---|---|---|---|
| | **Coefficient** | **SE** | **$\chi^2$** | **df** | **p** |
| **Intercept** | 80.03 | 4 | -[f] | - | - |
| **Treatment[a]** | | | -[f] | - | - |
| P | -1.61 | 4.92 | -[f] | - | - |
| S | 5.78 | 4.96 | - | - | - |
| ST | -1.77 | 4.75 | - | - | - |
| T | 14.23 | 4.99 | - | - | - |
| **Phase[b]** | | | -[f] | - | - |
| Phase 2 | 0.79 | 2.95 | - | - | - |
| Phase 3 | -4.22 | 3.03 | - | - | - |
| **Herd[c]** | | | -[f] | - | - |
| Herd 2 | -0.74 | 6.50 | - | - | - |
| **Origin[d]** | | | - | - | - |
| Kremesberg | 3.02 | 2.24 | 1.78 | 1 | 0.18 |
| **Duration of rumination[e]** | 0.75 | 0.58 | 1.68 | 1 | 0.20 |
| **Changes of squares[e]** | 4.72 | 0.64 | 42.73 | 1 | 0.00 |
| **Age[e]** | -0.72 | 2.15 | 0.11 | 1 | 0.74 |

Table A 7 (continued): Statistics of the full model for heart rate (HR)

| **Full model HR** | | | | | |
|---|---|---|---|---|---|
| | **Coefficient** | **SE** | **$\chi^2$** | **df** | **p** |
| **Treatment * Phase** | | | -[f] | - | - |
| P * phase 2 | -2.84 | 3.96 | - | - | - |
| S * phase 2 | -5.81 | 4.02 | - | - | - |
| ST * phase 2 | -1.40 | 3.81 | - | - | - |
| T * phase 2 | -16.29 | 3.97 | - | - | - |
| P * phase 3 | 2.35 | 4.06 | - | - | - |
| S * phase 3 | -1.66 | 4.08 | - | - | - |
| ST * phase 3 | 4.23 | 3.95 | - | - | - |
| T * phase 3 | -6.22 | 4.06 | - | - | - |
| **Treatment * Herd** | | | -[f] | - | - |
| P * herd 2 | 14.66 | 7.76 | - | - | - |
| S * herd 2 | 2.42 | 8.66 | - | - | - |
| ST * herd 2 | 13.47 | 7.59 | - | - | - |
| T * herd 2 | -12.94 | 7.66 | - | - | - |
| 2 * herd 2 | -3.26 | 4.58 | - | - | - |
| 3 * herd 2 | 2.71 | 4.66 | - | - | - |
| **Treatment * Phase * Herd** | | | 15.73 | 8 | **0.05** |
| P * phase 2 * herd 2 | 3.53 | 6.21 | - | - | - |
| S * phase 2 * herd 2 | -3.05 | 7.01 | - | - | - |
| ST * phase 2 * herd 2 | -0.51 | 6.09 | - | - | - |
| T * phase 2 * herd 2 | 17.69 | 6.08 | - | - | - |
| P * phase 3 * herd 2 | -2.46 | 6.26 | - | - | - |
| S * phase 3 * herd 2 | -6.70 | 7.01 | - | - | - |
| ST * phase 3 * herd 2 | -6.37 | 6.18 | - | - | - |
| T * phase 3 * herd 2 | 4.83 | 6.30 | - | - | - |

[a] dummy coded ('C' as reference category)

[b] dummy coded (‘Phase 1’ as reference category)

[c] dummy coded ('Herd 1'as reference category)

[d] dummy coded (Origin 'tenant' as reference category)

[e] variables were centered

[f] not shown because of having a very limited interpretation

Table A 8: Estimated means (EMM), standard errors (SE) and the lower and upper confidence levels ($CL_{lower}$, $CL_{upper}$) of the full model for heart rate (HR) of heifers of herd 1 (n = 26) and herd 2 (n = 18 ) belonging to five different treatment groups in an isolation test with three phases (phase 1, 5 min in isolation; phase 2, 5 min with familiar human present; phase 3, 5 min in isolation): C – control (herd 1: n = 5; herd 2: n = 3), P – human presence (herd 1: n = 5; herd 2: n = 4), S – stroking (herd 1: n = 5; herd 2: n = 2), ST – stroking and talking in a gentle voice (herd 1: n = 6; herd 2: n = 4), T – talking in a gentle voice (herd 1: n = 5; herd 2: n = 5). Statistics: Linear mixed model: interaction treatment * phase * herd, p = 0.05.

| **Herd 1** | | | | | | **Herd 2** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treat-ment** | **Phase** | **EMM** | **SE** | **$CL_{lower}$** | **$CL_{upper}$** | **Treat-ment** | **Phase** | **EMM** | **SE** | **$CL_{lower}$** | **$CL_{upper}$** |
| C | 1 | 81.55 | 4.51 | 72.59 | 90.50 | C | 1 | 80.81 | 5.61 | 69.67 | 91.95 |
| C | 2 | 82.34 | 4.40 | 73.60 | 91.08 | C | 2 | 78.34 | 5.63 | 67.15 | 89.52 |
| C | 3 | 77.33 | 4.47 | 68.46 | 86.19 | C | 3 | 79.29 | 5.61 | 68.14 | 90.44 |
| P | 1 | 79.94 | 4.42 | 71.15 | 88.73 | P | 1 | 93.86 | 5.42 | 83.09 | 104.63 |
| P | 2 | 77.89 | 4.41 | 69.14 | 86.65 | P | 2 | 92.08 | 5.40 | 81.35 | 102.81 |
| P | 3 | 78.07 | 4.38 | 69.36 | 86.77 | P | 3 | 92.22 | 5.39 | 81.51 | 102.94 |
| S | 1 | 87.33 | 4.42 | 78.54 | 96.12 | S | 1 | 89.02 | 6.98 | 75.15 | 102.88 |
| S | 2 | 82.31 | 4.41 | 73.54 | 91.08 | S | 2 | 77.68 | 7.02 | 63.74 | 91.63 |
| S | 3 | 81.45 | 4.41 | 72.68 | 90.22 | S | 3 | 79.15 | 6.98 | 65.28 | 93.01 |
| ST | 1 | 79.77 | 3.97 | 71.89 | 87.66 | ST | 1 | 92.50 | 5.16 | 82.24 | 102.77 |
| ST | 2 | 79.16 | 3.98 | 71.26 | 87.07 | ST | 2 | 88.13 | 5.18 | 77.84 | 98.41 |
| ST | 3 | 79.79 | 3.97 | 71.91 | 87.67 | ST | 3 | 88.85 | 5.19 | 78.55 | 99.16 |
| T | 1 | 95.77 | 4.90 | 86.03 | 105.51 | T | 1 | 82.09 | 4.92 | 72.31 | 91.87 |
| T | 2 | 80.27 | 4.87 | 70.59 | 89.96 | T | 2 | 81.02 | 4.97 | 71.15 | 90.90 |
| T | 3 | 85.33 | 4.87 | 75.65 | 95.01 | T | 3 | 79.18 | 5.06 | 69.14 | 89.23 |

## Annex 4: RMSSD- statistics of the full model, estimated means (EMM), standard errors (SE) and the lower and upper confidence levels ($CL_{lower}$, $CL_{upper}$) of the full model

Table A 9: Statistics of the full model for square root of the mean squared differences of successive inter-beat intervals (RMSSD) of heifers belonging to five different treatment groups in an isolation test with three phases (phase 1, 5 min in isolation; phase 2, 5 min with familiar human present; phase 3,5 min in isolation). C – control (n = 8), P – human presence (n = 9), S – stroking (n = 7), ST – stroking and talking in a gentle voice (n = 10), T – talking in a gentle voice (n = 10). Data were averaged across 1-min segments. Statistics: Linear mixed model. Significant results appear bold; trends appear in bold and italics.

| **Full model RMSSD** | | | | | |
|---|---|---|---|---|---|
| | **Coefficient** | **SE** | **$\chi^2$** | **df** | **p** |
| **Intercept** | 4.25 | 0.39 | -[f] | - | - |
| **Treatment**[a] | | | -[f] | - | - |
| P | -0.08 | 0.22 | - | - | - |
| S | -0.29 | 0.23 | - | - | - |
| ST | 0.03 | 0.21 | - | - | - |
| T | 0.36 | 0.23 | - | - | - |
| **Phase**[b] | | | -[f] | - | - |
| Phase 2 | 0.46 | 0.16 | - | - | - |
| Phase 3 | 0.34 | 0.16 | - | - | - |
| **Herd**[c] | | | -[f] | - | - |
| Herd 2 | -0.46 | 0.29 | - | - | - |
| **Origin**[d] | | | - | - | - |
| Kremesberg | -0.09 | 0.10 | 0.81 | 1 | 0.37 |
| **Duration of rumination**[e] | -0.04 | 0.03 | 2.03 | 1 | 0.15 |
| **Changes of squares**[e] | 0.10 | 0.04 | 6.80 | 1 | **0.01** |
| **Age**[e] | -0.20 | 0.09 | 4.61 | 1 | **0.03** |
| **Heart rate**[e] | -0.02 | 0.00 | 16.97 | 1 | 0.00 |

