Evaluation of plasma substance P concentrations in healthy German Simmental cows and calves

von
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Meiner Familie

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ABBREVIATIONS

°C	degree Celsius
BHBA	beta-hydroxybutyrate
BJV	blood taken from the Vena jugularis externa
CALF	female calves
CALM	male calves
CI	confidence interval
COW	cows
D1	first day of the trial
D2	second day of the trial
dl	deciliters
EDTA	Ethylene Diamine Tetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
G	Gauge
g	gravity
GSHPX	glutathione peroxidase
µg	microgram
H_0	null hypothesis
H_A	alternative hypothesis
Hb	hemoglobin concentration
kg	kilogram
l	liters
LEUC	mildley increased leucocyte count
µl	microliters
mg	milligram
ml	milliliters
n	number
NEFA(s)	non-esterified fatty acid(s)
NSAID(s)	non-steroidal-anti-inflammatory drug(s)
P-value	probability-value
PATH	pathological laboratory parameters
PCV	packed cell volume
pg	picogram
PHYS	physiological laboratory parameters
PSPC(s)	plasma substance P concentration(s)
RED	mildley increased hemoglobin concentrations or packed cell volume
SD	standard deviation
SP	substance P
TP	total protein
TV	blood taken from the Vena caudalis mediana
TV1 – TV5	blood taken from the Vena caudalis mediana at time point 1 – 5

I. INTRODUCTION

Pain is “an aversive sensory and emotional experience representing an awareness by the animal of damage or threat to the integrity of its tissues. It changes the animal’s physiology and behavior to reduce or avoid the damage, to reduce the likelihood of recurrence and to promote recovery” (MOLONY and KENT, 1997). Assessment of pain is quite a challenge in bovine animals, which are stoic patients and are able to mask pain behavior and discomfort to a certain degree. Masking pain is part of their nature as flight and prey animals, so that cattle only show signs of pain when the pain stimulus is severe (HUDSON et al., 2008).

The assessment of painful states can be done using subjective and objective methods (WEARY et al., 2006; HUDSON et al., 2008). Especially the assessment of behavioral parameters depends on the subjective judgment of the observer (PRUNIER et al., 2012). For a long time, cortisol was considered to be the predominant objective parameter for the assessment of stress and pain in bovine medicine (COETZEE et al., 2008). Today we know that using cortisol has its limitations. Immediate changes of cortisol concentrations are induced by acute pain related stress, but also by human presence, handling, and restraint (KARLEN et al., 2019), as well as individual and anxiety related behavior (BRISTOW and HOLMES, 2007), and different external environments and management techniques (OGINO et al., 2014).

Substance P (SP) is a sensory neurotransmitter, belonging to the family of tachykinins, and is composed of 11 amino acids (CHANG et al., 1971). SP is released both from the central and the peripheral nervous system and interacts with neurokinin receptors during painful states (HÖKFELT et al., 1975; RIBEIRO-DA-SILVA and HÖKFELT, 2000). Since a pain-related increase in its concentrations could be demonstrated during painful states in calves and cows (COETZEE et al., 2008; ALLEN et al., 2013; VAN ENGEN et al., 2014; BUSTAMANTE et al., 2015; RODRIGUEZ et al., 2018; TSCHONER et al., 2018; KARLEN et al., 2019), it gained attention as a sensitive and objective indicator for pain and nociception in research in bovine medicine (COETZEE et al., 2008).

Additionally to the increase of SP due to pain, research in human medicine showed that the secretion of SP is strongly related to acute psychological stress and phobia

(GERACIOTI et al., 2006; MICHELGÅRD et al., 2007). Increased concentrations of SP could also be demonstrated in context of inflammation, for example in patients with inflammatory bowel disease (MANTYH et al., 1988) and rheumatoid arthritis (MARSHALL et al., 1990).

So far, research in SP in bovine medicine mainly focused on the evaluation of plasma substance P concentrations (PSPCs) in animals which were exposed to a painful as well as stressful event. Changes in concentrations of SP in blood plasma or serum during castration (COETZEE et al., 2008; DOCKWEILER et al., 2013), dehorning (ALLEN et al., 2013; KARLEN et al., 2019), transportation (VAN ENGEN et al., 2014), or umbilical surgery (TSCHONER et al., 2018) have been described. Further studies also examined plasma SP concentrations in animals suffering from inflammation or infection such as clinical metritis (BARRAGAN et al., 2018) or lameness (BUSTAMANTE et al., 2015; RODRIGUEZ et al., 2018). Throughout these studies, SP concentrations of cattle showed high inter- and intraindividual variances (COETZEE et al., 2008; TSCHONER et al., 2018).

To our knowledge, no evaluation of SP concentrations in healthy cows and calves, which were neither exposed to a painful nor to a stressful environmental influence, or suffered from an inflammation, have been conducted to this day. Therefore, the aims of the present study were to

1) evaluate PSPCs in healthy untreated cows and calves of the breed German Simmental
2) compare PSPCs of blood taken from the jugular vein and from the tail vein in cows
3) evaluate PSPCs in the tail vein of cows every 6 hours over a period of 24 hours
4) compare PSPCs in male and female calves
5) assess the influence of age on PSPCs in adult cows and calves.

II. LITERATURE

1. Pain

1.1. Definition of pain

In 1979, pain was defined as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (MERSKEY, 1979). About 20 years later, MOLONY and KENT (1997) published a study about the assessment of acute pain in farm animals and proposed another definition of pain, approaching the understanding of pain at that time by considering sensory and emotional features as well as changes in behavior. According to MOLONY and KENT (1997), pain is “an aversive sensory and emotional experience representing an awareness by the animal of damage or threat to the integrity of its tissues. It changes the animal’s physiology and behavior to reduce or avoid the damage, to reduce the likelihood of recurrence and to promote recovery”. However, the understanding of pain is challenging because subjectivity is high, and there is no way of communication especially in organisms that are incapable of self-report. Therefore these definitions are of limited use in animals (ANAND and CRAIG, 1996).

There have been major advances in our understanding of pain since these definitions were established. Therefore, WILLIAMS and CRAIG (2016) captured the essence of what we presently understand of pain as the following: “Pain is a distressing experience with an actual or potential tissue damage with sensory, emotional, cognitive, and social components.” By specifying both sensory and emotional features accompanied with pain, this definition facilitates the multidimensional nature of pain (WILLIAMS and CRAIG, 2016).

1.2. Nociception

Nociception is the perception of a noxious stimulus announcing the presence of a potentially damaging stimulus. The neural pathways of a painful event can be separated into four physiological processes (WOOLF, 2004).

1. Pain receptors (nociceptors), which are located on the peripheral terminals of nociceptive sensory fibers, are activated by noxious stimuli. The recorded stimuli are transduced into an electrical signal (action potential) mediated

by specific receptor ion channels. These ion channels are nonselective cation or sodium channels, which are opened by temperature, chemical ligands, and mechanical shearing forces. A depolarization of the membrane is the result of a sodium and calcium ion flow into the peripheral ending of the nociceptor with the effect of several action potentials (WOOLF, 2004).

2. After this transduction, the action potential is transmitted in the peripheral nervous system along the sensory neuron axon via afferent A- or C-fibers (ANDERSON and MUIR, 2005b).
3. Reaching the central nervous system through the dorsal root ganglion and into the dorsal horn of the spinal cord, the signal is modulated from one neuron to another. Depending on the intensity of the peripheral noxious stimulus, action potentials vary in frequency and duration. Release of neuropeptides like SP results from high frequency action potentials (WOOLF, 2004).
4. The input from nociceptors is finally projected to the brain, mediated by direct monosynaptic contact or through multiple interneurons. Perception of pain is accomplished when the thalamus is transferring the sensory information to the brain and leads to a conscious subjective pain experience (WOOLF, 2004; ANDERSON and MUIR, 2005b).

Sensory nerve fibers, which are responsible for the transmission of a painful information, can be grouped into three classes, according to their function, anatomy (myelinization), and the speed with which they conduct electrical impulses (HENKE and ERHARDT, 2001; MUIR and WOOLF, 2001).

1. Aβ-fibers, which are thickly myelinated and activated by low intensity stimuli (low threshold) with a conduction time of 30 to 70 meters/second. Examples for Aβ- fibers are mechanoreceptors stimulated by pressure and vibration.
2. Aδ-fibers are minimally myelinated and are activated by high and low noxious stimulation with a conduction time of 2.5 to 30 meters/second. Examples for Aδ- fibers are mechanoreceptors and nociceptors.
3. Nonmyelinated C-fibers are high threshold mechano- and thermoreceptors and nociceptors with a conduction time of 0.5 to 2 meters/second.

1.3. Types of pain

Pain can essentially be divided into acute pain and chronic pain, considering the onset and duration of pain perception and tissue damage. An acute painful state usually does not outlast the healing period. To avoid recurrence of a painful experience, acute pain is associated with automatic behavioral changes through learning experiences in the animal (MOLONY and KENT, 1997). In contrast to that, chronic pain lasts beyond the predicted healing period of an injury (MOLONY & KENT, 1997).

If supposed that there is a benefit from a painful event, other classes of classification would be adaptive pain and maladaptive pain. As animals have learned to avoid stimuli associated with potential tissue damage, adaptive pain contributes to survival by protecting the animal from injury and by supporting the healing process (WOOLF, 2004). Adaptive pain is associated with surgical procedures like castration, dehorning, and laparotomy (ANDERSON and EDMONDSON, 2013). Maladaptive pain is a result of a pathological process uncoupled from a noxious stimulus or healing tissue (WOOLF, 2004). Diseases like septic arthritis, deep digital infections, and fracture with tissue destruction are accompanied by maladaptive pain (ANDERSON and EDMONDSON, 2013).

Another differentiation between several distinct types of pain can be made at a pathophysiological level, and according to the stimuli which cause the pain. These categories include nociceptive, inflammatory, neuropathic, and functional pain.

Nociceptive or physiologic pain is of great importance, as it is understood as a vital physiologic sensation (WOOLF, 2004). When nociceptors receive a chemical, thermal, or mechanical stimulus, the noxious information is transduced by sensory neurons in the dorsal horn of the spinal cord, transmitted, modulated, projected, and then precepted in the brain, resulting in an automatic response, for example a withdrawal reflex (WOOLF, 2004; ANDERSON and MUIR, 2005b,2005a).

Inflammatory pain is accompanied by a local inflammatory event, for example a trauma, surgery, or an inflammation. Peripheral nociceptors, which show an increased sensitivity at this time, are activated by multiple chemical mediators, and initiate healing by processing the noxious information. This process results in behavioral changes, for example protection or prevention of contact of the injured part until the end of the healing period (WOOLF, 2004).

In contrast to nociceptive and inflammatory pain, neuropathic and functional pain belong to the class of maladaptive pain, as they are uncoupled from a noxious stimulus or the healing tissue. Neuropathic pain may occur because of lesions of the peripheral or central nervous system. Functional pain may be a result of malfunction of the nervous system (WOOLF, 2004).

2. Pain assessment

Assessment of pain can be a challenge in farm animals, as they do not show obvious pain behavior and discomfort. Masking pain is a defense mechanism and cattle only show signs of pain when the noxious stimulus is severe (HUDSON et al., 2008). Assessment of pain is essential for animal welfare, pain prevention, and pain alleviation (GUATTEO et al., 2012; PRUNIER et al., 2012). Nowadays, the welfare of food-producing farm animals is given an increased attention from the general public, who demand freeness of pain in animals and consider animal welfare a priority in food production (WEARY et al., 2006; GUATTEO et al., 2012). This requirement does not only refer to animal agriculture; it is also a main concern in biomedical research (WEARY et al., 2006). In regard to achieving sustainable pain assessment, reliable information about the animals` affective state are required urgently (MCLENNAN, 2018). It is essential to recognize and quantify the amount of pain that animals are experiencing, and to be able to provide pain relief (WEARY et al., 2006).

Methods of assessing pain in cattle can be categorized into subjective and objective methods. Both subjective and objective parameters have their advantages and disadvantages (WEARY et al., 2006; HUDSON et al., 2008). Because of multifactorial influences on some of these parameters, it is recommended to use a combination of objective and subjective parameters for a comprehensive and reliable assessment of pain (WEARY et al., 2006; HUDSON et al., 2008). The methods of pain assessment in calves with their individual benefits and limitations were reviewed in detail by TSCHONER (2021). As of now, there is no objective parameter that can be used exclusively for the assessment of pain and nociception in calves and cows.

2.1. Subjective assessment of pain

Because of differences in perception and interpretation by observers (PRUNIER et al., 2012), subjective pain assessment heavily relies on the experience and

evaluation of the observer (HUDSON et al., 2008). Therefore it is essential that the observer is well trained and experienced (HUDSON et al., 2008) and that the behaviors which are assessed are clearly defined (FRASER and BROOM, 1990; HUDSON et al., 2008). Subjective assessment of behavior has to be consistent, to make sure that the same physiological and behavioral patterns are judged in individual animals (HUDSON et al., 2008). Furthermore, subjective assessment of pain requires a large number of animals due to the variability in behavior (FRASER and BROOM, 1990; JOHNSON et al., 2008). However, subjective methods are very popular in pain research in bovine medicine as they are considered practicable and relatively easy to apply (WEARY et al., 2006). Additionally, behavioral analysis is considered to be inexpensive (JOHNSON et al., 2008). Methods for subjective pain assessment in bovine medicine are given in Table 1.

Table 1: Methods for subjective pain assessment in calves and adult cattle. For each parameter, a short description of the method of evaluation, as well as references, are presented.

Parameter	Method	References
Ethogram	Analysis of behavioral patterns by an observer or video recordings	MAYER et al. (2020) STILWELL et al. (2008a) MELÉNDEZ et al. (2017) TSCHONER et al. (2020b)
Visual Analogue Scale	Description of pain limits on a 100 mm horizontal line, e.g. from 0 (no pain) to 10 (worst pain imaginable)	OLSON et al. (2016) MELÉNDEZ et al. (2017)
Numerical Rating Scale	Scale for pain with two end points, “no pain” and “worst pain imaginable”; mostly used for surveys	HUXLEY and WHAY (2006) REMNANT et al. (2017) TSCHONER et al. (2020a)
Facial Grimace Scale	Changes in facial expressions due to pain	GLEERUP et al. (2015) RÄÄF and OLSEN (2017)

2.2. Objective assessment of pain

Methods used for objective pain assessment in bovine medicine are presented in Table 2. Objective evaluation of painful states can be done by assessing physiological and production parameters (WEARY et al., 2006). Physiological parameters include biomarkers in the blood plasma and serum, for example cortisol (COETZEE et al., 2008; KARLEN et al., 2019) or SP (COETZEE et al., 2008; COETZEE et al., 2012), as well as clinical parameters such as heart rate and heart rate variability (BUSTAMANTE et al., 2015; STOCK et al., 2015). Production parameters include but are not limited to daily milk yield (BARRAGAN et al.,

2018) and daily weight gain in beef cattle (KARLEN et al., 2019). There are some feasible tools for measuring production parameters on farm-level with no or little influence on the animal, for example automatic calf feeding systems or pedometers (SUTHERLAND et al., 2018).

Table 2: Methods for objective pain assessment in calves and adult cattle. For each parameter, a short description of the method of evaluation, as well as references, are presented.