Table A 9 (continued): Statistics of the full model for RMSSD

| **Full model RMSSD** | | | | | |
|---|---|---|---|---|---|
| | **Coefficient** | **SE** | **$\chi^2$** | **df** | **p** |
| **Treatment * Phase** | | | -[f] | - | - |
| P * phase 2 | -0.39 | 0.21 | - | - | - |
| S * phase 2 | -0.17 | 0.22 | - | - | - |
| ST * phase 2 | -0.54 | 0.21 | - | - | - |
| T * phase 2 | -0.29 | 0.23 | - | - | - |
| P * phase 3 | -0.24 | 0.22 | - | - | - |
| S * phase 3 | -0.17 | 0.22 | - | - | - |
| ST * phase 3 | -0.35 | 0.21 | - | - | - |
| T * phase 3 | -0.19 | 0.22 | - | - | - |
| **Treatment * Herd** | | | -[f] | - | - |
| P * herd 2 | 0.21 | 0.36 | - | - | - |
| S * herd 2 | 1.24 | 0.39 | - | - | - |
| ST * herd 2 | 0.07 | 0.35 | - | - | - |
| T * herd 2 | 0.00 | 0.35 | - | - | - |
| **Phase * Herd** | | | -[f] | - | - |
| phase 2 * herd 2 | -0.29 | 0.25 | - | - | - |
| phase 3 * herd 2 | -0.31 | 0.25 | - | - | - |
| **Treatment * Phase * Herd** | | | 21.47 | 8 | **0.01** |
| P * phase 2 * herd 2 | 0.43 | 0.34 | - | - | - |
| S * phase 2 * herd 2 | -0.84 | 0.38 | - | - | - |
| ST * phase 2 * herd 2 | 0.68 | 0.33 | - | - | - |
| T * phase 2 * herd 2 | 0.37 | 0.34 | - | - | - |
| P * phase 3 * herd 2 | 0.62 | 0.34 | - | - | - |
| S * phase 3 * herd 2 | -0.58 | 0.38 | - | - | - |
| ST * phase 3 * herd 2 | 0.60 | 0.34 | - | - | - |
| T * phase 3 * herd 2 | 0.29 | 0.34 | - | - | - |

[a] dummy coded ('C' as reference category)

[b] dummy coded (‘Phase 1’ as reference category)

[c] dummy coded ('Herd 1'as reference category)

[d] dummy coded (Origin 'tenant' as reference category)

[e] variables were centered

[f] not shown because of having a very limited interpretation

Table A 10: Estimated means (EMM), standard errors (SE) and the lower and upper confidence levels ($CL_{lower}$, $CL_{upper}$) of the full model for square root of the mean squared differences of successive inter-beat intervals (RMSSD) of heifers of herd 1 (n = 26) and herd 2 (n = 18 ) belonging to five different treatment groups in an isolation test with three phases (phase 1, 5 min in isolation; phase 2, 5 min with familiar human present; phase 3, 5 min in isolation): C – control (herd 1: n = 5; herd 2: n = 3), P – human presence (herd 1: n = 5; herd 2: n = 4), S – stroking (herd 1: n = 5; herd 2: n = 2), ST – stroking and talking in a gentle voice (herd 1: n = 6; herd 2: n = 4), T – talking in a gentle voice (herd 1: n = 5; herd 2: n = 5). Statistics: Linear mixed model: interaction treatment * phase * herd, p = 0.01.

| **Herd 1** | | | | | | **Herd 2** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treat-ment** | **Phase** | **EMM** | **SE** | **$CL_{lower}$** | **$CL_{upper}$** | **Treat-ment** | **Phase** | **EMM** | **SE** | **$CL_{lower}$** | **$CL_{upper}$** |
| C | 1 | 14.11 | 2.88 | 9.42 | 21.16 | C | 1 | 8.87 | 2.24 | 5.38 | 14.63 |
| C | 2 | 22.29 | 4.40 | 15.07 | 32.96 | C | 2 | 10.50 | 2.67 | 6.34 | 17.39 |
| C | 3 | 19.93 | 4.06 | 13.31 | 29.84 | C | 3 | 9.15 | 2.32 | 5.54 | 15.11 |
| P | 1 | 13.06 | 2.62 | 8.79 | 19.43 | P | 1 | 10.14 | 2.52 | 6.20 | 16.59 |
| P | 2 | 14.01 | 2.81 | 9.42 | 20.84 | P | 2 | 12.51 | 3.06 | 7.70 | 20.33 |
| P | 3 | 14.45 | 2.87 | 9.74 | 21.43 | P | 3 | 15.20 | 3.72 | 9.36 | 24.69 |
| S | 1 | 10.51 | 2.10 | 7.07 | 15.62 | S | 1 | 22.81 | 7.17 | 12.23 | 42.54 |
| S | 2 | 14.00 | 2.78 | 9.45 | 20.74 | S | 2 | 9.83 | 3.12 | 5.24 | 18.42 |
| S | 3 | 12.50 | 2.48 | 8.44 | 18.52 | S | 3 | 11.09 | 3.48 | 5.96 | 20.66 |
| ST | 1 | 14.50 | 2.60 | 10.16 | 20.69 | ST | 1 | 9.74 | 2.29 | 6.11 | 15.54 |
| ST | 2 | 13.28 | 2.40 | 9.29 | 18.99 | ST | 2 | 13.14 | 3.06 | 8.28 | 20.86 |
| ST | 3 | 14.45 | 2.59 | 10.13 | 20.61 | ST | 3 | 12.92 | 3.02 | 8.12 | 20.54 |
| T | 1 | 20.24 | 4.61 | 12.88 | 31.80 | T | 1 | 12.67 | 2.77 | 8.21 | 19.55 |
| T | 2 | 23.87 | 5.19 | 15.51 | 36.74 | T | 2 | 16.25 | 3.61 | 10.46 | 25.25 |
| T | 3 | 23.64 | 5.13 | 15.37 | 36.36 | T | 3 | 14.50 | 3.31 | 9.22 | 22.80 |

**Annex 5: SDNN- statistics of the full and the reduced model, estimated means (EMM), standard errors (SE) and the lower and upper confidence levels ($CL_{lower}$, $CL_{upper}$) of the full and the reduced model**

Table A 11: Statistics of the full model for standard deviation of the inter-beat intervals (SDNN) of heifers belonging to five different treatment groups in an isolation test with three phases (phase 1, 5 min in isolation; phase 2, 5 min with familiar human present; phase 3,5 min in isolation). C – control (n = 8), P – human presence (n = 9), S – stroking (n = 7), ST – stroking and talking in a gentle voice (n = 10), T – talking in a gentle voice (n = 10). Data were averaged across 1-min segments. Statistics: Linear mixed model. Significant results appear bold; trends appear in bold and italics.

| **Full model SDNN** | | | | | |
|---|---|---|---|---|---|
| | **Coefficient** | **SE** | **$\chi^2$** | **df** | **p** |
| **Intercept** | 4.19 | 0.29 | -[f] | - | - |
| **Treatment[a]** | | | -[f] | - | - |
| P | -0.19 | 0.16 | - | - | - |
| S | -0.13 | 0.16 | - | - | - |
| ST | -0.17 | 0.16 | - | - | - |
| T | 0.25 | 0.17 | - | - | - |
| **Phase[b]** | | | -[f] | - | - |
| Phase 2 | 0.26 | 0.13 | - | - | - |
| Phase 3 | 0.08 | 0.13 | - | - | - |
| **Herd[c]** | | | -[f] | - | - |
| Herd 2 | -0.67 | 0.21 | - | - | - |
| **Origin[d]** | | | - | - | - |
| Kremesberg | -0.03 | 0.07 | 0.18 | 1 | 0.67 |
| **Duration of rumination[e]** | -0.06 | 0.02 | 6.21 | 1 | **0.01** |
| **Changes of squares[e]** | 0.09 | 0.03 | 7.96 | 1 | 0.00 |
| **Age[e]** | -0.21 | 0.06 | 9.62 | 1 | 0.00 |
| **Heart rate[e]** | 0.00 | 0.00 | 0.54 | 1 | 0.46 |

Table A 11 (continued): Statistics of the full model for SDNN

**Full model SDNN**

| | Coefficient | SE | $\chi^2$ | df | p |
|---|---|---|---|---|---|
| **Treatment * Phase** | | | -[f] | - | - |
| P * phase 2 | -0.14 | 0.17 | - | - | - |
| S * phase 2 | -0.47 | 0.18 | - | - | - |
| ST *phase 2 | -0.18 | 0.17 | - | - | - |
| T * phase 2 | -0.33 | 0.18 | - | - | - |
| P * phase 3 | 0.13 | 0.18 | - | - | - |
| S * phase 3 | -0.10 | 0.18 | - | - | - |
| ST * phase 3 | 0.14 | 0.17 | - | - | - |
| T * phase 3 | 0.07 | 0.18 | - | - | - |
| **Treatment * Herd** | | | -[f] | - | - |
| P * herd 2 | 0.15 | 0.26 | - | - | - |
| S * herd 2 | 0.83 | 0.28 | - | - | - |
| ST * herd 2 | 0.01 | 0.25 | - | - | - |
| T * herd 2 | -0.11 | 0.25 | - | - | - |
| **Phase * Herd** | | | -[f] | - | - |
| phase 2 * herd 2 | -0.24 | 0.20 | - | - | - |
| phase 3 * herd 2 | 0.19 | 0.20 | - | - | - |
| **Treatment * Phase * Herd** | | | 9.88 | 8 | 0.27 |
| P * phase 2 * herd 2 | 0.47 | 0.27 | - | - | - |
| S * phase 2 * herd 2 | 0.02 | 0.31 | - | - | - |
| ST * phase 2 * herd 2 | 0.54 | 0.27 | - | - | - |
| T * phase 2 * herd 2 | 0.54 | 0.27 | - | - | - |
| P * phase 3 * herd 2 | -0.01 | 0.27 | - | - | - |
| S * phase 3 * herd 2 | -0.36 | 0.31 | - | - | - |
| ST * phase 3 * herd 2 | -0.03 | 0.27 | - | - | - |
| T * phase 3 * herd 2 | -0.03 | 0.27 | - | - | - |

[a] dummy coded ('C' as reference category)
[b] dummy coded (‘Phase 1’ as reference category)
[c] dummy coded ('Herd 1'as reference category)
[d] dummy coded (Origin 'tenant' as reference category)
[e] variables were centered
[f] not shown because of having a very limited interpretation

Table A 12: Estimated means (EMM), standard errors (SE) and the lower and upper confidence levels ($CL_{lower}$, $CL_{upper}$) of the full model for standard deviation of the inter-beat intervals (SDNN) of heifers of herd 1 (n = 26) and herd 2 (n = 18 ) belonging to five different treatment groups in an isolation test with three phases (phase 1, 5 min in isolation; phase 2, 5 min with familiar human present; phase 3, 5 min in isolation): C – control (herd 1: n = 5; herd 2: n = 3), P – human presence (herd 1: n = 5; herd 2: n = 4), S – stroking (herd 1: n = 5; herd 2: n = 2), ST – stroking and talking in a gentle voice (herd 1: n = 6; herd 2: n = 4), T – talking in a gentle voice (herd 1: n = 5; herd 2: n = 5). Statistics: Linear mixed 55 model: interaction treatment * phase * herd, p = 0.27.

| **Herd 1** | | | | | | **Herd 2** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treat-ment** | **Phase** | **EMM** | **SE** | **$CL_{lower}$** | **$CL_{upper}$** | **Treat-ment** | **Phase** | **EMM** | **SE** | **$CL_{lower}$** | **$CL_{upper}$** |
| C | 1 | 52.93 | 7.83 | 39.51 | 70.92 | C | 1 | 27.02 | 4.91 | 18.86 | 38.71 |
| C | 2 | 68.74 | 9.76 | 51.90 | 91.05 | C | 2 | 27.51 | 5.05 | 19.13 | 39.56 |
| C | 3 | 57.30 | 8.44 | 42.81 | 76.68 | C | 3 | 35.26 | 6.43 | 24.58 | 50.59 |
| P | 1 | 43.99 | 6.36 | 33.05 | 58.56 | P | 1 | 26.19 | 4.67 | 18.40 | 37.29 |
| P | 2 | 49.60 | 7.18 | 37.25 | 66.05 | P | 2 | 37.04 | 6.50 | 26.17 | 52.42 |
| P | 3 | 54.42 | 7.80 | 40.98 | 72.27 | P | 3 | 38.52 | 6.75 | 27.23 | 54.51 |
| S | 1 | 46.26 | 6.66 | 34.79 | 61.51 | S | 1 | 54.26 | 12.28 | 34.67 | 84.91 |
| S | 2 | 37.61 | 5.37 | 28.36 | 49.87 | S | 2 | 35.24 | 8.05 | 22.42 | 55.39 |
| S | 3 | 45.48 | 6.50 | 34.29 | 60.34 | S | 3 | 44.93 | 10.14 | 28.75 | 70.23 |
| ST | 1 | 44.87 | 5.81 | 34.73 | 57.97 | ST | 1 | 23.06 | 3.90 | 16.49 | 32.23 |
| ST | 2 | 48.72 | 6.35 | 37.65 | 63.04 | ST | 2 | 33.72 | 5.64 | 24.22 | 46.94 |
| ST | 3 | 55.69 | 7.20 | 43.12 | 71.92 | ST | 3 | 33.47 | 5.62 | 24.00 | 46.68 |
| T | 1 | 68.16 | 11.17 | 49.28 | 94.29 | T | 1 | 31.29 | 4.89 | 22.96 | 42.64 |
| T | 2 | 63.40 | 9.86 | 46.60 | 86.26 | T | 2 | 39.16 | 6.23 | 28.58 | 53.66 |
| T | 3 | 78.89 | 12.24 | 58.02 | 107.28 | T | 3 | 42.35 | 6.97 | 30.58 | 58.64 |

Table A 13: Statistics of the reduced model for standard deviation of the inter-beat intervals (SDNN) of heifers belonging to five different treatment groups in an isolation test with three phases (phase 1, 5 min in isolation; phase 2, 5 min with familiar human present; phase 3,5 min in isolation). C – control (n = 8), P – human presence (n = 9), S – stroking (n = 7), ST – stroking and talking in a gentle voice (n = 10), T – talking in a gentle voice (n = 10). Data were averaged across 1-min segments. Statistics: Linear mixed model. Significant results appear bold; trends appear in bold and italics.

**Model SDNN reduced**

| | **Coefficient** | **SE** | **$\chi^2$** | **df** | **p** |
|---|---|---|---|---|---|
| **Intercept** | 4.12 | 0.28 | -[f] | - | - |
| **Treatment[a]** | | | -[f] | - | - |
| P | -0.24 | 0.15 | - | - | - |
| S | -0.12 | 0.16 | - | - | - |
| ST | -0.23 | 0.15 | - | - | - |
| T | 0.16 | 0.16 | - | - | - |
| **Phase[b]** | | | -[f] | - | - |
| Phase 2 | 0.13 | 0.11 | - | - | - |
| Phase 3 | 0.12 | 0.11 | - | - | - |
| **Herd[c]** | | | -[f] | - | - |
| Herd 2 | -0.76 | 0.18 | - | - | - |
| **Origin[d]** | | | - | - | - |
| Kremesberg | -0.03 | 0.07 | 0.24 | 1 | 0.62 |
| **Duration of rumination[e]** | -0.07 | 0.02 | 7.02 | 1 | **0.01** |
| **Changes of squares[e]** | 0.09 | 0.03 | 7.48 | 1 | **0.01** |
| **Age[e]** | -0.20 | 0.06 | 9.07 | 1 | 0.00 |
| **Heart rate[e]** | 0.00 | 0.00 | 0.12 | 1 | 0.73 |

Table A 13 (continued): Statistics of the reduced model for SDNN

**Model SDNN reduced**

| | Coefficient | SE | $\chi^2$ | df | p |
|---|---|---|---|---|---|
| **Treatment * Phase** | | | 14.16 | 8 | ***0.08*** |
| P * phase 2 | 0.05 | 0.14 | - | - | - |
| S * phase 2 | -0.43 | 0.15 | - | - | - |
| ST * phase 2 | 0.03 | 0.14 | - | - | - |
| T * phase 2 | -0.10 | 0.14 | - | - | - |
| P * phase 3 | 0.13 | 0.14 | - | - | - |
| S * phase 3 | -0.21 | 0.15 | - | - | - |
| ST * phase 3 | 0.12 | 0.14 | - | - | - |
| T * phase 3 | 0.06 | 0.14 | - | - | - |
| **Treatment * Herd** | | | 11.1 | 4 | **0.03** |
| P * herd 2 | 0.28 | 0.21 | - | - | - |
| S * herd 2 | 0.72 | 0.22 | - | - | - |
| ST * herd 2 | 0.16 | 0.20 | - | - | - |
| T * herd 2 | 0.08 | 0.20 | - | - | - |
| **Phase * Herd** | | | 2.22 | 2 | 0.33 |
| phase 2 * herd 2 | 0.11 | 0.09 | - | - | - |
| phase 3 * herd 2 | 0.12 | 0.09 | - | - | - |

[a] dummy coded ('C' as reference category)

[b] dummy coded (‘Phase 1’ as reference category)

[c] dummy coded ('Herd 1'as reference category)

[d] dummy coded (Origin 'tenant' as reference category)

[e] variables were centered

[f] not shown because of having a very limited interpretation

Table A 14: Estimated means (EMM), standard errors (SE) and the lower and upper confidence levels ($CL_{lower}$, $CL_{upper}$) of the reduced model for standard deviation of the inter-beat intervals (SDNN) of heifers of herd 1 (n = 26) and herd 2 (n = 18 ) belonging to five different treatment groups in an isolation test with three phases (phase 1, 5 min in isolation; phase 2, 5 min with familiar human present; phase 3, 5 min in isolation): C – control (herd 1: n = 5; herd 2: n = 3), P – human presence (herd 1: n = 5; herd 2: n = 4), S – stroking (herd 1: n = 5; herd 2: n = 2), ST – stroking and talking in a gentle voice (herd 1: n = 6; herd 2: n = 4), T – talking in a gentle voice (herd 1: n = 5; herd 2: n = 5). Statistics: Linear mixed model: interaction treatment * phase, p = 0.08; treatment * herd, p = 0.03; phase * herd, p = 0.33.