Parameter	Method	Reference
Cortisol	Laboratory analysis of blood plasma, blood serum, saliva, milk, or faeces	COETZEE et al. (2008) KLEINHENZ et al. (2016) KARLEN et al. (2019) TSCHONER et al. (2020b)
Heart rate Heart rate variability	Auscultation Heart rate recorder Electrocardiogram	OLSON et al. (2016) STOCK et al. (2015) BUSTAMANTE et al. (2015)
Body weight Daily weight gain	Weighting per scale	KARLEN et al. (2019)
Feed and water intake Rumination time	Weighing of the feed and leftovers Halters	HEINRICH et al. (2010) TSCHONER et al. (2020b) BRAUN et al. (2015)
Milk yield	Different milking technologies	BARRAGAN et al. (2018)
Activity	Recording of activity patterns with accelerometer Recordings of lying time, number of steps etc. with a pedometer	OLSON et al. (2016) MELÉNDEZ et al. (2017) BARRAGAN et al. (2018) HEINRICH et al. (2010)
Thermography	Measuring temperature via infrared thermography Ocular temperature Scrotal temperature	 KLEINHENZ et al. (2018) KARLEN et al. (2019) MELÉNDEZ et al. (2017)
Algometry	Measuring pain sensitivity as mechanical nociceptive threshold	KLEINHENZ et al. (2016) KARLEN et al. (2019) HEINRICH et al. (2010); STOCK et al. (2015)

Some of the objective physiological biomarkers have to be determined via blood samples and require invasive sampling procedures (KARLEN et al., 2019). Human presence, handling, and restraint during sampling may most likely result in altered and distorted measurement results (KARLEN et al., 2019). Additionally, physiological parameters in the blood plasma and serum, such as cortisol, have to be interpreted with caution, since they are influenced by stress or diseases (PRUNIER et al., 2012). PRUNIER et al. (2012) and WEARY et al. (2006) also considered measuring objective biomarkers as difficult on farms, because special equipment or specialized laboratory techniques for analysis of these indicators are

needed. However, physiological parameters can be a useful tool for research purposes. The benefit here lies in the possibility to compare the level of painfulness of different procedures, and the efficacy of pain management (PRUNIER et al., 2012).

3. Substance P

3.1. From cortisol to substance P

In pain research, cortisol was considered as the predominant indicator for (pain related) distress for a long time (BRISTOW and HOLMES, 2007). COETZEE et al. (2008) first introduced SP as an appropriate and objective pain indicator. Only in the last 15 years research did focus on SP as an indicator of pain in cattle (VAN ENGEN et al., 2014; BUSTAMANTE et al., 2015; TSCHONER et al., 2020b).

The search for a new and more objective biomarker for pain was deemed necessary because using cortisol as a biomarker for stress has its limitations. Immediate changes of cortisol concentrations are not only observed due to stress caused by acute pain, but also due to human presence, handling, and restraint (KARLEN et al., 2019). It was also proven that increase of cortisol concentrations in the blood plasma is influenced by individual and anxiety related behavior in cattle (BRISTOW and HOLMES, 2007). Furthermore, cortisol secretion is also affected by different external environments and management techniques (OGINO et al., 2014). Based on these limitations and influence factors, it is recommended nowadays to assay cortisol and SP in combination to distinguish between pain-related distress or handling-related stress (COETZEE et al., 2008; KARLEN et al., 2019).

3.2. History and molecular structure of substance P

SP was first mentioned in 1931, when VON EULER and GADDUM (1931) published the discovery of an unidentified depressor substance isolated from the equine brain and gut, which retained the activity of muscle and blood pressure. In 1934, GADDUM and SCHILD (1934) named the unidentified substance “substance P”, referring to the dry powder obtained after the extraction procedure. LEMBECK (1953) localized SP in the dorsal horn of the spinal cord and described its far-reaching similarity with other transmitter substances of sensitive nerves, defining SP to be a sensory neurotransmitter. CHANG et al. (1971) identified the

amino acid sequence of SP in 1971 as the following: “Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2”. A benchmark was reached with the successful peptide synthesis of SP (TREGEAR et al., 1971), and with the radioimmunoassay (POWELL et al., 1973), since the synthesis of SP made it possible to produce this peptide in larger quantities than naturally found. Based on this, further research of its physiological function in the nervous system could be conducted (TREGEAR et al., 1971).

3.3. Substance P in the central and peripheral nervous system

SP is present in the central and peripheral nervous system (RIBEIRO-DA-SILVA and HÖKFELT, 2000). It is released by sensory neurons in the spinal dorsal horn and in the brain (HÖKFELT et al., 1975). Several studies showed that the release of immunoreactive SP is induced by peripheral inflammation or noxious stimulation (OKU et al., 1987; DUGGAN et al., 1988; SCHAIBLE et al., 1990). Early on, PERNOW (1953) showed that SP concentrations are higher in the grey than in the white substance of the brain, as well as that there are high levels of SP in the dorsal root of the spinal cord. Also, the presence of SP in the peripheral nerves, particularly in autonomic nerves, spinal ganglia, and in the sympathetic trunk, was confirmed (PERNOW, 1953; HÖKFELT et al., 1975).

In the peripheral nervous system, SP is released by intrinsic enteric or extrinsic afferent neurons in the gastrointestinal tract. A mechanical or thermal trigger of the mucosa or distension of the muscles stimulates primary afferent neurons. By interacting with one of the three tachykinin receptors, SP can trigger multiple functions. The induced functions include changes in motility, electrolyte-, and fluid secretion, as well as influence on the vascular, and immune system. The tachykinin receptors are expressed by cells of enteric neurons, intestinal muscle, and by cells of the vascular, and immune system. SP does not exclusively show activating features but also inhibitory functions by descending inhibitory neuronal pathways and release of inhibitory transmitters such as nitric oxide. It is also known that SP synergizes with acetylcholine in some transmission processes (HOLZER and HOLZER-PETSCHE, 1997a,1997b).

3.4. Substance P in human medicine

Contrary to bovine medicine, SP has been the subject of research work in a large number of studies in human medicine.

3.4.1. Substance P in context of stress

It is known that stressful situations have an influence on the release of SP in the central nervous system. Significantly increased concentrations of SP in the cerebrospinal fluid could be found in patients with major depressions and posttraumatic stress disorders (GERACIOTI et al., 2006). Veterans with posttraumatic stress disorder and healthy volunteers were subject of this study and did undergo a lumbar puncture. Concentrations of SP in cerebrospinal fluid were measured during and after a symptom-provoked or neutral stimulus. A symptom-provoked stimulus triggered an increase in SP concentrations; compared to that, a neutral stimulus resulted in no changes in SP concentrations. These results indicate that the release of SP is strongly related to acute psychological stress and phobia (GERACIOTI et al., 2006). MICHELGÅRD et al. (2007) supported these findings. They showed an increased release of SP during fear provocation.

SP and NK-1 receptors are highly distributed in those areas of the brain which are associated with stress, anxiety, and mood responses (RIBEIRO-DA-SILVA and HÖKFELT, 2000; HÖKFELT et al., 2001). In these areas of the brain, SP coexists and interacts with other classical messenger molecules like acetylcholine and serotonin within single nerve cells (MERIGHI, 2002).

EBNER et al. (2004, 2008) demonstrated that a central NK-1-receptor-blockade reduced the release of SP in the key brain areas for emotional stress in knockout mice, leading to a mitigation of the stress response. They also described that the extent of SP release is influenced by the intensity of the emotional stressor. Therefore, humans with stress and psychiatric illness can benefit from therapeutical use of NK-1 receptor antagonists.

3.4.2. Substance P in context of inflammation

Inflammatory events are associated with changes in SP concentrations. Due to inflammatory processes, SP is released from sensitive neurons and the central nervous system, resulting in the release of histamine. The chemotaxis of neutrophil and eosinophil granulocytes into inflamed tissues is induced. This cellular migration is either proceeded directly or through chemokines, receptors, and adhesion molecules (HUNT and MANTYH, 2001; MASHAGHI et al., 2016). SP is secreted to a certain extent both by monocytes and macrophages. Thus, SP may play an important role for the pathogenesis of immune-mediated diseases (WEN-

ZHE et al., 1997).

Research in human medicine has identified some immune-mediated diseases, in which the presence and increase of SP has been proven. MANTYH et al. (1988) reported increased concentrations of SP and upregulated NK-1 receptor expression in the rectum and colon of patients with inflammatory bowel disease. Furthermore, elevated levels of SP were found in the synovial fluid and plasma of patients with rheumatoid arthritis (MARSHALL et al., 1990).

Asthma is a widespread disease with typical pathophysiological changes characterized by bronchoconstriction, oedema, and mucus in the airways. In the submucosa of patients with severe or fatal asthma, a higher number and length of SP immunoreactive nerve fibers could be found compared with patients who were not affected by asthma (OLLERENSHAW et al., 1991). Sarcoidosis is a granulomatous disorder in the lungs of unknown etiology. Increased levels of SP and NK-1 receptor upregulation in bronchoalveolar lavage fluid and endobronchial biopsies in patients with sarcoidosis could be detected compared with unaffected patients (O'CONNOR et al., 2003).

3.5. Substance P in laboratory animals

Studies about SP in laboratory animals can be found in large numbers. Laboratory animal models are an important tool in pain research for predicting analgesic efficacy, with the aim to develop clinical drugs (MOGIL, 2009). The studies cited are only intended to be an excerpt.

OKU et al. (1987) demonstrated an increase in immunoreactive SP in rats with polyarthritis after passive movement of inflamed joints compared with rats with sound joints. These results are in agreement with SCHAIBLE et al. (1990) in cats. Studies in pregnant mice showed significantly increased SP-mediated abortion rates after stressful situations (TOMETTEN et al., 2004). WONG et al. (2003, 2004) demonstrated that SP induces a NK-1 receptor mediated neurogenetic inflammation in rats, after inhalation of diesel exhaust and fire-smoke. HONORE et al. (2000) suggested that cancer induces unique persistent pain, as inflammatory, neuropathic, and cancer pain result in increased SP in the spinal cord in mice.

Other studies showed that central NK-1 receptor blockade reduced the release of SP in key brain areas for emotional stress (amygdala) in knockout mice, leading to a mitigation of stress response (EBNER et al., 2003, 2004).

3.6. Substance P in companion animals

Studies about SP in horses, cats, and dogs were conducted, but in a very manageable number. KIRKER-HEAD et al. (2000) reported a significant increase of SP and prostaglandin E_2 concentrations in synovial fluid in horses with osteoarthritic joints, compared with sound horses. For this study, arthritis was a natural process reflecting a chronic state of pain and was not induced by intra-articular injection of inflammatory factors. A correlation between SP, prostaglandin E_2, and severity or degree of disease could not be detected.

SCHAIBLE et al. (1990) induced experimental arthritis in the knee joint of cats. After mechanical stimulation, release of immunoreactive SP could be measured in the spinal cord in 7 out of 10 cats. The authors also stated that immunoreactive SP was released by stimuli like flexion and pressure, which had no effect under normal conditions.

POLIDORO et al. (2017) showed a dense distribution of NK-1 receptors in the smooth muscle cells and mucosal immunocytes in the ileum of dogs with inflammation. Consequently there is a therapeutical approach to use NK-1 receptor antagonists to reduce the severity of intestinal inflammation.

3.7. Substance P in bovine medicine

In the existing literature, one can find a manageable number of studies on SP in bovine medicine. Most of these studies were conducted using painful husbandry procedures in calves; there are not that many studies published about SP in adult cattle. An overview of studies on SP in bovine medicine in research literature is given in Table 3.

Table 3: Overview of studies on substance P in bovine medicine listed chronologically in research literature. Publication year, reference, type of painful procedure, and parameters for pain assessment with results are given in the columns.

Calves			
Year	**Author**	**Procedure**	**Results**
2008	COETZEE et al. (2008)	Surgical castration vs. sham castration	PSPCs[1] were significantly higher in castrated calves compared with sham castrated calves No difference in cortisol concentration between groups
2012	COETZEE et al. (2012)	Dehorning (scoop)	PSPCs were lower (with no significant difference) in meloxicam treated calves compared with placebo treated calves
2013	ALLEN et al. (2013)	Dehorning (hot-iron)	Significant reduction of PSPCs by administration of NSAID[2]
	DOCKWEILER et al. (2013)	Castration (band, cut-and-clamp, cut-and-pull)	Higher PSPCs in 6 months old calves compared with 8 weeks old calves regardless of the castration method
	REPENNING et al. (2013)	Castration (band)	No difference of PSPCs between calves without or with NSAID treatment after band castration
	THEURER et al. (2013)	Induced pneumonia	PSPCs were significantly higher in calves with *Mannheimia haemolytica* induced pneumonia compared to noninoculated controls
2015	STOCK et al. (2015)	Disbudding (cautery)	No difference of PSPCs between firocoxib treated and placebo treated calves
2016	OLSON et al. (2016)	Castration (band and surgical)	Higher PSPCs in placebo treated calves compared with meloxicam treated calves after surgical and band castration
	KLEINHENZ et al. (2016)	Dehorning (Electrocautery)	No difference of PSPCs between firocoxib treated and placebo treated calves
2018	TSCHONER et al. (2018)	Umbilical surgery with 2 analgesic treatments CON: meloxicam MET: meloxicam + metamizole	PSPCs increased in both groups (CON and MET) during surgery, without significant difference between groups, PSPCs were lower in MET than in CON
	MELÉNDEZ et al. (2018)	Castration (surgical and band)	Substance P concentrations in serum were higher in placebo treated calves compared with meloxicam treated calves after either surgical or band castration
	PARK et al. (2018)	Castration (surgical)	PSPCs were higher in placebo treated calves compared with meloxicam treated calves after surgical castration
	KLEINHENZ et al. (2018)	Castration (surgical)	No difference in PSPCs between placebo treated and flunixin meglumine treated calves after castration

2019	KARLEN et al. (2019)	Disbudding (caustic paste)	Significant reduction of PSPCs after administration of meloxicam
	PEARSON et al. (2019)	Assisted calving during parturition	No significant difference of PSPCs in meloxicam treated calves compared to placebo treated calves after assistance in birth
2020	MAYER et al. (2020)	Tail docking	No difference of PSPCs in tail docked calves compared to control calves after tail docking

Adult cows

Year	Author	Procedure	Results
2012	WHITLOCK et al. (2012)	Electroejaculation	No increase of PSPCs during electroejaculation Significant increase in vocalization and cortisol and progesterone concentrations
2014	VAN ENGEN et al. (2014)	Long distance transport	Increased PSPCs in beef steers after long-distance transportation and oral meloxicam treatment No difference of PSPCs between meloxicam treated and placebo treated steers
2015	BUSTAMANTE et al. (2015)	Lameness (acute, induced)	All plasma concentrations of the investigated biomarkers increased significantly after oligofructose-induced lameness in dairy heifers
2018	RODRIGUEZ et al. (2018)	Lameness (chronic)	All plasma concentrations were significantly higher in cows with lameness and in cows with high locomotion scores compared with sound cows
	BARRAGAN et al. (2018)	Metritis	Cows with diagnosed clinical metritis had higher PSPCs compared with cows with no clinical uterus diagnosis
	KASIMANICKAM et al. (2018)	Embryotransfer	PSPCs were higher in excited cows with no flunixin meglumine treatment
	SICKINGER et al. (2018b)	Intrapartum uterine torsion	PSPCs were significantly higher in cows without uterine torsion compared to cows with uterine torsion
2019	KASIMANICKAM et al. (2019)	Embryotransfer	PSPCs increased in excited cows with no flunixin meglumine treatment
2020	TSCHONER et al. (2020a)	Laparoscopic abomasopexy	No difference of PSPCs between xylazine treated cows and placebo treated cows
	LAUDER et al. (2020)	Ovariectomy (spaying)	No difference of PSPCs between spayed heifers without analgesia, spayed heifers with analgesia and non-spayed control animals

[1]plasma substance P concentrations, [2] non-steroidal-anti-inflammatory drug

3.7.1. Substance P in calves

3.7.1.1. Substance P in calves undergoing castration

In 2008, COETZEE et al. (2008) investigated changes in plasma substance P concentrations and cortisol levels in beef calves undergoing surgical castration compared with calves undergoing simulated castration (sham castration). In this study, blood samples were taken before, at the time of, and at several time intervals after the castration or sham castration. To create a context between expression of pain and changes in physiological indicators, vocalization and attitude scores were determined. The results showed that cortisol concentrations did not differ between castrated and sham castrated calves. Mean PSPCs were significantly higher in castrated compared with sham castrated calves at all times of blood sampling. Additionally, there was no significant difference in cortisol concentration in calves with high compared with calves with low vocalization scores. However, in calves with high vocalization scores, significantly higher concentrations of SP were found, compared with calves with low vocalization scores (COETZEE et al., 2008).