| **Herd 1** | | | | | | **Herd 2** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treat-ment** | **Phase** | **EMM** | **SE** | **$CL_{lower}$** | **$CL_{upper}$** | **Treat-ment** | **Phase** | **EMM** | **SE** | **$CL_{lower}$** | **$CL_{upper}$** |
| C | 1 | 54.69 | 7.70 | 41.38 | 72.28 | C | 1 | 25.47 | 4.28 | 18.24 | 35.55 |
| C | 2 | 62.58 | 8.54 | 47.75 | 82.01 | C | 2 | 32.60 | 5.53 | 23.29 | 45.63 |
| C | 3 | 61.47 | 8.55 | 46.67 | 80.95 | C | 3 | 32.22 | 5.49 | 22.99 | 45.16 |
| P | 1 | 42.90 | 5.89 | 32.68 | 56.30 | P | 1 | 26.55 | 4.51 | 18.97 | 37.17 |
| P | 2 | 51.36 | 7.12 | 39.03 | 67.57 | P | 2 | 35.56 | 5.91 | 25.57 | 49.46 |
| P | 3 | 54.68 | 7.45 | 41.74 | 71.63 | P | 3 | 38.11 | 6.33 | 27.42 | 52.97 |
| S | 1 | 48.60 | 6.83 | 36.79 | 64.19 | S | 1 | 46.57 | 9.52 | 31.04 | 69.89 |
| S | 2 | 36.31 | 5.03 | 27.60 | 47.76 | S | 2 | 38.92 | 8.00 | 25.88 | 58.53 |
| S | 3 | 44.49 | 6.16 | 33.81 | 58.53 | S | 3 | 48.00 | 9.83 | 31.96 | 72.09 |
| ST | 1 | 43.58 | 5.39 | 34.12 | 55.67 | ST | 1 | 23.92 | 3.78 | 17.49 | 32.71 |
| ST | 2 | 51.23 | 6.38 | 40.04 | 65.55 | ST | 2 | 31.46 | 5.01 | 22.93 | 43.15 |
| ST | 3 | 55.07 | 6.83 | 43.09 | 70.39 | ST | 3 | 34.03 | 5.36 | 24.90 | 46.50 |
| T | 1 | 64.50 | 10.02 | 47.41 | 87.74 | T | 1 | 32.38 | 4.88 | 24.01 | 43.67 |
| T | 2 | 66.87 | 9.97 | 49.75 | 89.88 | T | 2 | 37.56 | 5.78 | 27.69 | 50.95 |
| T | 3 | 77.18 | 11.42 | 57.55 | 103.51 | T | 3 | 43.63 | 6.75 | 32.10 | 59.29 |

## Annex 6: HF- statistics of the full model, estimated means (EMM), standard errors (SE) and the lower and upper confidence levels ($CL_{lower}$, $CL_{upper}$) of the full model

Table A 16: Statistics of the normalized high frequency power (HF) of heifers belonging to five different treatment groups in an isolation test with three phases (phase 1, 5 min in isolation; phase 2, 5 min with familiar human present; phase 3,5 min in isolation). C – control (n = 8), P – human presence (n = 9), S – stroking (n = 7), ST – stroking and talking in a gentle voice (n = 10), T – talking in a gentle voice (n = 10). Data were averaged across 1-min segments. Statistics: Linear mixed model. Significant results appear bold; trends appear in bold and italics.

**Full model HF**

| | Coefficient | SE | $\chi^2$ | df | p |
|---|---|---|---|---|---|
| **Intercept** | 5.35 | 0.60 | -[f] | - | - |
| **Treatment**[a] | | | -[f] | - | - |
| P | 0.30 | 0.34 | - | - | - |
| S | 0.15 | 0.35 | - | - | - |
| ST | -0.21 | 0.33 | - | - | - |
| T | 0.07 | 0.36 | - | - | - |
| **Phase**[b] | | | -[f] | - | - |
| Phase 2 | 0.01 | 0.32 | - | - | - |
| Phase 3 | -0.12 | 0.32 | - | - | - |
| **Herd**[c] | | | -[f] | - | - |
| Herd 2 | -0.21 | 0.43 | - | - | - |
| **Origin**[d] | | | - | - | - |
| Kremesberg | -0.16 | 0.12 | 1.76 | 1 | 0.18 |
| **Duration of rumination**[e] | -0.01 | 0.05 | 0.07 | 1 | 0.79 |
| **Changes of squares**[e] | -0.04 | 0.07 | 0.45 | 1 | 0.50 |
| **Age**[e] | -0.24 | 0.12 | 3.94 | 1 | **0.05** |
| **Heart rate**[e] | -0.04 | 0.01 | 29.74 | 1 | 0.00 |

Table A 16 (continued): Statistics of the HF

**Full model HF**

| | Coefficient | SE | $\chi^2$ | df | p |
|---|---|---|---|---|---|
| **Treatment * Phase** | | | -[f] | - | - |
| P * phase 2 | -0.28 | 0.43 | - | - | - |
| S * phase 2 | -0.55 | 0.44 | - | - | - |
| ST * phase 2 | -0.34 | 0.41 | - | - | - |
| T * phase 2 | 0.29 | 0.44 | - | - | - |
| P * phase 3 | -0.15 | 0.44 | - | - | - |
| S * phase 3 | -0.24 | 0.44 | - | - | - |
| ST * phase 3 | -0.15 | 0.43 | - | - | - |
| T * phase 3 | -0.16 | 0.44 | - | - | - |
| **Treatment * Herd** | | | -[f] | - | - |
| P * herd 2 | 0.17 | 0.55 | - | - | - |
| S * herd 2 | 0.55 | 0.60 | - | - | - |
| ST * herd 2 | 0.56 | 0.53 | - | - | - |
| T * herd 2 | 0.16 | 0.53 | - | - | - |
| **Phase * Herd** | | | -[f] | - | - |
| phase 2 * herd 2 | 0.53 | 0.50 | - | - | - |
| phase 3 * herd 2 | -0.11 | 0.50 | - | - | - |
| **Treatment * Phase * Herd** | | | 5.8 | 8 | 0.67 |
| P * phase 2 * herd 2 | -0.23 | 0.67 | - | - | - |
| S * phase 2 * herd 2 | -0.74 | 0.76 | - | - | - |
| ST * phase 2 * herd 2 | -0.61 | 0.66 | - | - | - |
| T * phase 2 * herd 2 | -0.71 | 0.67 | - | - | - |
| P * phase 3 * herd 2 | 0.51 | 0.68 | - | - | - |
| S * phase 3 * herd 2 | -0.58 | 0.76 | - | - | - |
| ST * phase 3 * herd 2 | 0.30 | 0.67 | - | - | - |
| T * phase 3 * herd 2 | 0.48 | 0.68 | - | - | - |

[a] dummy coded ('C' as reference category)

[b] dummy coded (‘Phase 1’ as reference category)

[c] dummy coded ('Herd 1'as reference category)

[d] dummy coded (Origin 'tenant' as reference category)

[e] variables were centered

[f] not shown because of having a very limited interpretation

Table A 17: Estimated means (EMM), standard errors (SE) and the lower and upper confidence levels ($CL_{lower}$, $CL_{upper}$) of the full model for normalized high frequency power (HF) of heifers of herd 1 (n = 26) and herd 2 (n = 18 ) belonging to five different treatment groups in an isolation test with three phases (phase 1, 5 min in isolation; phase 2, 5 min with familiar human present; phase 3, 5 min in isolation): C – control (herd 1: n = 5; herd 2: n = 3), P – human presence (herd 1: n = 5; herd 2: n = 4), S – stroking (herd 1: n = 5; herd 2: n = 2), ST – stroking and talking in a gentle voice (herd 1: n = 6; herd 2: n = 4), T – talking in a gentle voice (herd 1: n = 5; herd 2: n = 5). Statistics: Linear mixed model: interaction treatment * phase * herd, p = 0.67.

| **Herd 1** | | | | | | **Herd 2** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treat-ment** | **Phase** | **EMM** | **SE** | **$CL_{lower}$** | **$CL_{upper}$** | **Treat-ment** | **Phase** | **EMM** | **SE** | **$CL_{lower}$** | **$CL_{upper}$** |
| C | 1 | 6.60 | 2.05 | 3.57 | 12.19 | C | 1 | 5.32 | 2.02 | 2.51 | 11.27 |
| C | 2 | 6.66 | 1.97 | 3.71 | 11.96 | C | 2 | 9.16 | 3.53 | 4.29 | 19.59 |
| C | 3 | 5.84 | 1.81 | 3.17 | 10.75 | C | 3 | 4.23 | 1.61 | 1.99 | 8.99 |
| P | 1 | 8.87 | 2.69 | 4.88 | 16.13 | P | 1 | 8.52 | 3.15 | 4.11 | 17.69 |
| P | 2 | 6.80 | 2.06 | 3.73 | 12.39 | P | 2 | 8.87 | 3.21 | 4.34 | 18.11 |
| P | 3 | 6.78 | 2.03 | 3.75 | 12.25 | P | 3 | 9.79 | 3.54 | 4.79 | 20.01 |
| S | 1 | 7.65 | 2.30 | 4.22 | 13.86 | S | 1 | 10.69 | 5.05 | 4.21 | 27.16 |
| S | 2 | 4.44 | 1.32 | 2.47 | 8.00 | S | 2 | 5.04 | 2.41 | 1.96 | 12.95 |
| S | 3 | 5.33 | 1.59 | 2.96 | 9.61 | S | 3 | 3.76 | 1.77 | 1.48 | 9.53 |
| ST | 1 | 5.37 | 1.45 | 3.15 | 9.17 | ST | 1 | 7.6 | 2.66 | 3.8 | 15.18 |
| ST | 2 | 3.86 | 1.06 | 2.25 | 6.63 | ST | 2 | 5.05 | 1.75 | 2.55 | 10.02 |
| ST | 3 | 4.11 | 1.11 | 2.41 | 7.01 | ST | 3 | 7.05 | 2.46 | 3.54 | 14.04 |
| T | 1 | 7.10 | 2.41 | 3.63 | 13.88 | T | 1 | 6.74 | 2.16 | 3.58 | 12.72 |
| T | 2 | 9.58 | 3.06 | 5.09 | 18.02 | T | 2 | 7.66 | 2.51 | 4.01 | 14.64 |
| T | 3 | 5.35 | 1.71 | 2.84 | 10.05 | T | 3 | 7.34 | 2.51 | 3.74 | 14.43 |

## Annex 7: RMSSD/SDNN- statistics of the full model, Estimated means (EMM), standard errors (SE) and the lower and upper confidence levels ($CL_{lower}$, $CL_{upper}$) of the full model

Table A 18: Statistics of ration of RMSSD and SDNN of heifers belonging to five different treatment groups in an isolation test with three phases (phase 1, 5 min in isolation; phase 2, 5 min with familiar human present; phase 3,5 min in isolation). C – control (n = 8), P – human presence (n = 9), S – stroking (n = 7), ST – stroking and talking in a gentle voice (n = 10), T – talking in a gentle voice (n = 10). Data were averaged across 1-min segments. Statistics: Linear mixed model. Significant results appear bold; trends appear in bold and italics.