Further studies dealing with SP concentrations during castration in calves were conducted with similar experimental designs and protocols. In these studies, calves were treated with a systematic non-steroidal-anti-inflammatory drug (NSAID) in different forms of administration. Subsequently either surgical castration (KLEINHENZ et al., 2018; PARK et al., 2018), band castration (REPENNING et al., 2013), or a combination of both (OLSON et al., 2016; MELÉNDEZ et al., 2018) was performed. For analgesic treatment, OLSON et al. (2016), as well as MELÉNDEZ et al. (2018) and REPENNING et al. (2013) used meloxicam. PARK et al. (2018) treated calves with flunixin meglumine for systemic analgesia in addition to lidocaine for local anesthesia. KLEINHENZ et al. (2018) chose flunixin meglumine as an analgesic treatment. Physiological parameters including SP were measured and behavioral patterns were assessed in all studies.

MELÉNDEZ et al. (2018) showed that PSPCs were higher in placebo treated calves after surgical and band castration, compared with calves which were administered meloxicam. It was also observed that PSPCs did not increase in sham castrated calves. These results are in agreement with OLSON et al. (2016) and PARK et al. (2018). Both studies also reported increased PSPCs in placebo treated compared with NSAID treated calves after castration. Therefore, it can be assumed that PSPC

may be an indicator for nociception and pain during castration.

Contrary to that, neither REPENNING et al. (2013) nor KLEINHENZ et al. (2018) confirmed a difference in PSPCs between placebo treated and NSAID treated calves after different methods of castration.

3.7.1.2. Substance P in calves undergoing dehorning

COETZEE et al. (2012) conducted a study about scoop dehorning and thermocautery in calves. The results of the study showed that mean PSPCs were lower (even if not significantly) in meloxicam treated compared with placebo treated calves. Subsequent studies about dehorning with a similar experimental setup were in agreement with the previous study, showing a significant reduction of PSPCs after administration of an NSAID in context of dehorning (ALLEN et al., 2013; KARLEN et al., 2019). However, not all studies investigating the relationship between dehorning and SP agreed with the decrease of PSPCs after analgesic treatment. STOCK et al. (2015) and KLEINHENZ et al. (2016) could not observe significantly lower PSPCs in firocoxib treated compared with placebo treated calves after cautery disbudding.

3.7.1.3. Substance P during other procedures

TSCHONER et al. (2018) described increased PSPCs in calves undergoing umbilical surgery under general anesthesia, both in calves treated either with meloxicam or with meloxicam and metamizole, with no significant difference between both groups (TSCHONER et al., 2018). PEARSON et al. (2019) investigated the effect of either a meloxicam or placebo treatment on calves which required assistance at birth on physiological indicators of pain and inflammation. They could not confirm a significant effect of analgesic treatment on PSPCs (PEARSON et al., 2019).

THEURER et al. (2013) published significantly higher PSPCs in calves with *Mannheimia haemolytica* induced pneumonia compared to noninoculated controls. In a recent study by MAYER et al. (2020), no significant effect of tail docking in calves on PSPCs was found.

3.7.2. Substance P in adult cattle

There are only a few studies on the evaluation of SP concentrations performed in adult cattle. WHITLOCK et al. (2012) conducted a study about the painfulness of

electroejaculation, using angus bulls. The results of the study showed a significant increase in vocalization and concentrations of cortisol and progesterone in bulls during electroejaculation, but no increase in PSPCs. Because of the lack of difference in PSPCs, the authors assumed that electroejaculation may be a stressful, but not a painful event (WHITLOCK et al., 2012). VAN ENGEN et al. (2014) described increased PSPCs in beef steers after long-distance transportation and oral meloxicam treatment. However, there was no difference in PSPCs between meloxicam treated and placebo treated steers.

To assess acute painful states, BUSTAMANTE et al. (2015) conducted a study about oligofructose-induced lameness in dairy heifers. Measurement of clinical parameters and plasma biomarkers like cortisol, haptoglobin, norepinephrine, beta-endorphin, and SP were performed. The results showed that the plasma concentrations of all the investigated biomarkers increased significantly, with PSPCs increasing significantly with a peak at 12 hours after induction of lameness (BUSTAMANTE et al., 2015). Comparable results were achieved in a study published by RODRIGUEZ et al. (2018), where dairy cows with lameness and different locomotion scores were subject of a trial. The authors showed that PSPCs, just like norepinephrine and beta-endorphin, were significantly higher in lame cows and in cows with high locomotion scores compared with cows with low locomotion scores (RODRIGUEZ et al., 2018).

BARRAGAN et al. (2018) published a study including lactating dairy cows with clinical metritis. Assessment of daily activity patterns and measurement of SP concentrations showed that cows diagnosed with clinical metritis had significantly higher PSPCs compared with cows with no clinical diagnosis (BARRAGAN et al., 2018). It can be assumed that clinical metritis is associated with visceral pain (STOJKOV et al., 2015), and that the increased PSPCs are the result of nociception resulting from visceral pain in cows with clinical metritis (BARRAGAN et al., 2018). The aims of the studies of KASIMANICKAM et al. (2018, 2019) were to show an effect of flunixin meglumine in various forms of administration on pregnancy rate in embryo recipient beef cows, and on proportion of non-pregnant cows returning to estrus. With the determination of different plasma parameters, including SP, it could be demonstrated that SP concentrations were increased in excited cows which were not treated with flunixin meglumine, compared with cows which received flunixin meglumine within the same experimental environment

(KASIMANICKAM et al. (2018, 2019). TSCHONER et al. (2020b) published a study about laparoscopic abomasopexy in dairy cows which received either xylazine or a placebo before surgical correction of left displaced abomasum. Investigated blood parameters were plasma cortisol concentrations and PSPCs. The results of the study showed a significant increase in cortisol concentration in placebo treated compared with xylazine treated cows. It was concluded that animals experience less stress during the laparoscopic procedure of abomasopexy after administration of xylazine compared with a placebo. However, PSPCs did not differ between groups, and did not reflect the stress situation in xylazine treated cows (TSCHONER et al., 2020b).

SICKINGER et al. (2018b) investigated serum neuropeptides including SP concentrations in cows with intrapartum uterine torsion. Their results showed significantly higher SP concentrations in cows undergoing parturition without uterine torsion compared to cows with a uterine torsion, concluding that uterine torsion is not associated with more pain than normal calving (SICKINGER et al., 2018b).

LAUDER et al. (2020) published a study about ovariectomy (spaying) and its behavioral and physiological responses to pain mitigation in beef heifers. Blood samples were collected before, after, and at the time of the procedure. The results showed no significant difference between animals spayed without analgesia, spayed with analgesia, and non-spayed control animals. Therefore the authors concluded that SP is not an appropriate pain biomarker for the assessment of pain during ovariectomy, or that the pain induced by ovariectomy is not sufficient to release SP (LAUDER et al., 2020).

Nevertheless, in summary, most studies show an increase of PSPCs during the exposure to a painful stimulus, in calves as well as in adult cattle (COETZEE et al., 2008; ALLEN et al., 2013; KARLEN et al., 2019). Therefore, it can be concluded that SP may be an appropriate indicator for nociception and pain during painful procedures in bovine medicine. However, systematic studies about the effect of different influences (e.g. stress or inflammation) on SP are missing.

3.8. Limitations and influencing factors of substance P

The studies on SP in bovine medicine support the idea that SP can be used as an indicator for pain and nociception. However, some study results are contradictory

about the suitability of using SP as a biomarker for pain; therefore, SP should be assayed in combination with cortisol to better differentiate between stressful and painful procedures as recommended by COETZEE et al. (2008) and KARLEN et al. (2019).

There are several limitations to the use of SP as a specific and objective biomarker for pain. Release of SP is influenced by a variety of physical and psychological stressors (VAN ENGEN et al., 2014). Due to inflammatory processes, SP is released from sensory neurons and the central nervous system (HUNT and MANTYH, 2001; MASHAGHI et al., 2016). Also, stress can result in changes of SP concentrations (GERACIOTI et al., 2006; MICHELGÅRD et al., 2007). It needs to be considered that SP concentrations might vary due to the chosen time intervals of blood sampling and sample handling, as cortisol does (LEFCOURT et al., 1993). To reduce the enzymatic degradation of SP, it is important that sample tubes are spiked with a protease-inhibitor (e.g. aprotonin). Furthermore, time for sample processing after blood collection is limited; samples should be processed in laboratories within 1 hour (MOSHER et al., 2014) to 2 hours (TSCHONER et al., 2018) after blood collection. Additionally, plasma samples must be stored at -70°C (degree Celsius) (MOSHER et al., 2014). There is also evidence that the age of animals might have an influence on SP concentrations. DOCKWEILER et al. (2013) described higher concentrations of SP in 6 months old calves compared with 8 weeks old calves, regardless of the castration method. Additionally, various studies showed that there are high inter-and intraindividual variations in mean PSPC in cows and calves (Table 4).

In summary, it can be assumed that stressful (VAN ENGEN et al., 2014) and painful events (DOCKWEILER et al., 2013), as well as a state of inflammation (BARRAGAN et al., 2018), sample handling (MOSHER et al., 2014), and inter-, and intraindividual differences (COETZEE et al., 2008; TSCHONER et al., 2018) result in changes of SP concentrations in the blood plasma. It is notable that to this day, there are no studies investigating the PSPCs in healthy and untreated animals, which are neither exposed to a stressful nor to a painful environment. Above mentioned studies, among these COETZEE et al. (2008), published baseline PSPCs of animals, which were subject of a sham procedure and experienced the same stressful experimental environment as animals which were subject of a surgical procedure. Other studies only published results of PSPCs in animals which were

treated with NSAIDs like meloxicam and metamizole (TSCHONER et al., 2018) or with a placebo (STOCK et al., 2015).

Therefore, there is a massive lack of knowledge concerning basic research about SP as a biomarker for pain in cattle.

Table 4: Overview of studies assessing substance P, demonstrating the high variation in plasma substance P concentrations (PSPCs) in calves and adult cattle. Parameters are presented as mean with standard deviation, or median. Units are given in picogram (pg)/ml or nanogram (ng)/ml.

Reference	Painful procedure/condition	Mean/median PSPCs
Calves		
COETZEE et al. (2008)	Sham or surgical castration	386.42 ± 40.09 pg/ml in uncastrated control calves 506.43 ± 38.11 pg/ml in castrated calves
STOCK et al. (2015)	Cautery disbudding	20.8 ±0.4 pg/ml in placebo treated calves 22.7 ± 0.7 pg/ml in firocoxib treated calves
TSCHONER et al. (2018)	Umbilical surgery	690.0 pg/ml in meloxicam treated calves 560.3 pg/ml in meloxicam + metamizole treated calves
Adult cattle		
WHITLOCK et al. (2012)	Electroejaculation	93.4 ± 17.2 pg/ml in control bulls 77.2 ± 17.2 pg/ml in bulls following electroejaculation
BUSTAMANTE et al. (2015)	Oligofructose induced-lameness	0.26 – 0.42 ng/ml in control heifers 2.20 ± 0.47 ng/ml in lameness induced heifers
BARRAGAN et al. (2018)	Clinical metritis	37.73 ± 5.41 pg/ml in cows diagnosed with no clinical metritis 47.15 ± 5.38 pg/ml in cows diagnosed with clinical metritis
RODRIGUEZ et al. (2018)	Lameness (chronic)	0.25 ± 0.09 ng/ml in non-lame cows 0.61 ± 0.12 ng/ml in severely lame cows
TSCHONER et al. (2020b)	Laparoscopic abomasopexy	555.37 ± 252.77 pg/ml in placebo treated cows 490.60 ± 219.62 pg/ml in xylazine treated cows

III. MATERIAL & METHODS

The research project consisted of two trials. Subject of the first trial were 54 cows (COW) of the breed German Simmental. The second trial consisted of 52 female calves (CALF) and 49 male calves (CALM) of the breed German Simmental. Both trials were conducted at the Research- and Teaching Center Achselschwang, located at 86919 Utting am Ammersee, Germany. The trials were approved by the ethics committee of the government of Upper Bavaria (reference number 55.2-1-54-2532-12-13).

Blood samples for the experimental group COW were collected from December 2019 to June 2021. For the experimental groups CALF and CALM blood sampling was done from December 2019 to December 2021.

1. Cows

1.1. Housing and feeding

The experimental group COW consisted of 54 adult German Simmental cows aged 5.0 ± 1.3 (3.4 – 9.1) years. Further animal data (number of lactations, days postpartum, days pregnant, daily milk yield) were collected. Animals were housed at the Research- and Teaching Center Achselschwang; the study was conducted at the same location. All cows were kept in loose housing with solid ground. The resting area consisted of deep bed cubicles with straw bedding. The cows were milked twice daily. A functional claw trimming was performed three times a year.

Cows were kept in three different housing and feeding groups, based on their lactation stage and milk yield. Animals of the present study population were of group 1 (n (number) = 19, 35.2% of cows, high-yielding animals) and group 3 (n = 35, 64.8% of cows, animals participating in feeding studies conducted by the Bavarian State Research Center for Agriculture in 2021 (ETTLE et al., 2020; ETTLE et al., 2021a; ETTLE et al., 2021b; RIEPL et al., 2021)). For another study about the influence of the ruminant adequacy on milk yield and body condition, which was also conducted during the sample period for the present study, no publication exists so far according to information from the Bavarian State Research Center for Agriculture. Group assignment in individual cows is given in Supplemental 1.

All cows which were part both of this research project and of the above mentioned feeding studies were not exposed to any influences during these feeding studies which affected our results.

For the trial, cows from group 1 were separated into a smaller area of the stable (Figure 1) and were released from the feeding fence into this area after the blood collection and clinical examination was completed. During this time, visual and tactile contact with the rest of the herd was possible at all times. All blood sampling of cows of group 1 were performed in the separated area. Cows of group 3 were caught in the feeding fence of the loose housing system for the first blood sample. All further blood sampling at the respective time points was carried out with the animals caught in their cubicles.

Figure 1: Housing of the study animals during the sampling procedure. Cows of group 1 (n = 19) were separated into a small area of the stable with visual and tactile contact to the rest of the herd. Group 3 animals (n = 35) remained in the herd for the entire duration of the study.

1.2. Selection of cows

1.2.1. Inclusion criteria

Cows had to be of the breed German Simmental (at least 75%). Additionally, they had to be in their second or higher lactation and be pregnant (day 60 to 250 of

lactation).

All cows had to be clinically healthy with no previous treatment with an antibiotic or analgesic drug within the last 14 days before the start of the trial. Cows had to stay healthy after the sampling period and were excluded from the trial if they were diagnosed with a disease and/or treated as stated above within a period of 7 days after blood sample collection.

1.2.2. Clinical examination

A clinical examination as described by DIRKSEN et al. (1979) was performed after collection of blood samples. The examination included assessment of heart rate, respiratory rate, rumen motility, and rectal temperature, as well as assessment of the general behavior, posture, and feed intake of the cows. Additionally, a fecal sample was taken from every cow. Study protocol of the clinical examination in individual animals is given in Supplemental 2.

1.2.3. Blood parameters

For the confirmation to include only clinically healthy animals into the trial, defined blood parameters (leucocyte count, packed cell volume (PCV), hemoglobin concentration (Hb), total protein (TP), glutaraldehyde test, beta-hydroxybutyrate (BHBA), non-esterified fatty acids (NEFA) and glutathione peroxidase (GSHPX)) were additionally analyzed.