**Full model RMSSD/SDNN**

| | Coefficient | SE | $\chi^2$ | df | p |
|---|---|---|---|---|---|
| **Intercept** | 0.83 | 0.11 | -[f] | - | - |
| **Treatment**[a] | | | -[f] | - | - |
| P | 0.04 | 0.06 | - | - | - |
| S | -0.01 | 0.07 | - | - | - |
| ST | 0.04 | 0.06 | - | - | - |
| T | 0.04 | 0.07 | - | - | - |
| **Phase**[b] | | | -[f] | - | - |
| Phase 2 | 0.05 | 0.06 | - | - | - |
| Phase 3 | 0.04 | 0.06 | - | - | - |
| **Herd**[c] | | | -[f] | - | - |
| Herd 2 | 0.04 | 0.08 | - | - | - |
| **Origin**[d] | | | - | - | - |
| Kremesberg | -0.01 | 0.02 | 0.37 | 1 | 0.54 |
| **Duration of rumination**[e] | 0.02 | 0.01 | 2.76 | 1 | ***0.10*** |
| **Changes of squares**[e] | 0.00 | 0.01 | 0.003 | 1 | 0.96 |
| **Age**[e] | -0.01 | 0.02 | 0.24 | 1 | 0.62 |
| **Heart rate**[e] | -0.01 | 0.001 | 23.13 | 1 | 0.00 |

Table A 18 (continued): Statistics of RMSSD/SDNN

**Full model RMSSD/SDNN**

| | Coefficient | SE | $\chi^2$ | df | p |
|---|---|---|---|---|---|
| **Treatment * Phase** | | | -[f] | - | - |
| P * phase 2 | -0.08 | 0.08 | - | - | - |
| S * phase 2 | 0.07 | 0.09 | - | - | - |
| ST * phase 2 | -0.11 | 0.08 | - | - | - |
| T * phase 2 | 0.07 | 0.09 | - | - | - |
| P * phase 3 | -0.06 | 0.09 | - | - | - |
| S * phase 3 | -0.04 | 0.09 | - | - | - |
| ST * phase 3 | -0.11 | 0.08 | - | - | - |
| T * phase 3 | -0.04 | 0.09 | - | - | - |
| **Treatment * Herd** | | | -[f] | - | - |
| P * herd 2 | 0.04 | 0.10 | - | - | - |
| S * herd 2 | 0.16 | 0.11 | - | - | - |
| ST * herd 2 | 0.06 | 0.10 | - | - | - |
| T * herd 2 | 0.04 | 0.10 | - | - | - |
| **Phase * Herd** | | | -[f] | - | - |
| phase 2 * herd 2 | 0.06 | 0.10 | - | - | - |
| phase 3 * herd 2 | -0.10 | 0.10 | - | - | - |
| **Treatment * Phase * Herd** | | | 8.71 | 8 | 0.37 |
| P * phase 2 * herd 2 | -0.08 | 0.13 | - | - | - |
| S * phase 2 * herd 2 | -0.33 | 0.15 | - | - | - |
| ST * phase 2 * herd 2 | -0.06 | 0.13 | - | - | - |
| T * phase 2 * herd 2 | -0.11 | 0.13 | - | - | - |
| P * phase 3 * herd 2 | 0.12 | 0.13 | - | - | - |
| S * phase 3 * herd 2 | -0.15 | 0.15 | - | - | - |
| ST * phase 3 * herd 2 | 0.14 | 0.13 | - | - | - |
| T * phase 3 * herd 2 | 0.07 | 0.13 | - | - | - |

[a] dummy coded ('C' as reference category)

[b] dummy coded (‘Phase 1’ as reference category)

[c] dummy coded ('Herd 1'as reference category)

[d] dummy coded (Origin 'tenant' as reference category)

[e] variables were centered

[f] not shown because of having a very limited interpretation

Table A 19: Estimated means (EMM), standard errors (SE) and the lower and upper confidence levels ($CL_{lower}$, $CL_{upper}$) of the ratio of RMSSD/SDNN of heifers of herd 1 (n = 26) and herd 2 (n = 18 ) belonging to five different treatment groups in an isolation test with three phases (phase 1, 5 min in isolation; phase 2, 5 min with familiar human present; phase 3, 5 min in isolation): C – control (herd 1: n = 5; herd 2: n = 3), P – human presence (herd 1: n = 5; herd 2: n = 4), S – stroking (herd 1: n = 5; herd 2: n = 2), ST – stroking and talking in a gentle voice (herd 1: n = 6; herd 2: n = 4), T – talking in a gentle voice (herd 1: n = 5; herd 2: n = 5). Statistics: Linear mixed model: interaction treatment * phase * herd, p = 0.67.

| **Herd 1** | | | | | | **Herd 2** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treat-ment** | **Phase** | **EMM** | **SE** | **$CL_{lower}$** | **$CL_{upper}$** | **Treat-ment** | **Phase** | **EMM** | **SE** | **$CL_{lower}$** | **$CL_{upper}$** |
| C | 1 | 52.93 | 7.83 | 39.51 | 70.92 | C | 1 | 27.02 | 4.91 | 18.86 | 38.71 |
| C | 2 | 68.74 | 9.76 | 51.90 | 91.05 | C | 2 | 27.51 | 5.05 | 19.13 | 39.56 |
| C | 3 | 57.30 | 8.44 | 42.81 | 76.68 | C | 3 | 35.26 | 6.43 | 24.58 | 50.59 |
| P | 1 | 43.99 | 6.36 | 33.05 | 58.56 | P | 1 | 26.19 | 4.67 | 18.40 | 37.29 |
| P | 2 | 49.60 | 7.18 | 37.25 | 66.05 | P | 2 | 37.04 | 6.50 | 26.17 | 52.42 |
| P | 3 | 54.42 | 7.80 | 40.98 | 72.27 | P | 3 | 38.52 | 6.75 | 27.23 | 54.51 |
| S | 1 | 46.26 | 6.66 | 34.79 | 61.51 | S | 1 | 54.26 | 12.28 | 34.67 | 84.91 |
| S | 2 | 37.61 | 5.37 | 28.36 | 49.87 | S | 2 | 35.24 | 8.05 | 22.42 | 55.39 |
| S | 3 | 45.48 | 6.50 | 34.29 | 60.34 | S | 3 | 44.93 | 10.14 | 28.75 | 70.23 |
| ST | 1 | 44.87 | 5.81 | 34.73 | 57.97 | ST | 1 | 23.06 | 3.90 | 16.49 | 32.23 |
| ST | 2 | 48.72 | 6.35 | 37.65 | 63.04 | ST | 2 | 33.72 | 5.64 | 24.22 | 46.94 |
| ST | 3 | 55.69 | 7.20 | 43.12 | 71.92 | ST | 3 | 33.47 | 5.62 | 24.00 | 46.68 |
| T | 1 | 68.16 | 11.17 | 49.28 | 94.29 | T | 1 | 31.29 | 4.89 | 22.96 | 42.64 |
| T | 2 | 63.40 | 9.86 | 46.60 | 86.26 | T | 2 | 39.16 | 6.23 | 28.58 | 53.66 |
| T | 3 | 78.89 | 12.24 | 58.02 | 107.28 | T | 3 | 42.35 | 6.97 | 30.58 | 58.64 |

## Annex 8: Cortisol- statistics of the full model, estimated means (EMM), standard errors (SE) and the lower and upper confidence levels ($CL_{lower}$, $CL_{upper}$) of the full model

Table A 20: Statistics of the full model for cortisol concentrations of heifers of herd 1 (n = 30) and herd 2 (n = 27) belonging to five different treatment groups. C – control (n = 11), P – human presence (n = 12), S – stroking (n = 10), ST – stroking and talking in a gentle voice (n = 12), T – talking in a gentle voice (n = 12). Comparison of the mean cortisol concentrations between the timepoints A and B for sampling (A – before isolation test, B – after isolation test). Statistics: Linear mixed model. Significant results appear bold; trends appear in bold and italics

**Full model cortisol concentrations**

| | Coefficient | SE | $\chi^2$ | df | p |
|---|---|---|---|---|---|
| **Intercept** | -1.93 | 0.31 | -[f] | - | - |
| **Treatment**[a] | | | -[f] | - | - |
| P | 0.27 | 0.43 | - | - | - |
| S | 0.18 | 0.43 | - | - | - |
| ST | 0.05 | 0.43 | - | - | - |
| T | -0.08 | 0.43 | - | - | - |
| **Timepoint**[b] | | | -[f] | - | - |
| timepoint B | 1.25 | 0.32 | - | - | - |
| **Herd**[c] | | | -[f] | - | - |
| herd 2 | 0.38 | 0.45 | - | - | - |
| **Origin**[d] | | | 294.18 | 6.85 | **0.01** |
| Rehgras | 0.49 | 0.18 | - | - | - |
| **Changes of squares**[e] | -0.01 | 0.09 | 287.36 | 0.03 | 0.87 |
| **Duration of rumination**[e] | -1.07 | 0.16 | 322.94 | 35.61 | 0.00 |

Table A 20 (continued): Statistics of the full model for cortisol concentrations

**Full model cortisol concentrations**

| | Coefficient | SE | $\chi^2$ | df | p |
|---|---|---|---|---|---|
| **Treatment * Timepoint** | | | -[f] | - | - |
| P * timepoint B | 0.43 | 0.44 | - | - | - |
| S * timepoint B | -0.01 | 0.45 | - | - | - |
| ST * timepoint B | -0.14 | 0.45 | - | - | - |
| T * timepoint B | 0.5 | 0.44 | - | - | - |
| **Treatment * Herd** | | | -[f] | - | - |
| P * herd 2 | -0.53 | 0.62 | - | - | - |
| S * herd 2 | -0.03 | 0.66 | - | - | - |
| ST * herd 2 | -0.26 | 0.62 | - | - | - |
| T * herd 2 | 0.2 | 0.62 | - | - | - |
| **Timepoint * Herd** | | | -[f] | - | - |
| timepoint B * herd 2 | -0.85 | 0.47 | - | - | - |
| **Treatment * Timepoint * Herd** | | | 1.94 | 4 | 0.75 |
| P * timepoint B * herd 2 | 0.58 | 0.64 | - | - | - |
| S * timepoint B * herd 2 | 0.39 | 0.68 | - | - | - |
| ST * timepoint B * herd 2 | 0.86 | 0.66 | - | - | - |
| T * timepoint B * herd 2 | 0.27 | 0.65 | - | - | - |

[a] dummy coded ('C' as reference category)

[b] dummy coded ('timepoint A' as reference category)

[c] dummy coded ('Herd 1' as reference category)

[d] dummy coded (Origin 'Kremesberg' as reference category)

[e] variables were centered

[f] not shown because of having a very limited interpretation

Table A 21: Estimated means (EMM), standard errors (SE) and the lower and upper confidence levels ($CL_{lower}$, $CL_{upper}$) of the full model for cortisol concentrations of herd 1 (n = 30) and herd 2 (n = 27) belonging to five different treatment groups. C – control (n = 11), P – human presence (n = 12), S – stroking (n = 10), ST – stroking and talking in a gentle voice (n = 12), T – talking in a gentle voice (n = 12). Comparison of the mean cortisol concentrations between the timepoints A and B for sampling (A – before isolation test, B – after isolation test). Statistics: Linear mixed model: interaction treatment * timepoint * herd, p = 0.75.

| **Herd 1** | | | | | | **Herd 2** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treat-ment** | **Time-point** | **EMM** | **SE** | **$CL_{lower}$** | **$CI_{upper}$** | **Treat-ment** | **Time-point** | **EMM** | **SE** | **$CL_{lower}$** | **$CI_{upper}$** |
| C | A | 0.11 | 0.04 | 0.06 | 0.21 | C | A | 0.16 | 0.06 | 0.08 | 0.33 |
| C | B | 0.38 | 0.13 | 0.19 | 0.75 | C | B | 0.24 | 0.09 | 0.11 | 0.51 |
| P | A | 0.14 | 0.05 | 0.07 | 0.28 | P | A | 0.12 | 0.04 | 0.06 | 0.24 |
| P | B | 0.76 | 0.26 | 0.39 | 1.50 | P | B | 0.50 | 0.18 | 0.25 | 1.02 |
| S | A | 0.13 | 0.04 | 0.07 | 0.25 | S | A | 0.18 | 0.08 | 0.08 | 0.43 |
| S | B | 0.45 | 0.16 | 0.22 | 0.90 | S | B | 0.40 | 0.17 | 0.17 | 0.94 |
| ST | A | 0.11 | 0.04 | 0.06 | 0.22 | ST | A | 0.13 | 0.04 | 0.07 | 0.25 |
| ST | B | 0.34 | 0.12 | 0.17 | 0.69 | ST | B | 0.39 | 0.13 | 0.20 | 0.77 |
| T | A | 0.10 | 0.03 | 0.05 | 0.20 | T | A | 0.18 | 0.06 | 0.09 | 0.35 |
| T | B | 0.57 | 0.20 | 0.29 | 1.15 | T | B | 0.57 | 0.20 | 0.29 | 1.14 |

**Annex 9: Line graphs of all parameters and boxplots of SDNN**

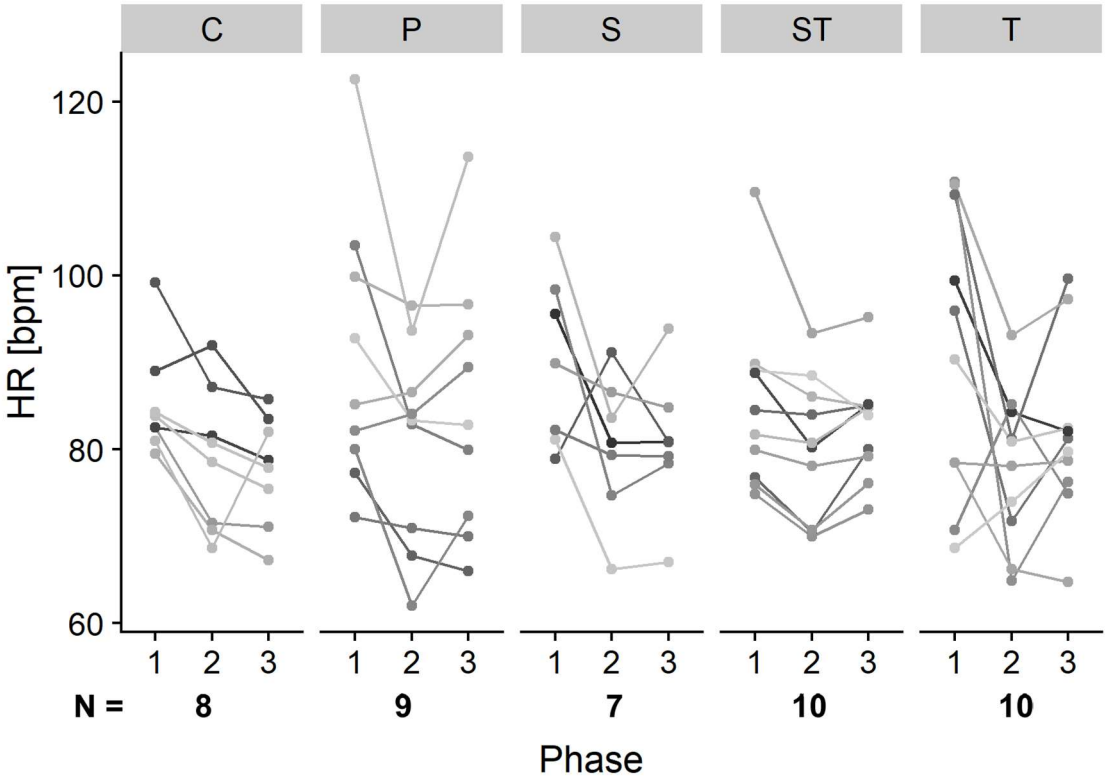


Figure A 1: Means of heart rate [bpm] of heifers belonging to five different treatment groups in an isolation test with three phases (phase 1, 5 min in isolation; phase 2, 5 min with familiar human present; phase 3, 5 min in isolation). C – control (herd 1: n = 5; herd 2: n = 3), P – human presence (herd 1: n = 5; herd 2: n = 4), S – stroking (herd 1: n = 5; herd 2: n = 2), ST – stroking and talking in a gentle voice ( herd 1: n = 6; herd 2: n = 4), T – talking in a gentle voice (herd 1: n = 5; herd 2: n = 5). Data were averaged across 1-min segments. Statistics: Linear mixed model: interaction treatment * phase * herd, p = 0.05.