Based on the health status, which was assessed with the clinical examination in combination with the laboratory findings, 54 cows out of a total of 77 sampled cows were included in this study. Clinically healthy adult cows with no deviations from the reference ranges defined by the Clinic for Ruminants with Ambulatory and Herd Health Services were included in group PHYS (physiological laboratory parameters, n = 23, 42.6% of cows). To be able to include a higher number of animals into the statistical model, we also included clinically healthy animals with blood parameters with minor deviations from the defined reference ranges (ranges as defined for the present study are given in brackets). Therefore, clinically healthy cows with deviations in leucocyte count (3 – 15 x10^3/microliters (µl), n = 2), in Hb (8 – 15 g/deciliters (dl), n = 5), in PCV (26 – 42%, n = 4), in TP (40 – 85 g/l, n = 3), in glutaraldehyde test (14 – 15 min, n = 1), and cows with more than one parameter within the expanded reference ranges (n = 16) were included into group PATH (pathological laboratory parameters, n = 31, 57.4% of cows) .

1.3. Experimental design and sampling

The experimental period covered two days. Sampling was conducted according to a defined schedule. The study protocol is given in Figure 2. Blood samples were taken from the Vena jugularis externa (jugular vein, BJV) and the Vena caudalis mediana (tail vein, TV).

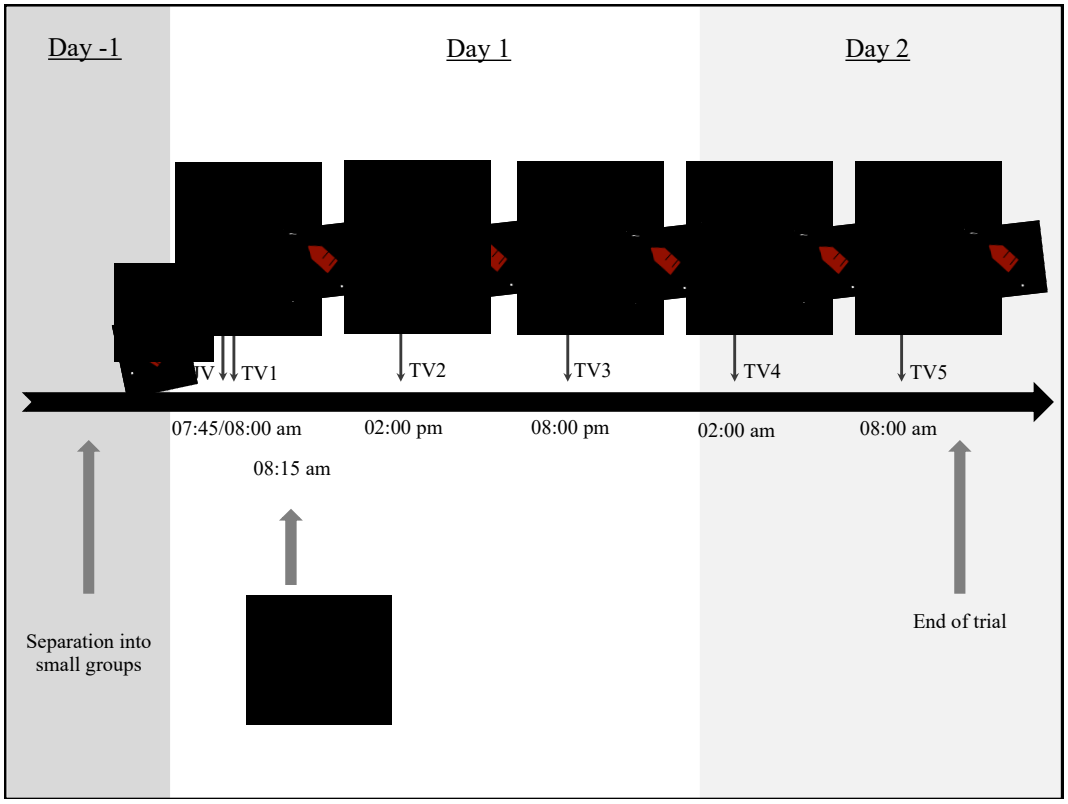

Figure 2: Study protocol for blood sampling in 77 cows for the analysis of substance P (SP) concentrations in healthy German Simmental cows. The trial was conducted over a period of 2 days. Basal SP concentrations were taken at 7:45 a.m. in the morning of the first study day (D1) at the Vena jugularis (jugular vein, BVJ). For the determination of a possible circadian rhythm of SP additional blood samples were taken at the Vena caudalis mediana (tail vein (TV)) with intervals of six hours (8:00 a.m., 14:00 p.m., and 20:00 p.m. on day 1 (D1), and 2:00 a.m. and 8:00 a.m. on day 2 (D2) of the study, TV1 to TV5 respectively). Clinical examination and fecal sampling were performed after sampling of the BJV and TV1 at 8:15 a.m. on D1.

At the first day of the trial (D1), blood sampling started at 7:45 a.m. The preparations for sampling included separation of the cows from the rest of the herd into smaller groups for group 1, catching them in the feeding fence (cows of groups 1 and 3), and tethering them with a halter with the head bent to one side. A total of four blood samples was taken at this time point with a 14 Gauge (G) syringe. The puncture site was disinfected with alcohol. For analysis of the base

concentration of SP at the jugular vein, the first sample was collected with a 10 ml Ethylene Diamine Tetraacetic Acid (EDTA) tube containing 45 µl aprotonin per tube. Aprotonin is a protease-inhibitor which prevents the decay of SP. For analysis of concentrations of the laboratory parameters, the remaining three samples were taken with an EDTA tube (leucocyte count, Hb, PCV, glutaraldehyde test), a blood gas tube (GSHPX), and a serum tube (TP, NEFA, BHBA).

After collection of the BJV, blood sampling at the tail vein (TV1) was conducted at 8:00 a.m., with the cows remaining in the feeding fence.

The puncture site of the tail vein was cleaned and disinfected with alcohol. The vein was then punctuated with a 20 G syringe and a vacutainer system (4 milliliters (ml) EDTA tubes). Immediately after blood extraction, 18 µl aprotonin was added to each EDTA tube on site. Further blood samples at the tail vein were collected with time intervals of six hours, at 2:00 p.m. and 8:00 p.m. on D1, and 2:00 a.m. and 8:00 a.m. at the second day (D2) of the trial (TV2 to TV5, respectively). The cows were either caught in the feeding fence, or else in their cubicles for sample collection of TV2 to TV5. If the blood collection at the tail vein was not successful at the first attempt, a second attempt was done. In case of an unsuccessful second attempt, sampling was aborted. If more than two samples from the tail vein were missing, animals were excluded from the trial. This was the case for one animal.

Throughout the whole sampling time, all samples for the analysis of SP were kept on ice to stabilize the protease-inhibitor aprotonin. The time slot for processing the samples was kept as short as possible, so that blood samples were processed within 2 hours after collection at the Research- and Teaching Center Achselschwang. Blood samples for the analysis of SP were centrifuged at 4°C for 15 min (1600x gravity (g)) and the extracted blood plasma for analysis of substance P was kept frozen at -20°C at the Research- and Teaching Center Achselschwang and at the Clinic for Ruminants with Ambulatory and Herd Health Services until the end of the experimental period. Serum samples were centrifuged at 4°C for 10 min (1000xg). The blood gas, EDTA, and fecal samples were stored in a refrigerator until the following day, on which they were transferred to the Clinic for Ruminants with Ambulatory and Herd Health Services for further analysis. Subsequently for the analysis of leucocyte count, Hb, PCV, and glutaraldehyde test, the EDTA samples were processed with a hematology analysis device (Vetscan® HM5,

Abaxis, Union City, USA). For analysis of GSHPX the blood gas samples were processed with the RAPIDPoint® 450 (Siemens, Munich, Germany). For analysis of TP, NEFA, and BHBA the serum samples were processed with the cobas® c 311 Analyzer (Roche, Basel, Switzerland). The fecal samples were evaluated using a Microscope (Olympus, Tokyo, Japan) for parasitological examination at the laboratory of Clinic for Ruminants with Ambulatory and Herd Health Services. For the diagnosis of a fasciolosis and paramphistomosis a sedimentation, for the exclusion of an infection with gastro-intestinal helminths a flotation, and for the exclusion of a dictyocaulosis infection the Baermann method was performed.

2. Calves

2.1. Housing and feeding

The experimental group of calves consisted of 52 female (CALF) and 49 male (CALM) calves of the breed German Simmental. Calves were born and raised at the Research- and Teaching Center Achselschwang located at 86919 Utting am Ammersee, Germany. A total of n = 7 male and n = 13 female calves were housed in individual pens (19.8% of calves), whereas a total of n = 42 male and n = 39 female calves were housed in groups (80.2% of calves) with an average group size of 5.8 ± 1.9 (2.0 – 9.0) animals. Housing of calves is given in Supplemental 3. The calves were kept on straw and had ad libitum access to milk, a total mixed ration, water, and hay. Calves born and sampled in the period from December 2019 to October 2021 were fed with whole milk ad libitum. From November 2021 till the end of the trial, calves were fed with a milk replacer (Sprayfo Delta®, 125g/liters (l) water). Blood sampling was done at the calves' habitual environment.

At the time of sampling, calves of CALM were 17.2 ± 2.1 (14 – 21) days old, and calves of CALF were 17.1 ± 2.3 (14 – 21) days old. Birth weight was 44.7 ± 4.9 (32 – 58) kilogram (kg) in CALM and 41.4 ± 6.3 (28 – 54) kg in CALF. After parturition, the calves of group CALM received 2.6 ± 1.0 (0.5 – 7.0) l of colostrum, and calves of group CALF received 2.3 ± 0.7 (0.5 – 4.0) l of colostrum within the first hours of life. The information whether a calf received colostrum was missing in 7.9% of calves (n = 8 in total calves; n = 5 for male and n = 3 for female calves). The amount of colostrum intake could not be documented in 3.0% of calves

(n = 3 in total calves; n = 1 for male and n = 2 for female calves) because they remained with their dam after parturition. Data of birth weight is missing in 2.0% of calves (n = 2 in total calves; n = 1 in male and female calves each).

All calves were treated subcutaneously with 5 ml Alpha-Tocopherolacetet and Natriumselenit (Vitamin E-Selen®, 100 milligram (mg)/ml + 0.658 mg/ml, cp-pharma) on their first day of life. Additionally, 100 microgram (µg) per kg of body weight Halofuginon (Halocur®, 0,5 mg/ml, MSD) was administered orally for seven consecutive days, beginning with the first day of life. At the time of sampling calves were neither dehorned (CALM and CALF) nor castrated (CALM).

2.2. Selection and inclusion criteria

2.2.1. Inclusion criteria

Calves had to be of the breed German Simmental (at least 75%). Furthermore, they had to be between 14 and 21 days old. Calves were only included into the present study if they were neither dehorned nor castrated.

Calves had to be clinically healthy, with no treatment with an antibiotic or analgesic drug within the last 14 days before sampling. Furthermore, calves were excluded from the study if they were diagnosed with a disease and/or treated as stated above within seven days after the day of the experiment.

2.2.2. Clinical examination

A clinical examination as described by DIRKSEN et al. (1979) was performed after the collection of the blood samples, including assessment of heart rate, respiratory rate, rectal temperature, general behavior, quality of the feces, and umbilical abnormalities. Study protocol of the clinical examination in individual animals is given in Supplemental 4.

2.2.3. Blood parameters

Defined blood parameters (leucocyte count, PCV, Hb, TP, and GSHPX) were analyzed additionally to the clinical examination in every calf. Calves were assigned to four different groups according to the findings in their laboratory analysis. Clinically healthy calves with no deviations from the reference ranges defined by the Clinic for Ruminants with Ambulatory and Herd Health Services were included in group PHYS (n = 16, 15.8% of calves). To be able to include

more animals into the statistical analysis, clinically healthy calves with blood parameters with minor deviations (ranges as defined for the present study are given in brackets) from the reference ranges as given by the Clinic for Ruminants with Ambulatory and Herd Health Services were included in the study. Calves of the group LEUC (n = 23, 22.8% of calves) had deviations in leucocyte count (3 – 15 x10³/µl), calves of the group RED (n = 24, 23.8% of calves) had either deviations in Hb (8 – 15 g/dl) or PCV (26 – 42%). Calves with more than one blood parameter deviating from the defined reference ranges were assigned to the group PATH (n = 38, 37.6% of calves).

2.3. Experimental design and sampling

Blood samples were taken at 6:00 a.m. Blood was taken with a 17 G syringe at the jugular vein after disinfection with alcohol. The hair was not clipped. A clinical examination was performed subsequently at 6:15 a.m.

Two blood samples were taken from each calf. Samples for analysis of SP were collected in a 2 ml EDTA tube containing 9 µl aprotonin per tube. SP sample tubes were kept on ice at all times. Blood samples for analysis of the defined blood parameters (leucocyte count, Hb, PCV, GSHPX, and TP) were collected with a 2 ml blood gas tube. All blood samples were transferred to the laboratory of the Clinic for Ruminants with Ambulatory and Herd Health Services within 2 hours after sampling.

For the determination of SP, EDTA blood samples were centrifuged within 2 hours after blood collection (4°C, 1600xg for 15 min). Blood plasma was frozen at -20°C at the laboratory of the Clinic for Ruminants with Ambulatory and Herd Health Services until the end of the experimental period. For the analysis of leucocyte count, Hb, PCV, GSHPX, and TP the EDTA samples were processed with the RAPIDPoint® 450 (Siemens, Munich, Germany).

3. Substance P analysis in cows and calves

For the analysis of the concentrations of SP in the blood plasma of cows and calves, a SP Enzyme-Linked Immunosorbent Assay (ELISA) kit (Substance P ELISA Kit, Enzo Life Sciences GmbH, Cologne, Germany) was used. Samples were analyzed at the laboratory of the Clinic for Ruminants with Ambulatory and Herd Health Services. The reading and evaluation of the plates was done by use of a Microplate

Reader (CLARIOstar®, BMG LABTECH GmbH, Ortenberg, Germany) and the corresponding software (MARS Data Analysis Software, BMG LABTECH GmbH, Ortenberg, Germany). Optical densities were assayed in duplicate, and means were used for the calculation of concentrations. Lower and upper limits for the quantification for the SP ELISA kit were 167.78 picogram (pg)/ml and 1000.00 pg/ml, respectively. The intra- and interassay coefficient of variation was calculated to be 20%.

For the analysis of the concentrations of SP a sensitivity of 167.78 pg/ml was reached in the laboratory of the Clinic for Ruminants with Ambulatory and Herd Health Services. This sensitivity is higher than the manufacturer's specifications of 5.30 pg/ml. In our analysis of SP concentrations in cows, 3 values fell below the specified sensitivity limit; in these cows, a concentration of 167.78 pg/ml was used for the present study. In the analysis of SP concentrations in calves no samples fell below the specified sensitivity limit.

4. Statistical analysis

Data analysis was performed using R (version 4.1.3; R Core Team, 2022). The median, mean, first and third quartiles, and standard deviation (SD) were determined descriptively for all measured variables in cows and calves. The results of the descriptive statistics were graphically presented by violin plots. Normality of the data was assessed via Shapiro-Wilk-Test. The null hypothesis (H_0) stated, "The data are normally distributed". Since the P-values (probability-value) were < 0.05, the H_0 was rejected in favor of the alternative hypothesis (H_A), which meant that data in cows and in calves were nonnormally distributed. For further statistical analysis non-parametric test methods were conducted for nonnormally distributed data.

4.1. Statistical analysis in cows

The data set consisted of a total of 308 samples. Number of samples were n = 54 for BJV, n = 54 for TV1, n = 52 for TV2, n = 50 for TV3, n = 49 for TV4, and n = 49 for TV5.