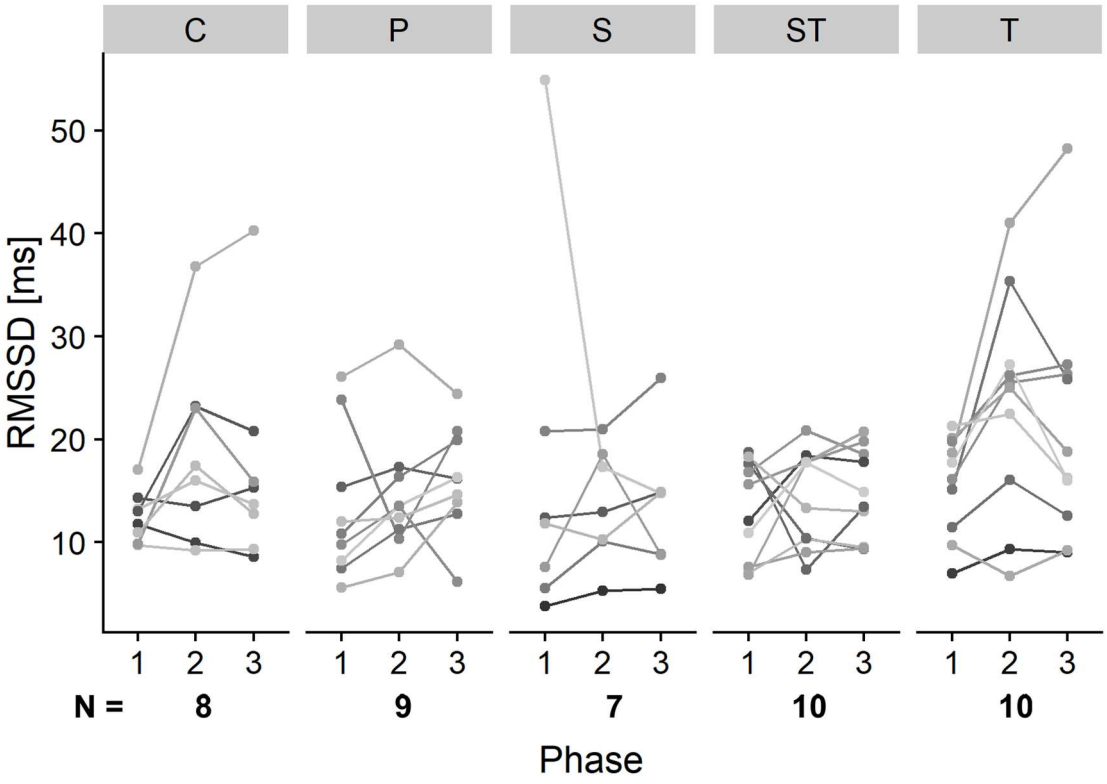


Figure A 2: Means of the square root of the mean squared differences of successive inter-beat intervals (RMSSD) [ms] of each heifer of herd 1 (n = 25) and herd 2 (n = 19) belonging to five different treatment groups: C – control, P – human presence, S – stroking, ST – stroking and talking in a gentle voice, T – talking in a gentle voice. From phase 1 to phase 2 the RMSSD decreased in 23 animals (S: n = 3, T: n = 6, C: n = 5, ST: n = 6, P: n = 3). And from phase 2 to phase 3 it decreased in 5 (S: n = 4, T: n = 6, C: n = 5, ST: n = 6, P: n = 4). (1 – first 5 min alone, 2 – 5 min with familiar human present, 3 – last 5 min alone). Data were averaged across 1-min segments. Statistics: Linear mixed model: interaction treatment * phase * herd, p = 0.01.

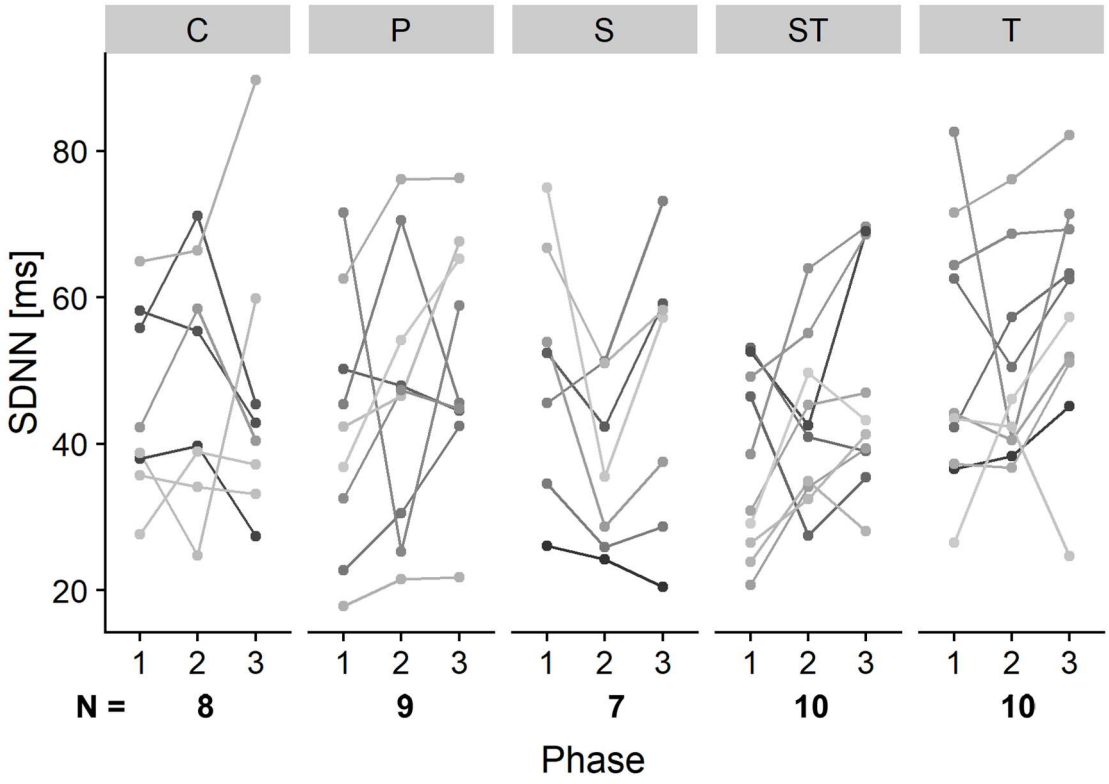


Figure A 3: Means of standard deviation of the inter-beat intervals (SDNN) [ms] of each heifer of herd 1 (n = 25) and herd 2 (n = 19) belonging to five different treatment groups in an isolation test with three phases (phase 1, 5 min in isolation; phase 2, 5 min with familiar human present; phase 3,5 min in isolation). C – control (n = 8), P – human presence (n = 9), S – stroking (n = 7), ST – stroking and talking in a gentle voice (n = 10), T – talking in a gentle voice (n = 10). Data were averaged across 1-min segments. Statistics: Linear mixed model: interaction treatment * phase * herd, p = 0.27.

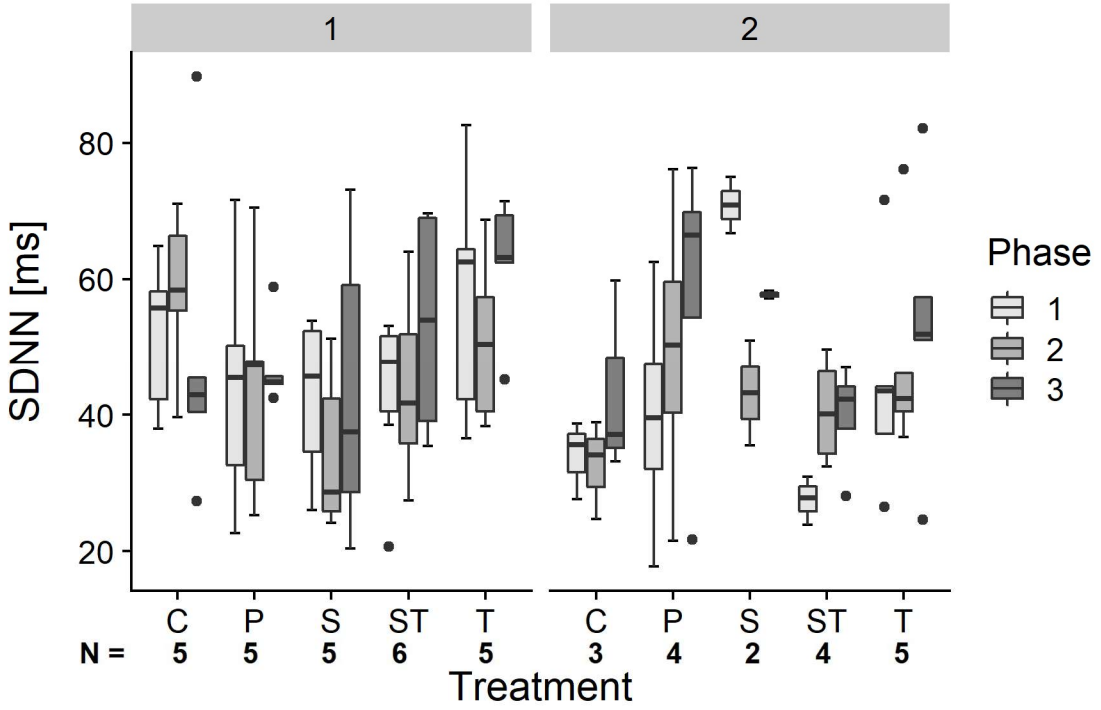


Figure A 4: Means of standard deviation of the inter-beat intervals (SDNN) [ms] of herd 1 (n = 26) and herd 2 (n = 19) for heifers of herd 1 (n = 26) and herd 2 (n = 18 ) belonging to five different treatment groups: C – control, P – human presence, S – stroking, ST – stroking and talking in a gentle voice, T – talking in a gentle voice, comparison of the mean SDNN between the phases 1, 2, 3 (1 – first 5 min alone, 2 – 5 min with familiar human present, 3 – last 5 min alone). Data were averaged across 1-min segments within phase and animal. Statistics: Linear mixed model: interaction treatment * phase * herd, p = 0.27.