Blood samples at the tail vein could not be collected in 3.7% (n = 2) of cows at 2:00 p.m. (TV2) and in 7.4% (n = 4) of cows at 8:00 p.m. (TV3) on D1 of the study, and in 9.3% (n = 5) of cows at 2:00 a.m. (TV4) and 1.9% (n = 5) of cows at

8:00 a.m. (TV5) of D2 of the study. This resulted in a total number of n = 12 samples for which sampling was not successful (successful and unsuccessful blood sampling in individual cows is given in Supplemental 5). Furthermore, samples were excluded from the data set because of a mix-up during sorting of the samples in the laboratory (n = 1), and because of concentrations of SP being > 10.000 pg/ml, for which we were unable to perform a further dilution for the determination (n = 2).

The data of cows were imputed by chained random forest (MAYER, 2021). The quality of imputation was checked visually. Since the imputed values were distributed within the distribution of existing data, the imputation was accepted.

4.1.1. Mann-Whitney U Test (= Wilcoxon Rank Sum Test)

For the determination of differences in PSPCs between BJV and TV1, between PHYS and PATH, and between feeding group 1 and group 3 both in BJV and in TV1 the Mann-Whitney U Test was conducted. The following hypotheses were stated:

$H_{0\ (BJV/TV1)}$: BJV and TV1 are similar.

$H_{A\ (BJV/TV1)}$: BJV and TV1 differ.

$H_{0\ (PHYS/PATH)}$: PHY and PATH are similar both in jugular and in tail vein.

$H_{A\ (PHYS/PATH)}$: PHY and PATH differ both in jugular and in tail vein.

$H_{0\ (group1/3)}$: Feeding group 1 and group 3 are similar both in jugular and tail vein.

$H_{A\ (group1/3)}$: Feeding group 1 and group 3 differ both in jugular and tail vein.

4.1.2. Friedman Test

For the determination of a circadian rhythm between different time points the Friedman Test was conducted. The following hypotheses were stated:

H_0: Time points TV1, TV2, TV3, TV4 and TV5 are similar.

H_A: Time points TV1, TV2, TV3, TV4 and TV5 differ from each other.

To answer the question which time point differ from each other, a pairwise comparison was conducted via Durbin-Conover tests with a Holm P-value correction for multiple comparisons.

4.1.3. Spearman Correlation

In order to analyze whether there was a correlation between PSPCs of BJV and TV1, and between PSPCs of BJV and age, respectively a Spearman correlation was conducted. The following hypotheses were stated:

$H_{0\ (BJV/TV1)}$: No correlation between PSPC of BJV and TV1.

$H_{A\ (BJV/TV1)}$: PSPC of BJV and TV1 are correlated.

$H_{0\ (BJV/age)}$: No correlation between PSPC of BJV and age.

$H_{A\ (BJV/age)}$: PSPC of BJV and age are correlated.

4.2. Statistical analysis in calves

The data set consisted of a total of 101 samples in calves. The results of one calf were excluded from the data set due to the SP concentration being almost 4000 pg/ml, resulting in the definition of this animal as an outlier.

4.2.1. Kruskal-Wallis Test

For the determination of a difference in PSPCs between calves of group PHYS, LEUC, RED and PATH the Kruskal-Wallis Test was conducted. The following hypotheses were stated:

H_0: Group PHYS, LEUC, RED and PATH are similar.

H_A: At least one group is different from at least one other group.

To answer the question which groups differ from each other, a pairwise comparison was conducted with a Holm P-values correction.

4.2.2. Mann-Whitney U Test (= Wilcoxon Rank Sum Test)

For the determination of a difference in PSPCs between female and male calves, single and group housed calves, adult cattle and (female) calves the Mann-Whitney U Test was conducted. The following hypotheses were stated:

$H_{0\ (female/male)}$: Female calves are similar with male calves.

$H_{A\ (female/male)}$: Female calves differ from male calves.

$H_{0\ (single/group\ housed)}$: Single housed calves are similar to group housed calves.

$H_{A\ (single/group\ housed)}$: Single housed calves differ from group housed calves.

$H_{0\ (adult\ cattle/\ (female)\ calves)}$: Adult cattle are similar to (female) calves.

$H_{A\ (adult\ cattle/\ (female)\ calves)}$: Adult cattle differ from female calves.

IV. RESULTS

1. Cows

1.1. Physiological findings

1.1.1. General animal data

Age, number of lactations, days post-partum, days pregnant, and daily milk yield in cows is given in Table 5. Findings in individual animals are presented in Supplemental 1.

Table 5: General findings in 54 clinically healthy adult cows sampled for the evaluation of plasma substance P concentrations in German Simmental cattle. Parameters are presented as mean and standard deviation (SD). Ranges are given in brackets.

Parameter	Mean	SD
Age in years	5.0 (3.4 – 9.1)	1.3
Number of lactations	3.2 (2.0 – 7.0)	1.3
Days post-partum	177.2 (117.0 – 241.0)	35.4
Days pregnant	65.6 (39.0 – 166.0)	29.1
Daily milk yield in kilogram	37.3 (29.3 – 51.6)	4.9

1.1.2. Clinical examination

Temperature, heart rate, respiratory rate, and rumen motility in cows is given in Table 6. Findings in individual animals are presented in Supplemental 1.

1.1.3. Laboratory findings

Laboratory findings were within the reference ranges determined by the Clinic for Ruminants with Ambulatory and Herd Health Services in 42.6% of cows (n = 23). Findings in individual animals are presented in Supplemental 6.

Table 6: Findings of clinical examination as described by DIRKSEN et al. (1979) in 54 clinically healthy adult cows sampled for the evaluation of plasma substance P concentrations in German Simmental cattle. Parameters are presented as mean and standard deviation (SD). Ranges are given in brackets.

Parameter	Mean	SD
Temperature (°C)	38.4 (38.0 – 39.4)	0.3
Heart Rate (beats/minute)	82.0 (68 – 100)	6.2
Respiratory Rate (breaths/minute)	34.1 (20 – 48)	7.0
Rumen Cycles (in 2 minutes)	2.2 (0 – 3)	0.8

Table 7: Laboratory findings of selected blood parameters in 54 clinically healthy adult cows sampled for the evaluation of plasma substance P concentrations in healthy German Simmental cattle. Parameters assessed were leucocyte count, packed cell volume (PCV), hemoglobin concentration (Hb), total protein (TP), glutaraldehyde test, beta-hydroxybutyrate (BHBA), non-esterified fatty acids (NEFA) and glutathione peroxidase (GSHPX). Parameters are presented as mean and standard deviation (SD). Ranges are given in brackets.

Blood Parameter (Reference range)[1]	Mean	SD
Leucocytes (4 – 10 x10³/µl)	7.0 (4.0 – 12.7)	1.7
Packed Cell Volume (30 – 36%)	32.5 (26.5 – 41.6)	3.1
Hemoglobin (10 – 13 g/dl)	10.3 (8.3 – 13.6)	1.1
Total Protein (40 – 80 g/l)	74.7 (67.0 – 83.6)	4.0
Glutaraldehyde Test[2] (> 15 minutes)	15.9 (14 – 16)	0.4
Beta-hydroxybutyrate (< 1.2 mmol/l)	0.6 (0.3 – 1.0)	0.2
Non-esterified fatty acids (< 0.57 mmol/l)	0.1 (0.0 – 0.3)	0.0
Glutathione Peroxidase (> 250)	607.1 (283.4 – 828.1)	117.7

[1]as defined by the Clinic for Ruminants with Ambulatory and Herd Health Services
[2]glutaraldehyde test is missing in 1 animal

1.2. Plasma substance P concentrations in the jugular vein and tail vein

Results of the evaluation of PSPCs in blood taken from the jugular vein and blood taken from the tail vein in 54 healthy cows are given in Table 8. PSPCs in individual animals are presented in Supplemental 5. There were significant differences in PSPCs between BJV and TV1 ($p < 0.01$, Figure 3). PSPCs did not differ

significantly between feeding groups 1 and 3, neither in BJV (p = 0.10), nor in TV1 (p = 0.74, Table 9). Also, there were no significant differences in PSPCs between cows of PHYS compared with PATH, both for BJV (p = 0.32) and TV1 (p = 0.13, Table 10).

Table 8: Plasma substance P concentrations (PSPCs) in pg/ml in the blood taken from the Vena jugularis externa (jugular vein, BVJ) and Vena caudalis mediana (tail vein, TV1) of 54 adult cows sampled for the evaluation of PSPCs in clinically healthy German Simmental cows at 8:00 a.m. Parameters are presented as mean with standard deviation (SD), and median. Ranges are given in brackets.

PSPC (pg/ml)	Mean	SD	Median
BJV (501.95 – 2337.25)	1076.61	433.19	983.78
TV1 (192.34 – 2531.00)	912.49	397.12	818.31

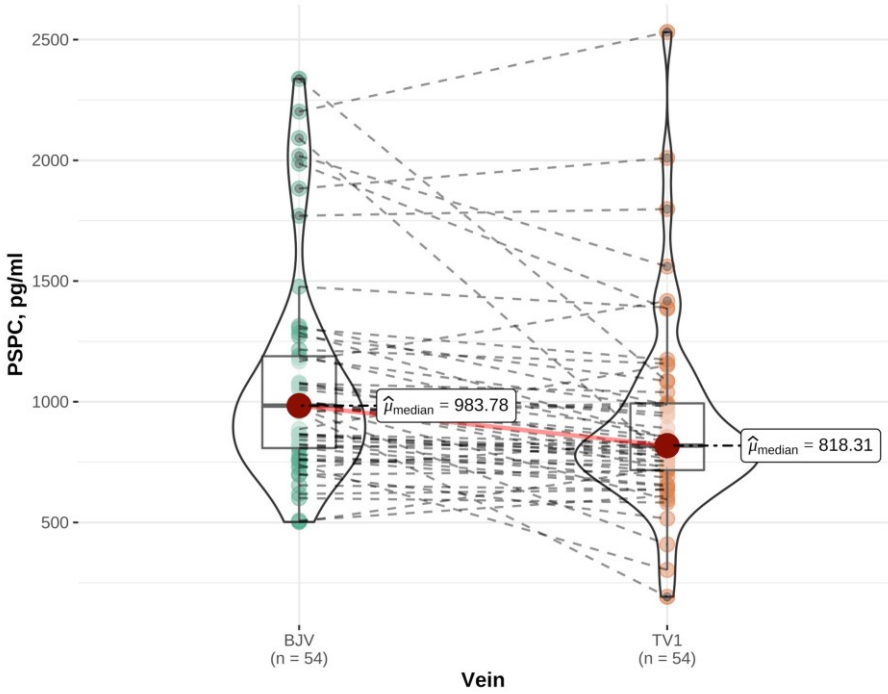

Figure 3: Median plasma substance P concentrations (PSPCs) in pg/ml in the blood taken from the Vena jugularis externa (jugular vein, BVJ) and Vena caudalis mediana (tail vein, TV1) of 54 adult cows sampled for the evaluation of PSPCs in clinically healthy German Simmental cows at 8:00 a.m. The dashed lines connect the individual PSPCs in the blood samples of BJV and TV1.There were significant differences in PSPCs between BJV and TV1 (p < 0.01).

Table 9: Plasma substance P concentrations (PSPCs) in pg/ml in the blood taken from the Vena jugularis externa (jugular vein, BVJ) and Vena caudalis mediana (tail vein, TV1) of cows in feeding groups 1 (n = 19, 35.2% of cows) and 3 (n = 35, 64.8% of cows). Animals were kept in two different feeding groups due to research purposes. PSPCs between groups did not differ significantly ($p \geq 0.10$). Parameters are presented as mean with standard deviation (SD), and median. Ranges are given in brackets.

	PSPC (pg/ml)		
	Mean	**SD**	**Median**
BJV			
Group 1 (501.95 – 1303.34)	907.53	205.19	886.64
Group 3 (508.19 – 2337.25)	1168.39	495.56	999.95
	Mean	**SD**	**Median**
TV1			
Group 1 (636.17 – 1174.20)	866.42	168.99	833.24
Group 3 (192.34 – 2531.00)	937.50	478.42	797.11

Table 10: Plasma substance P concentrations (PSPCs) in pg/ml in the blood taken from the Vena jugularis externa (jugular vein, BVJ) and Vena caudalis mediana (tail vein, TV1) of clinically healthy adult cows with no (PHYS, n = 23, 42.6% of cows) and with mild deviations (PATH, n = 31, 57.4% of cows) from the reference ranges as defined by the Clinic for Ruminants with Ambulatory and Herd Health Services. PSPCs between groups did not differ significantly ($p > 0.10$). Parameters are presented as mean with standard deviation (SD), and median. Ranges are given in brackets.

	PSPC (pg/ml)		
	Mean	**SD**	**Median**
BJV			
PHYS (508.19 – 2092.05)	1040.57	449.75	958.23
PATH (501.95 – 2337.25)	1103.35	425.97	1001.05
	Mean	**SD**	**Median**
TV1			
PHYS (303.49 – 1560.13)	840.58	332.01	779.60
PATH (192.34 – 2531.00)	965.98	436.84	850.28

1.3. Plasma substance P concentrations in the tail vein over 24 hours

Mean, SD, and median of PSPCs in the tail vein over a period of 24 hours are given in Table 11. PSPCs in individual animals are presented in Supplemental 5. There were significant differences between TV1 and TV3 ($p < 0.01$), TV1 and TV4 ($p < 0.01$), TV1 and TV5 ($p < 0.01$), TV2 and TV3 ($p = 0.02$), TV2 and TV4 ($p < 0.01$), TV2 and TV5 ($p < 0.01$), and TV3 and TV4 ($p = 0.03$, Figure 4).

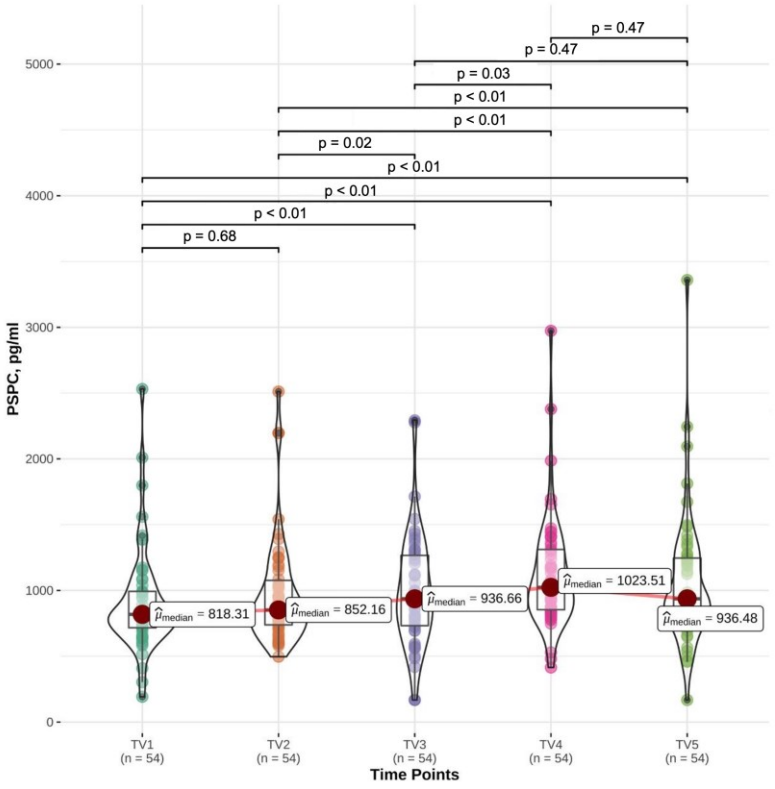

Figure 4: Median plasma substance P concentrations (PSPCs) in pg/ml in the blood taken from the Vena caudalis mediana (tail vein, TV) of 54 adult cows at the interval of 6 hours over the course of 24 hours (8:00 a.m. (TV1), 2:00 p.m. (TV2), 8:00 p.m. (TV3), 2:00 a.m. (TV4), and 8:00 a.m. (TV5). Differences of $p < 0.05$ were considered significant and are indicated.