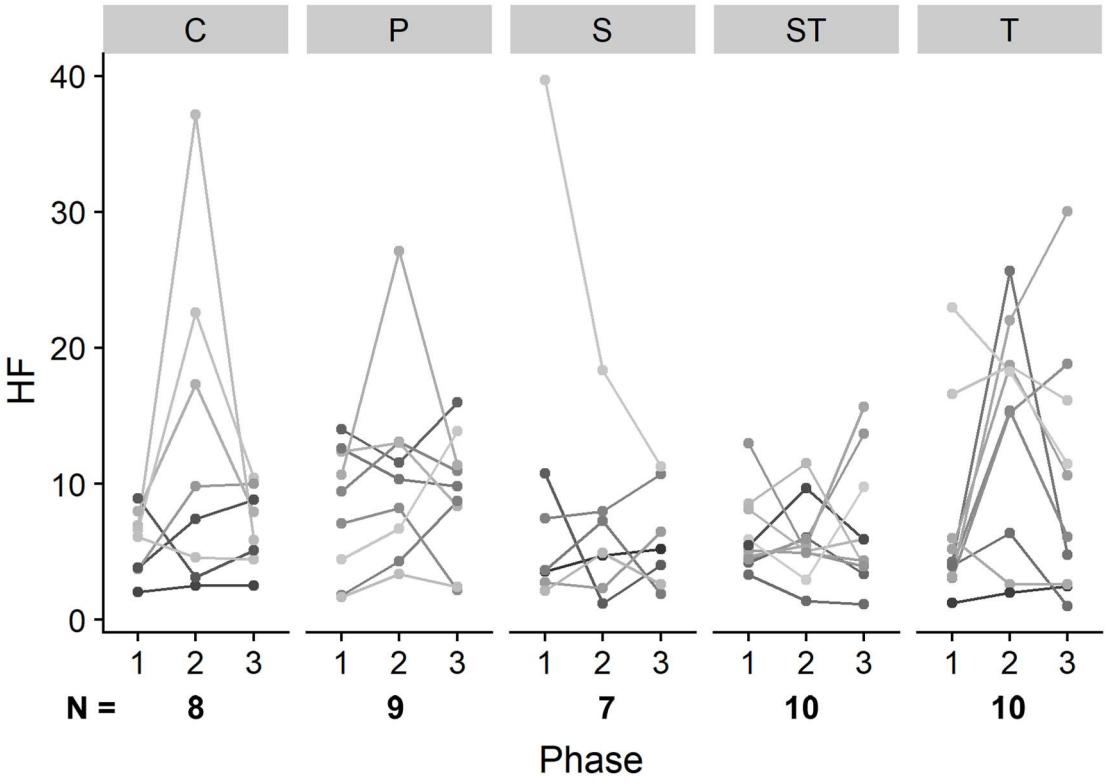


Figure A 5: Means of normalized high frequency power (HF) of each heifer of herd 1 (n = 25) and herd 2 (n = 19) belonging to five different treatment groups in an isolation test with three phases (phase 1, 5 min in isolation; phase 2, 5 min with familiar human present; phase 3,5 min in isolation). C – control (n = 8), P – human presence (n = 9), S – stroking (n = 7), ST – stroking and talking in a gentle voice (n = 10), T – talking in a gentle voice (n = 10). Data were averaged across 1-min segments. Statistics: Linear mixed model: interaction treatment * phase * herd, p = 0.67.

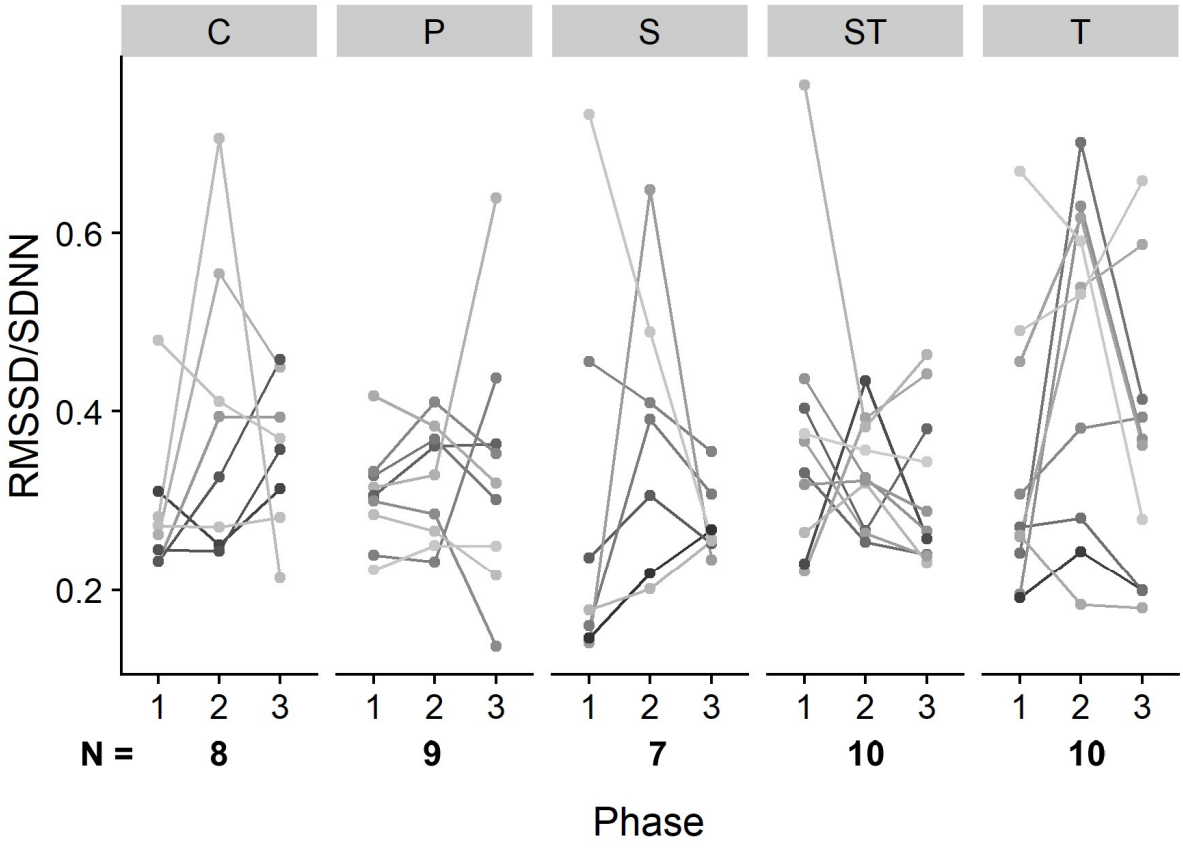


Figure A 6: Means of RMSSD/SDNN of each heifer of herd 1 (n = 25) and herd 2 (n = 18) belonging to five different treatment groups in an isolation test with three phases (phase 1, 5 min in isolation; phase 2, 5 min with familiar human present; phase 3,5 min in isolation). C – control (n = 8), P – human presence (n = 9), S – stroking (n = 7), ST – stroking and talking in a gentle voice (n = 10), T – talking in a gentle voice (n = 10). Statistics: Linear mixed model: interaction treatment * phase * herd, p = 0.67.

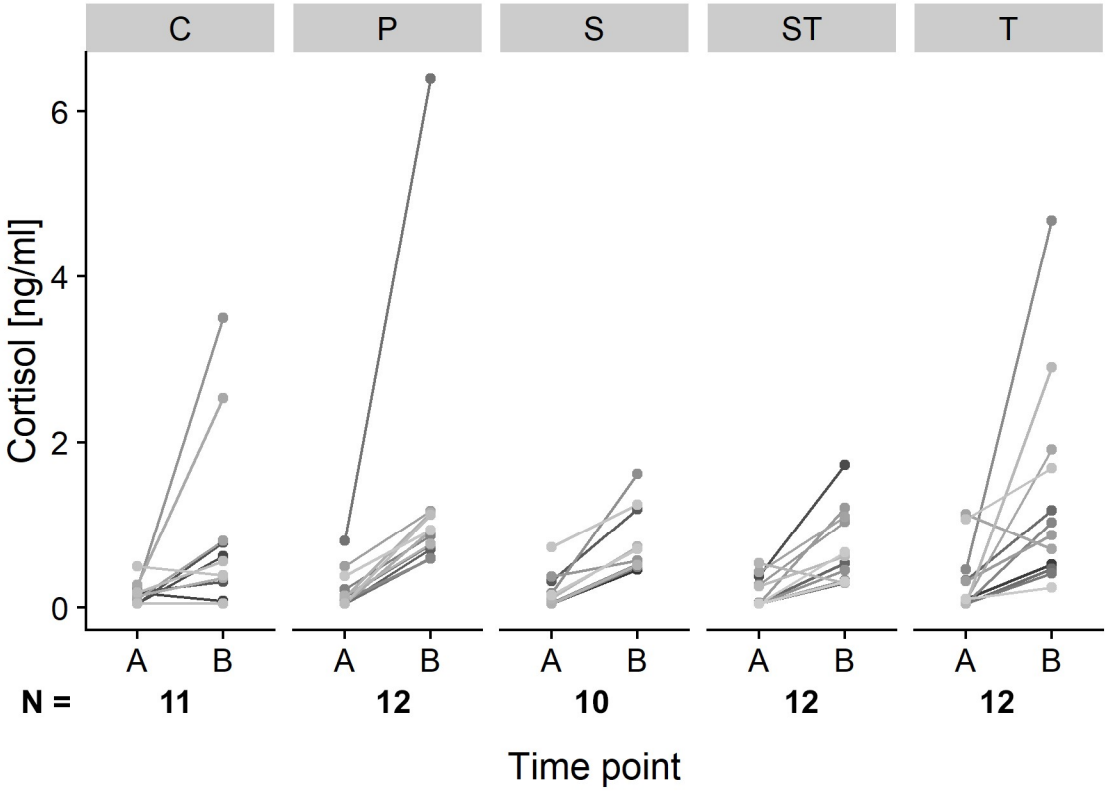


Figure A 7: Cortisol concentrations [ng/ml] of heifers of herd 1 (n = 30) and herd 2 (n = 27) belonging to five different treatment groups. C – control (n = 11), P – human presence (n = 12), S – stroking (n = 10), ST – stroking and talking in a gentle voice (n = 12), T – talking in a gentle voice (n = 12). Comparison of the mean cortisol concentrations between the timepoints A and B for sampling (A – before isolation test, B – after isolation test). Statistics: Linear mixed model: interaction treatment * timepoint * herd, p = 0.75.