Table 11: Plasma substance P concentrations (PSPCs) in pg/ml in the blood taken every 6 hours from the Vena caudalis mediana (tail vein, TV) of 54 clinically healthy adult cows to assess SP concentrations over a period of 24 hours. Blood was taken at 8:00 a.m. (TV1), 2:00 p.m. (TV2), 8:00 p.m. (TV3), 2:00 a.m. (TV4), and 8:00 a.m. (TV5). Parameters are presented as mean with standard deviation (SD), and median. Ranges are given in brackets. Blood samples were missing for n = 2 in TV2, n = 4 in TV3, and n = 5 for TV 4 and TV5, respectively.

PSPC (pg/ml)	Mean	SD	Median
TV1 (192.34 – 2531.00)	912.49	397.12	818.31
TV2 (496.87 – 2511.62)	972.83	404.25	852.16
TV3 (167.78 – 2292.84)	993.24	421.50	936.66
TV4 (414.85 – 2973.53)	1124.78	434.80	1023.51
TV5 (167.78 – 3359.53)	1066.80	503.22	936.48

1.4. Correlation of plasma substance P concentrations in the jugular vein and tail vein

There was a significantly positive correlation between PSPCs in the blood from the jugular vein (BVJ) and the blood from the tail vein (TV1, $p < 0.01$, Figure 5).

1.5. Plasma substance P concentrations and cows' age

There was no correlation between PSPCs in the blood from the jugular vein (BVJ) and the age of cows ($p = 0.70$, Figure 6).

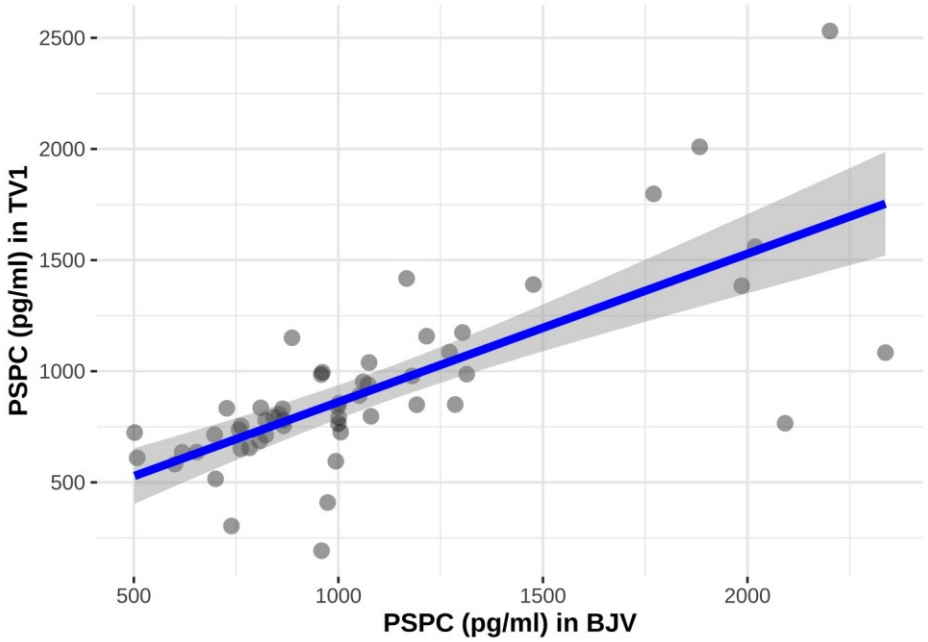

Figure 5: Plasma substance P concentrations (PSPCs) in pg/ml in the blood taken from the Vena jugularis externa (jugular vein, BVJ) in correlation to PSPC in the blood taken from the Vena caudalis mediana (tail vein, TV1) of 54 adult cows sampled for the evaluation of PSPCs in clinically healthy German Simmental cows at 8:00 a.m. There was a significantly positive correlation ($p < 0.01$) in PSPCs between both sampling sites.

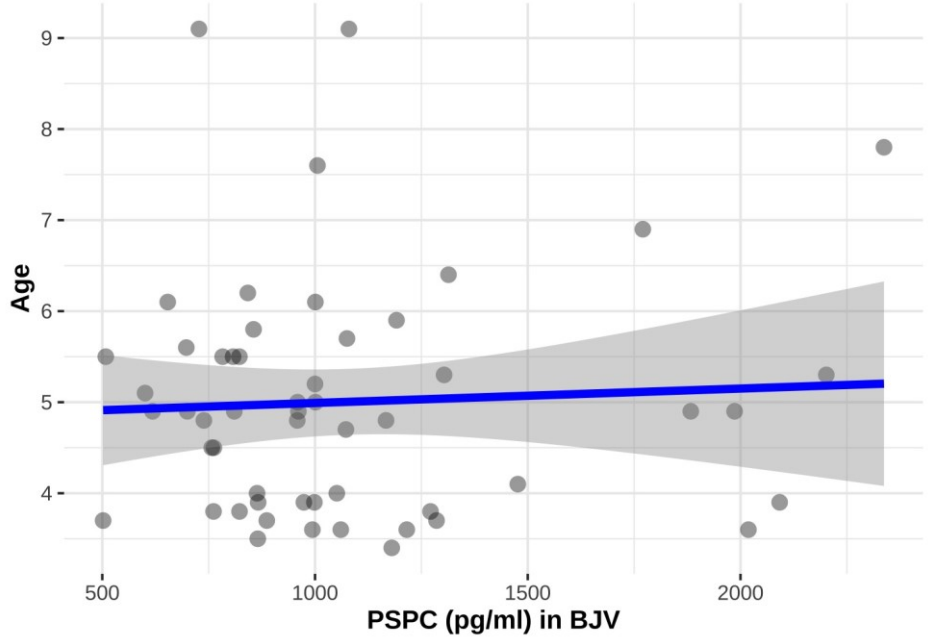

Figure 6: Plasma substance P concentrations (PSPCs) in pg/ml in the blood taken from the Vena jugularis externa (jugular vein, BVJ) in 54 cows sampled for the evaluation of PSPCs in clinically healthy German Simmental cows at 8:00 a.m. in correlation to the age of the cows. Age of the cows was 5.0 ± 1.3 (3.4 – 9.1) years. There was no correlation ($p = 0.70$) between PSPC and the age of cows.

2. Calves

2.1. Physiological findings

2.1.1. General animal data

Age, birth weight, and colostrum intake in CALF and CALM is given in Table 12. Findings in individual animals is presented in Supplemental 3.

2.1.2. Findings in clinical examination

Temperature, heart rate, and respiratory rate in CALF and CALM are given in Table 13. Findings in individual animals are presented in Supplemental 3.

Table 12: Age in days (d), birth weight in kilogram (kg), and liters (l) of colostrum intake in 49 male (CALM) and 52 female calves (CALF) sampled for the evaluation of plasma substance P concentrations in clinically healthy German Simmental calves. Parameters are presented as mean and standard deviation (SD). Ranges are given in brackets.

Parameter	Total (n = 101)	CALM (n = 49)	CALF (n = 52)
Age (d)	17.1 ± 2.1 (14 – 21)	17.2 ± 2.1 (14 – 21)	17.1 ± 2.3 (14 – 21)
Birth Weight (kg)[1]	43.0 ± 5.9 (28 ± 58)	44.7 ± 4.9 (32 – 58)	41.4 ± 6.3 (28 – 54)
Colostrum Intake (l)[2]	2.5 ± 0.9 (0.5 – 7.0)	2.7 ± 1.0 (0.5 – 7.0)	2.3 ± 0.7 (0.5 – 4.0)

[1]Birth weight is missing in n = 1 in group CALM and CALF each, [2]Colostrum intake is missing in n = 6 in group CALM and n = 5 in group CALF.

Table 13: Findings of clinical examination in 49 male (CALM) and 52 female calves (CALF) sampled for the evaluation of plasma substance P concentrations in clinically healthy German Simmental calves. Clinical examination was performed after blood sampling, and as described by DIRKSEN et al. (1979). Parameters are presented as mean and standard deviation (SD). Ranges are given in brackets.

Parameter	Total (n = 101)	CALM (n = 49)	CALF (n = 52)
Temperature (°C)	39.0 ± 0.4 (37.9 – 39.5)	39.0 ± 0.4 (37.9 – 39.5)	39.0 ± 0.3 (38.1 – 39.5)
Heart Rate (beats/minute)	138.1 ± 19.3 (100.0 – 176.0)	137.3 ± 18.2 (108.0 – 168.0)	139.5 ± 20.4 (100.0 – 176.0)
Respiratory Rate (breaths/minute)	38.5 ± 6.2 (20.0 – 49.0)	39.4 ± 6.7 (20.0 – 49.0)	37.7 ± 5.7 (24.0 – 48.0)

2.1.3. Laboratory findings

Laboratory findings including leucocyte count, PCV, Hb, TP, and GSHPX are given in Table 14. Laboratory findings were within the reference ranges determined by the Clinic for Ruminants with Ambulatory and Herd Health Services in 15.8% of calves (n = 16). Findings in individual animals are presented in Supplemental 7.

Table 14: Laboratory findings of selected blood parameters in 49 male (CALM) and 52 female calves (CALF) sampled for the evaluation of plasma substance P concentrations in clinically healthy German Simmental calves. Parameters assessed were leucocyte count, packed cell volume (PCV), hemoglobin concentration (Hb), total protein (TP), and glutathione peroxidase (GSHPX). Parameters are presented as mean and standard deviation (SD). Ranges are given in brackets.

Parameter (Reference range)[1]	**Total (n = 101)**	**CALM (n = 49)**	**CALF (n = 52)**
Leucocytes (4 – 10 x10³/µl)	9.9 ± 2.8 (3.2 – 14.9)	10.3 ± 2.7 (5.3 – 14.9)	9.6 ± 2.9 (3.2 – 14.7)
PCV (30 – 36%)	35.7 ± 3.0 (27.9 ± 41.4)	35.9 ± 2.8 (28.5 – 40.8)	35.5 – 3.1 (27.9 – 41.4)
Hemoglobin (10 – 13 g/dl)	11.5 ± 1.0 (9.0 – 14.2)	11.6 ± 1.0 (9.6 – 14.2)	11.5 ± 1.1 (9.0 – 14.0)
Total Protein (40 – 80 g/l)	57.3 ± 5.0 (47.5 – 71.8)	57.3 ± 5.2 (47.5 – 71.8)	57.3 ± 4.8 (47.6 – 69.4)
GSHPX (> 250)	577.7 ± 142.6 (297.0 – 995.1)	586.7 ± 137.0 (330.0 – 995.1)	569.2 ± 148.6 (297.0 – 983.7)

2.2. Evaluation of plasma substance P concentrations

Results of PSPCs in CALF, CALM, and in total are given in Table 15. PSPCs in individual animals are presented in Supplemental 7. PSPCs were significantly (p = 0.01) lower in female compared with male calves. PSPCs did not differ significantly between animals kept in individual compared with group housing (p = 0.87, Table 16).

Table 15: Plasma substance P concentrations (PSPCs) in pg/ml in the blood taken from the Vena jugularis externa (jugular vein) at 6:00 a.m. in 49 male and 52 female German Simmental calves. All calves were clinically healthy and were exposed to the same surroundings, feedstuff, and handling. PSPCs were significantly (p = 0.01) lower in female compared with male calves. PSPCs are given as mean, standard deviation (SD), and median.

PSPC (pg/ml)	**Range**	**Mean**	**SD**	**Median**
Total (n = 101)	229.27 – 1614.96	610.90	272.27	525.92
Male Calves (n = 49)	301.56 – 1614.96	678.81	286.23	583.71
Female Calves (n = 52)	229.27 – 1262.70	546.91	244.22	491.61

Table 16: Plasma substance P concentrations (PSPCs) in pg/ml in the blood taken from the Vena jugularis externa (jugular vein) in 49 male and 52 female German Simmental calves housed either in individual (n = 21, 20.8% of calves) or group (n = 80, 79.2% of calves) igloos. Group size was 5.9 ± 1.9 (2 – 9) calves. All calves were exposed to the same surroundings, feedstuff, and handling. PSPCs did not differ significantly (p = 0.87) between housing systems. PSPCs are given as mean, standard deviation (SD), and median.

	Individual Housing			Group Housing		
PSPC (pg/ml)	**Total** (n = 21)	**Male** (n = 8)	**Female** (n = 13)	**Total** (n = 80)	**Male** (n = 41)	**Female** (n = 39)
Mean	569.97	692.65	494.47	621.64	676.11	564.38
SD	192.37	219.10	132.14	289.67	299.77	270.71
Median	525.15	664.17	499.22	526.43	573.11	488.81
Range	236.71 – 1085.49	422.06 – 1085.49	236.71 – 755.13	229.27 – 1614.96	301.56 – 1614.96	229.27 – 1262.70

2.3. Plasma substance P concentrations according to laboratory findings

PSPCs in PHYS, LEUC, RED, and PATH are given Table 17. There were no significant differences in PSPCs between groups ($p > 0.10$), but a trend ($p = 0.08$, Figure 7) for PSPCs to be higher in PATH compared with PHYS.

Table 17: Plasma substance P concentrations (PSPCs) in pg/ml in the blood taken from the Vena jugularis externa (jugular vein) at 6:00 a.m. in clinically healthy German Simmental calves of groups PATH (n = 38, 37.6% of calves), LEUC (n = 23, 22.8% of calves), RED (n = 24, 23.8% of calves), and PHYS (n = 16, 15.8% of calves). All calves were exposed to the same surroundings, feedstuff, and handling. PSPCs are given as mean, standard deviation (SD), and median.

PSPC (pg/ml)	Range	Mean	SD	Median
PHYS (n = 16)	271.19 – 974.16	504.95	189.29	463.55
LEUC (n = 23)	229.27 – 1614.96	650.58	333.76	509.64
RED (n = 24)	236.71 – 1255.34	541.34	224.60	491.52
PATH (n = 38)	245.52 – 1382.60	675.43	273.85	600.18

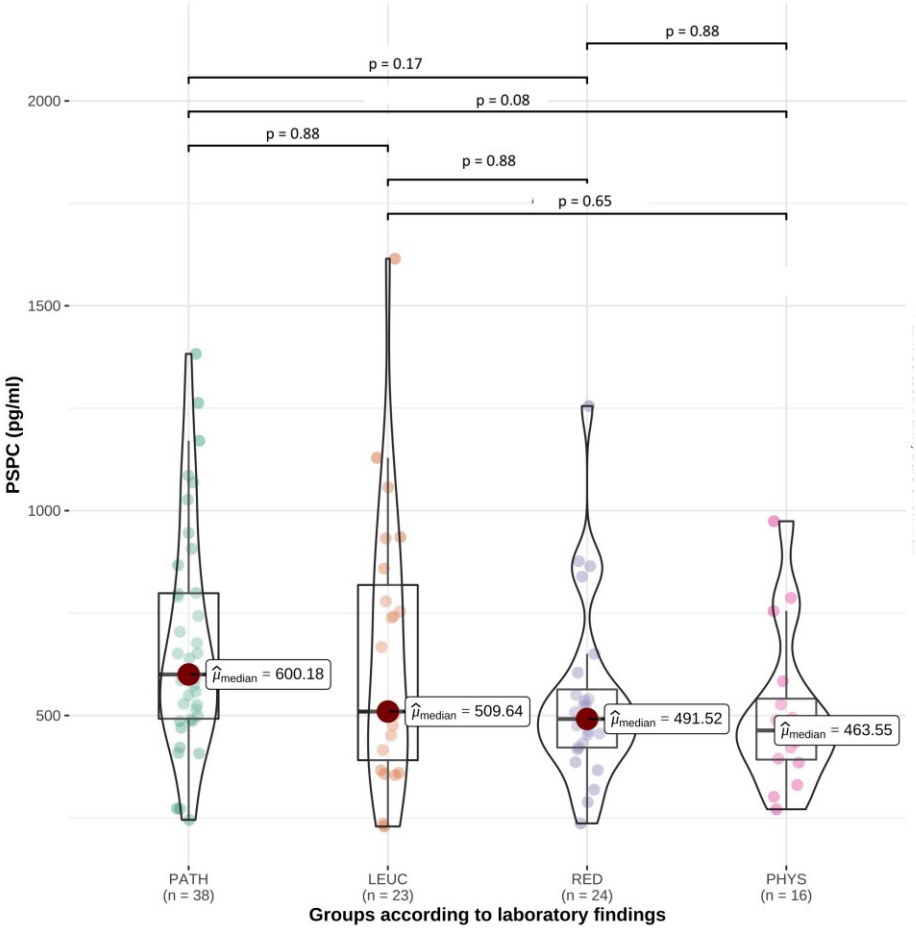

Figure 7: Median plasma substance P concentrations (PSPCs) in pg/ml in the blood taken from the Vena jugularis externa (jugular vein) at 6:00 a.m. in clinically healthy German Simmental calves. Clinically healthy calves with deviations in leucocyte count were included in group LEUC (n = 23, 22.8% of calves), calves with deviations in hemoglobine concentration or packed cell volume in group RED (n = 24, 23.8% of calves), calves with more than one blood parameter deviating from the defined reference ranges in group PATH (n = 38, 37.6% of calves), and calves with no deviations from the reference range in group PHYS (n = 16, 15.8% of calves), respectively. The reference ranges were defined by the Clinic for Ruminants with Ambulatory and Herd Health Services. All calves were exposed to the same surroundings, feedstuff, and handling. There were no significant differences between groups (p > 0.10), but a trend for PSPCs to be higher in PATH compared with PHYS (p = 0.08). Differences of p < 0.05 were considered significant.

2.4. Plasma substance P concentrations in calves and adult cows

PSPCs in calves (n = 101) were compared with the dataset of PSPCs in adult cows assessed from the jugular vein (BVJ; n = 54; Table 18). Median PSPCs of adult cows were significantly higher compared with median PSPC of calves (p < 0.01). PSPCs in female calves (CALF; n = 52) were compared with the same dataset of PSPCs in adult cows assessed from the jugular vein (BVJ; n = 54, Table 18). Median PSPCs in adult cows were significantly higher than median PSPCs of

female calves ($p < 0.01$).

Table 18: Plasma substance P concentrations (PSPCs) in pg/ml in the blood taken from the Vena jugularis externa (jugular vein) in adult cows (n = 54), total calves (n = 101), and female calves (n = 52) sampled at 8:00 a.m. in cows and at 6:00 a.m. in calves, respectively. All animals were clinically healthy and of the German Simmental breed. There were significant differences ($p < 0.01$) between PSPCs of adult cows and total calves, and female calves, respectively. PSPCs are given as mean, standard deviation (SD), and median.

PSPC (pg/ml)	Range	Mean	SD	Median
Adult cows (n = 54)	501.59 – 2337.25	1076.61	433.19	983.78
Total calves (n = 101)	229.27 – 1614.96	610.90	272.27	525.92
Female calves (n = 52)	229.27 – 1262.70	546.91	244.22	491.61

V. DISCUSSION

To our knowledge, no research exists about the evaluation of SP concentrations in healthy, not stressed, and untreated cows and calves. The present study has the role of a pilot study, as SP concentrations were evaluated in cows and calves which did not experience pain, were not diseased, and were not exposed to a stressful experimental environment. The combination of blood sample collection from the jugular vein and the tail vein, and the repeated blood sample collection from the tail vein in cows, was done to provide the most comprehensive knowledge about baseline concentrations of SP and the potential presence of a circadian rhythm in SP secretion.

1. Animals, clinical examination, and laboratory findings

Inclusion criteria for cows of the present study regarding lactation and milk yield were limited to cows being in their second or higher lactation and being pregnant. It would have added value to our results to include non-pregnant heifers and cows which were not milked as done by BUSTAMANTE et al. (2015), as well as cows of different stages of lactation and pregnancy, or dried off animals. This would have provided insight into whether there is an influence of performance or stage of pregnancy on PSPCs. As the dataset of animals in the present study is small, we did not include different lactation and pregnancy states into the statistical model.

Inclusion criteria for calves in the present study were that calves had to be from a physiological parturition and had to have received colostrum after birth. These criteria were especially important, as it is well known that a physiological birth and immediate access to colostrum decrease the risk of mortality in calves (TYLER et al., 1999; LOMBARD et al., 2007). It was published by LOMBARD et al. (2007) that dystocia has an acute negative impact on the calf´s vitality and passive immunity transfer. To avoid the influence of dystocia on calves, and to be able to obtain healthy and immunocompetent calves, these two requirements for the inclusion of calves into the present study were thoroughly checked for each individual calf.

Since we know from previous research work in humans and in laboratory animals that changes in PSPCs can result from pain (COETZEE et al., 2008), as well as

from inflammatory processes (HUNT and MANTYH, 2001; MASHAGHI et al., 2016), and from stressful situations (TOMETTEN et al., 2004; EBNER et al., 2008), it was particularly important that all cows and calves sampled for the present study were clinically healthy with no previous treatment because of an infection or disease. Therefore, based on the preliminary medical report, the clinical examination, and the laboratory findings, the goal was to identify animals with aberrance from the physiological ranges and thereby to exclude any possible interference of the study results. The preliminary medical report was of particular importance in calves. It is evident that the incidence of respiratory and enteric diseases in calves is higher in winter than in autumn and summer (LOMBARD et al., 2007), and most of the calves which were sampled for the evaluation of PSPCs were born and raised in winter. However, it must be considered that the preliminary medical report depended on the subjective perception and evaluation of abnormalities by the stockperson, in cows as well as in calves. Therefore, it is possible that animals judged to be healthy upon clinical examination showed mild deviations from the laboratory reference ranges due to prior mild infections not recognized by the stockpersons.

However it should also be mentioned that the results of the clinical examination depend on the subjective assessment of the examiner, similar to the assessment of pain (HUDSON et al., 2008; PRUNIER et al., 2012). In order to exclude as much subjectivity as possible, a standardized procedure for the clinical examination, which was described by DIRKSEN et al. (1979), was used. Also, the clinical examination was performed by only two veterinarians, one who was trained by the other, to exclude the interference of the data due to various veterinarians. Furthermore, the two examiners consulted on how to classify the clinical findings in ambiguous cases. Cases of umbilical hernia in calves were not classified as pathological. DIRKSEN (2006) described that umbilical hernias can close within 14 days to several weeks after birth. Calves from the present study, which were diagnosed with an umbilical hernia were between 14 and 21 days old. As it was most likely that the umbilical hernias would close with increasing age, these findings were considered as nonpathological. Mild to moderate auscultatory findings of the lungs of otherwise healthy calves were characterized as physiological findings in young animals as described by BAUMGARTNER (2014).

When implementing the experiment, the goal was to create a preferably stress-free

environment for the animals, and thus to avoid the bias of data by influence of stress (TOMETTEN et al., 2004; EBNER et al., 2008). Reduction of stress included that all animals originated from the same farm, so that they were already acclimatized to the setting before the start of the experiment. Another positive aspect of performing the experiment on the farm of origin was that they did not have to be transported or re-housed for the experimental period, as was done for other studies (WHITLOCK et al., 2012; BUSTAMANTE et al., 2015) and which could have resulted in stress. Since it is known that social isolation is a severe psychological stressor in cattle (BOISSY and LE NEINDRE, 1997), the cows in the present study were only separated from their herd by a grid, resulting in visual and tactile contact with the rest of the herd, and permanent contact with the other animals during and between sampling intervals.

Since the present study was done over a long period of time (December 2019 to December 2021), cows and calves were exposed to different environmental influences at different seasons. This might be reflected particularly in the results of animals sampled in summer. It is well known, that heat stress results in increased heart and respiratory rates, and rectal temperature (KOVÁCS et al., 2018; HERBUT et al., 2019). Furthermore, regardless of the influence of ambient temperature on vital parameters, heart rate may also be influenced by stress caused by routine management practice (KOVÁCS et al., 2014). Because an increase in heart rate might be due to heat stress and stress due to handling, individual animals with an increased heart rate but otherwise no pathological findings during clinical examination were not excluded from the study.

The assessment of the laboratory findings was done according to the reference ranges determined by the Clinic for Ruminants with Ambulatory and Herd Health Services. Even though the laboratory findings of some animals in the present study were not within the specified reference ranges provided by the laboratory, the animals were included in the study, as they were healthy upon clinical examination. CONSTABLE et al. (2017) defined reference ranges as a range of values of a test which are expected in a group of healthy animals. These reference ranges include a 5% false-positive rate, and there is a lower chance for minor differences from the reference ranges to indicate a disease than for larger differences (CONSTABLE et al., 2017). To assess if there was an influence of pathological findings in blood parameters on PSPCs, four and two different groups of animals with mild deviations

in blood parameters in clinically healthy calves and cows, respectively, were compared. As there were no significant differences in PSPCs between the two groups of cows with physiological compared with pathological laboratory findings, the mild deviations in laboratory findings are not likely to have influenced the study results, even if higher PSPCs were found in cows of the group PATH compared with cows of the group PHYS. The same applies to the results in calves, where no significant differences in PSPCs between the four groups with different laboratory findings could be shown. Therefore, we currently assume that slightly increased parameters of inflammation as in group LEUC, or in the red blood count (RED) are not associated with changes in PSPCs in calves. However, it is interesting that calves of all three groups including animals with mild deviations from the physiological ranges of the laboratory parameters had higher PSPCs than calves of PHYS. Also, it is not negligible that a trend ($p = 0.08$) for PSPCs in calves to be higher in PATH compared with PHYS was demonstrated. Taking this into consideration, it is possible that a statistically significant difference between PHYS and PATH in calves might have been shown in a larger population of animals. It is also possible that differences in PSPCs between groups of different laboratory findings could have been more prominent if the assessment of other inflammatory parameters had been included into the study protocols. Further inflammatory parameters commonly determined in cattle and belonging to the acute-phase proteins are haptoglobin and serum amyloid A (ALSEMGEEST et al., 1994; HORADAGODA et al., 1999). A specific inflammatory status of the animals and in particular a differentiation between acute and chronic inflammation would have been useful, but also would have generated additional financial costs to the present project, since analysis of haptoglobin and serum amyloid A require an ELISA analysis method (HORADAGODA et al., 1999), which is expensive.

2. Findings in plasma substance P concentrations

2.1. Substance P concentrations in the jugular vein of cows and calves

The mean PSPCs in the jugular vein in cows was 1076.61 ± 433.19 pg/ml. It is remarkable that PSPCs in animals of the present study are distinctively higher than the baseline concentrations of PSPCs in cows of previous studies. BARRAGAN et al. (2018) found concentrations of 37.73 ± 5.41 pg/ml in control cows without clinical metritis. In a study by BUSTAMANTE et al. (2015), in which the authors

investigated oligofructose-induced lameness in cattle, mean PSPCs of 0.26 ng/ml – 0.42 ng/ml (= 260 – 420 pg/ml) were described in healthy control heifers. Further concentrations published in other studies were 0.25 ± 0.09 ng/ml (= 250 ± 90 pg/ml) in non-lame cows (RODRIGUEZ et al., 2018), and 93.4 ± 17.2 pg/ml in control bulls for electroejaculation (WHITLOCK et al., 2012). It is known from previous studies in adult cows (TSCHONER et al., 2020b) as well as in calves (COETZEE et al., 2008) that there are high between- and within- animal variations in SP concentrations. However, we assume that several factors might have had an impact on our results.

Generally, it should be noted that SP concentrations in the animals of the present study were neither assessed in diseased (SICKINGER et al., 2018b; TSCHONER et al., 2020b) nor in analgesic- or placebo-treated animals (VAN ENGEN et al., 2014; KLEINHENZ et al., 2016; MELÉNDEZ et al., 2018; TSCHONER et al., 2018). Furthermore, the animals in the present study were neither submitted to pain induced by a husbandry procedure (COETZEE et al., 2008; ALLEN et al., 2013; LAUDER et al., 2020) nor were they experiencing stress due to any other procedure (WHITLOCK et al., 2012; KASIMANICKAM et al., 2018; KASIMANICKAM et al., 2019). Because of this, and since all animals were exposed to the same management, environment, and handling, it is difficult to compare the present data with results of previous research.

Differences in PSPCs in animals might also result both from the different analysis methods, and from different methods of sample handling (MOSHER et al., 2014). In a systematic review, TSCHONER and FEIST (2022) reviewed 36 papers using SP concentrations in adult cattle and calves, among other pain parameters, to assess pain during different procedures, conditions, and diseases. The authors found that the processing of blood samples was heterogenous throughout the publications. It can be assumed that hours until processing, centrifugation of blood samples, matrix (blood plasma or serum) used, temperature at which samples were kept until analysis, type of protease inhibitor, and types and charge number of ELISA kits, might result in differences in PSPCs (MOSHER et al., 2014; TSCHONER and FEIST, 2022). Comparing the different analysis methods for SP in various studies, it is remarkable that no gold standard has been established yet. For the analysis of PSPCs in the present study, an multispecies ELISA kit was used, whereas other authors described analysis of PSPCs in adult cattle with bovine ELISA kits

(BUSTAMANTE et al., 2015; RODRIGUEZ et al., 2018), competitive radioimmunoassay techniques (BARRAGAN et al., 2018), or competitive immunoassays (LAUDER et al., 2020). Therefore it can be concluded that it is difficult to compare PSPCs which have been assayed using different tests and laboratory methods, as has been described for serum pepsinogen concentrations in diagnosing ostertagiasis (BERGHEN et al., 1993; SCOTT et al., 1995).

Knowing that the release of SP is influenced by a wide variety of psychological and physical stressors (VAN ENGEN et al., 2014), it is interesting that the variance in PSPCs in the present study population was as high as it was, especially because all animals originated from the same farm. Animals were born, raised, and housed at the Research- and Teaching Center Achselschwang. The farm, where the study was conducted, is a state facility used for research and agricultural education. Since the farm is a research facility, adult cattle were not only subject to our study, but also to a feeding study conducted by the Bavarian State Research Center for Agriculture (ETTLE et al., 2020; ETTLE et al., 2021a; ETTLE et al., 2021b; RIEPL et al., 2021). However, a possible influence of the concurrent feeding studies on our results could be excluded, as no significant difference in PSPCs could be demonstrated between the different feeding groups. However, management could have influenced the PSPCs in the present study. We have to assume that calves, born and raised on the farm and being part of our study population as an adult cow, were exposed to various human influences, since agricultural students help in addition to the core training staff with the daily barn work. BOIVIN et al. (1994) described the importance of the first three month of life for the establishment of a human-cattle relationship. The imprinting by numerous different human contacts in early rearing could be a reason for the high variance in PSPCs in adult cattle.

It could be demonstrated that the hormone secretion rhythm of cortisol is influenced by different management techniques and external environment including different housing and feeding systems (OGINO et al., 2014). According to our results, PSPCs in calves housed either in individual or in in group igloos did not differ significantly. Therefore, we can assume that there seems to be no effect of housing on PSPCs, at least for calves. Most common diseases like diarrhea and infection of the respiratory tract are not consistently associated with group housing (COSTA et al., 2016). However, it is reasonable to think that calves housed in group igloos might have higher SP concentrations as a result of increased social contact and stimulation of

the immune system by potential pathogens, since it is known that diseases can be spread by horizontal calf – calf contact in group housing (MCGUIRK, 2008). It is also possible that injuries because of rank fights might have resulted in increased PSPCs due to the fact that inflammation (BARRAGAN et al., 2018) and pain (COETZEE et al., 2008) result in increased SP concentrations. This could not be confirmed by the results in calves of the present study. In cows, we cannot make a statement about the effect of group housing on PSPCs, since we did not have a control group of cows that were kept in tethered housing and thus not exposed to rank fights.

It must be considered that cows and calves included in the present study were all of the German Simmental breed, which might have had an influence on the PSPCs. It is known from previous research that the immunoreactive area for SP in the corpus abomasi is influenced by the genetic component of cows. The results of this study showed a significantly smaller immunoreactive area for SP in German Holstein than in German Simmental cows (SICKINGER et al., 2008). With this background information and the fact that the abomasal wall of German Simmental cows has a lower number of nerve fibers than that of German Holsteins cows (SICKINGER et al., 2008) releasing SP (HOLZER and HOLZER-PETSCHE, 1997a,1997b), it is suggested that an influence of breed on PSPCs exists. However, further research on the effect of breed on serum neuropeptide concentrations showed no significant difference in SP concentrations in blood serum between healthy German Holstein and German Simmental cows (SICKINGER et al., 2018a). Since these results concerning the influence of the breed on SP concentrations are inconsistent, it is very difficult to put our results into comparison with other studies. Further research is needed to answer the question which role the genetical component plays in the SP secretion in healthy and diseased cows.

Parity of cows seems to have an influence on PSPCs. BARRAGAN et al. (2020) found that circulating SP concentrations were significantly higher in primiparous, compared with multiparous cows ($p = 0.04$). These findings cannot be confirmed by the results of the present study, as only pluriparous cows (two or more calvings) were included in this study. Also, due to the small study population, cows were not divided into groups according to their number of calving for the analysis.

PSPCs can also depend on the temperament of an animal. KASIMANICKAM et al. (2018) found significantly higher SP concentrations in excitable compared with

calm cattle prior to weaning and breeding. As we did not divide animals into groups according to temperament, we cannot make a statement about this fact. However, the different temperaments of the animals could explain the high variability in PSPCs in our study population. The high variability in PSPC due to inter- and intraindividual variations has been reported previously in calves (COETZEE et al., 2008; TSCHONER et al., 2018) as well as in adult cows (TSCHONER et al., 2020b).

The high variability of PSPCs was also seen in calves in the present study. The mean and SD of PSPC in calves was 610.90 ± 272.27 pg/ml. Values ranged from 229.27 to 1614.96 pg/ml. It is remarkable that PSPCs in the present study are higher than the baseline concentrations in calves of previous studies. The higher SP values are likely to result from the fact that different batches of the SP ELISA kit or various devices for the evaluation of the plates are automatically used in different studies. COETZEE et al. (2008) also confirmed the high within- and between- calf variations of SP concentrations in calves undergoing castration. In a study by STOCK et al. (2015), mean SP concentrations of 20.8 ± 0.4 pg/ml were described in placebo (non-analgesic) treated calves after cautery disbudding. Median SP baseline concentrations of 560.3 pg/ml and 690.0 pg/ml were found in calves before treatment with either meloxicam or metamizole in addition to meloxicam, respectively (TSCHONER et al., 2018). Since the data collection in previous publications was conducted in calves which were exposed to a painful procedure (COETZEE et al., 2008; STOCK et al., 2015; TSCHONER et al., 2018) and since the data are presented differently in the publications (mean or median) we cannot directly compare our results with these data. It is very unlikely, that the PSPCs of the present study are that high because of a possible experience of pain induced by the single puncture of the jugular vein. Especially as authors from other studies also collected blood from the jugular vein. It is known that SP is primarily released from C-fibers (SNIJDELAAR et al., 2000) which conduct pain with a much slower speed than Aβ- fibers and Aδ- fibers (HENKE and ERHARDT, 2001; MUIR and WOOLF, 2001). Blood sample collection in the present study was performed within a very short time. Therefore, high PSPCs should not be associated with pain induced due to the blood sample collection. This can also be supported by the fact that blood for the assessment of PSPCs was taken immediately after puncture of the vein and before any other samples were collected. Additionally, PSPCs of the present study

were not influenced by any other painful event, since only calves which were neither dehorned nor castrated were subject to the present study population. These inclusion criteria were of particular importance, since several studies reported increased SP concentrations as a consequence of castration (COETZEE et al., 2008) and dehorning (ALLEN et al., 2013; KARLEN et al., 2019).

Release of cortisol due to handling and restraint in calves during blood sampling is evident, when the blood sampling procedure takes longer than two minutes (STILWELL et al., 2008b). For the evaluation of SP concentrations, calves were exposed to a sampling situation for which it cannot be stated how stressful the situation was for the animals, as no other parameter indicating stress (e.g., cortisol) was determined. It is possible that entering the housing system by the examiner, approaching and separating the calf from the rest of the group, restraining the animal, and performing a blood sampling, was experienced as a stressful situation. However, increase of PSPCs due to stress, as known for cortisol, can be excluded in the present study because the puncture of the jugular vein and blood collection in calves was limited to a very short time (< two minutes), and the blood samples for the determination of SP were collected immediately after fixation of the animals. Furthermore, it can be assumed that the stress caused by the sampling procedure did not cause an increase in PSPCs, since a previous study stated that PSPCs did not increase in cows put into lateral and dorsal recumbency for a laparoscopic abomasopexy, neither in the xylazine treated, nor in the placebo treated group (TSCHONER et al., 2020b).

2.2. Substance P concentrations in female and male calves

According to our results, median PSPCs are significantly lower in female calves compared with male calves (491.61 pg/ml and 538.71 pg/ml, respectively); this indicates that there is an influence of sex on SP concentrations. It has already been suggested in a study by TSCHONER et al. (2018) that the sex of an animal may have an influence on PSPCs. Research in experimental animals showed that testosterone regulates SP levels in areas of the brain which also regulate mating behavior in hamsters (SWANN and NEWMAN, 1992). Furthermore, it was demonstrated that anterior pituitaries in adult male rats contained a higher concentration of SP-like immunoreactivity than those of female. As a neonatal castration resulted in a decrease of SP-like immunoreactivity levels in adult males, it can be assumed that testosterone may have an influence on SP (YOSHIKAWA

and HONG, 1983), at least in areas of the brain in rats. It is possible that testosterone may also have an effect on PSPCs in male calves, resulting in significantly higher PSPCs in male compared with female calves in the present study. As we did not include the evaluation of PSPCs in adult bulls, a possible difference in SP concentration between cows and bulls and consequently an influence of testosterone in the sexually mature organism on the SP concentrations could not be investigated.

2.3. Influence of age on substance P concentrations

The age of animals seems to have an influence on PSPCs. According to the present data, PSPCs were significantly higher in adult cows compared with calves, indicating that SP concentrations are higher in older animals. These results are in agreement with DOCKWEILER et al. (2013), who found that SP concentrations during castration were higher in 6-month-old calves compared with 8-week old calves. Animals in the study of DOCKWEILER et al. (2013) were older compared with the calves in the present study (6- months and 8-weeks old, compared with 17.2 ± 2.1 days, respectively), as well as younger than the cows (5.0 ± 1.3 years old). As no animals between the age groups of calves and adult cows were included in the present study, a statement about PSPCs in heifers can't be made. For the present study, sample processing in calves was not performed immediately after sample collection at the Research- and Teaching Center Achselschwang, as was done for cows. Since the laboratory of the Clinic for Ruminants with Ambulatory and Herd Health Services is a one-hour drive away from the sample collection site at Achselschwang, more time passed for the calves` samples until centrifugation was done, compared with the cows` samples. However, both for the samples taken from cows and from calves, the centrifugation of EDTA samples for the determination of SP concentrations was carried out within the recommended time slot of two hours after sample collection (TSCHONER et al., 2018). Therefore, the possibility that the comparatively longer time until the calves` samples were processed resulted in a decay of the SP and therefore lower PSPCs in calves compared with cows seems to be unlikely. This thought is supported by the fact that both the cows` and the calves` sample tubes were spiked with aprotonin to reduce the decay of SP and were continuously kept on ice.

Our results showed no correlation between age and PSPCs in adult cows; therefore, it can be concluded that an age-related difference in PSPC in adult cows is

neglectable. Knowing from previous studies in human medicine that SP plays a role in the immune system (MANTYH et al., 1988; WEN-ZHE et al., 1997), it might be possible that PSPCs are significantly lower in juvenile animals because of a not yet fully developed immune system. However, since it is known that ruminants have a well-developed immune system at birth (CARDOSO et al., 2021) and passive immunity depends on the supply with colostrum (CASTRO et al., 2009), the influence of the development of the immune system on PSPCs in cattle is unclear. Therefore, it cannot be said why higher PSPCs are reached in adult cattle compared to calves.

2.4. Substance P concentrations in the jugular vein and tail vein

The median PSPCs in blood taken from the jugular and the tail vein in cows were 983.78 pg/ml and 818.31 pg/ml, respectively. Our results showed that PSPCs were significanty higher in blood taken from the jugular compared with the tail vein. Taking blood from the jugular vein is associated with measures of restraint and immobilization of the animal. The cow must be caught in a feeding fence and tethered with a halter with the head bent to one side, so that the jugular vein is accessible and a blood statis can be performed before the vein is punctuated (SCHWENDENWEIN, 2014). By choosing the tail vein for blood sample collection, there is a certain risk of infection and of creating a hematoma (SCHWENDENWEIN, 2014). However, an advantage for this injection site is the good accessibility in most types of cattle housing (SEARS et al., 1978). SEARS et al. (1978) demonstrated that tail vein canulation is a practical alternative method to jugular vein canulation. Our study yields new findings because the blood samples to assess PSPCs were taken at the tail vein in addition to the jugular vein. Previous studies (COETZEE et al., 2008; RODRIGUEZ et al., 2018) only described blood sampling at the jugular vein with an intravenous catheter. In the present study, we showed that there was a significantly positive correlation between PSPCs in the jugular vein and in the tail vein. The fact that PSPCs were significantly higher in blood taken from the jugular compared with concentrations in blood from the tail vein clearly support the hypothesis, that the puncture site has an influence on PSPCs - at least if only single blood samples are taken. Since the blood sampling interval between jugular (7:45 a.m.) and tail vein (8:00 a.m.) was within a period of 15 minutes, and higher PSPCs were found in the jugular vein, an increase in PSPCs caused by pain due to the previous punctation of the jugular vein can be excluded.

It is known that the handling of the blood samples, including time until processing of samples, keeping blood samples in an ice bath or at ambient temperature, or the kind of protease inhibitors used (MOSHER et al., 2014) can influence PSPCs. In the present study, care was taken to ensure that all samples were handled in the same manner. All sample tubes were put in an ice bath right after sampling and centrifuged within two hours. Furthermore aprotonin as a protease inhibitor was used, which is described to be the most effective inhibitor of SP degradation, if samples are put on ice until processing (MOSHER et al., 2014). However, the timing of adding the protease inhibitor to the EDTA tubes was different between samples taken from the jugular and tail vain. For blood samples taken from the jugular vein, aprotonin was added to the EDTA tubes before sampling. For blood sampled from the tail vein, aprotonin was added right after blood sampling, as samples were taken with a vacutainer system. It is possible that this delay in time of adding the protease inhibitor to the blood samples from the tail vein could have resulted in the lower PSPCs in TV1.

Another reason for the significantly lower PSPCs in the tail vein could be the mixture of arterial and venous blood. It is possible that the blood sampled from the tail vein not only obtained venous but also arterial blood due to the anatomical structures and the small lumen of the Vena caudalis mediana (resulting in mixed blood). There are no studies describing differences of PSPCs in venous, aterial, or mixed blood. However, BONNET et al. (2016) found that puncture site and mixed levels of blood have significant effects on plasma concentrations of metabolites, ions, hormones and enzymes in turtles. These findings are in contrast with another study, where the metabolic profile in the blood serum taken from two different superficial and well-identified veins in ostriches (jugular and wing vein) were compared and no effect of puncture site was reported (MONIELLO et al., 2005). However, ŠAMANC et al. (2014) observed partly significant differences in concentrations of several variables (concentrations of insulin, glucose, NEFAs, BHB, and urea) measured in blood taken from either the jugular or the mammary vein in dairy cattle. However, the differences in variables are apparently due to their utilization in the mammary gland. Although nothing is yet known about any utilization of SP, one possible explanation for the differences in PSPCs between BJV and TV1 could be the location of the puncture site.

2.5. Substance P concentrations in the tail vein at different time points

It is well known that the secretion of cortisol is subject to diurnal fluctuations with lowest concentrations at night and highest concentrations in the daytime, underlying a circadian rhythm (LEFCOURT et al., 1993). One aim of the present study was to investigate the possible presence of a circadian rhythm in PSPCs in healthy lactating cows over a period of 24 hours. Therefore, blood sample collection was performed every 6 hours within a 24 hours time span (TV1 – TV5). Our results showed an increase in PSPCs with every sampling time with the highest measured median concentration at TV4 (1023.51 pg/ml). Additionally, significant differences in PSPCs at the different time points could be demonstrated. Furthermore, according to our results, the median PSPC at TV1 on D1 and TV5 on D2 of the study differed significantly (818.31 pg/ml and 937.36 pg/ml, respectively).

It is very likely that the increase in PSPCs during the sampling period is due to repeated and invasive puncture of the same vein. The successive puncture of the tail vein might have led to tissue damage and consequently to inflammation at the injection site. It is known from human medicine that SP is released in the course of inflammation, and that SP regulates the chemotaxis of neutrophil and eosinophil granulocytes, as well as the migration of cells to the site of inflammation (HONORE et al., 2000; HARRISON and GEPPETTI, 2001; HUNT and MANTYH, 2001). Therefore, it is possible, that in the present study, PSPC increased due to the local irritation and inflammation of the tail vein, because of the repeated puncture of the vein. It would have been useful to include the assessment of more inflammatory blood parameters e.g. haptoglobin and serum amyloid A (ALSEMGEEST et al., 1994; HORADAGODA et al., 1999) into our study protocol to investigate the presence of a possible state of inflammation. However, for the present study the tail vein was assessed visually for indicators of inflammation. Minor signs of inflamed tissue may not yet have been visible and therefore were not detected.

Furthermore, one must assume that successive sampling is associated with a stressful situation for cows. It is imaginable that the cows already knew to expect stress and possible pain caused by the repeated sampling procedure at the advanced sampling time points. Because of this and since we know from research in laboratory animals that SP is released into the uterine tissue of pregnant mice following the exposition to a stressor (TOMETTEN et al., 2004), it is also possible that the increase of PSPC was due to stress. However, according to TSCHONER et

al. (2020b), PSPCs were not influenced by putting cows in lateral and dorsal recumbency for a laparoscopic abomasopexy, contrary to cortisol concentrations. Cortisol is considered to be the predominant indicator for (pain related) distress (BRISTOW and HOLMES, 2007). To confirm our presumption that successive blood sample collection might be experienced as a stressful situation, it would have been necessary to determine cortisol concentrations in addition to PSPCs. HOPSTER et al. (1999) showed increased cortisol concentrations in successive blood sample collection within 15 to 20 minutes at the jugular vein. This increase was not demonstrated in blood samples within 1 minute after approaching the cows and in cows which were used of being handled and being restrained (HOPSTER et al., 1999).

Since it has been stated that SP might be an appropriate and objective pain indicator in bovine medicine (COETZEE et al., 2008), it is also possible that the increase in PSPCs within the 24 hour sampling period might be due to the experience of pain in cows following the progressive blood sample collection. However, this is in contrast to investigations of MAYER et al. (2020), who found that the amputation of beef calves´ tail ends using elastic rubber rings is not associated with an increase in PSPCs due to pain. LAUDER et al. (2020) could not show an increase in PSPCs during ovariectomy in beef heifers, which is assumed to be a painful intervention. Both studies are consistent with our assumption that pain induced by the current procedure of blood sampling does not reach the pain threshold necessary for SP release. This is supported by the fact that hyperalgesia (increased sensitivity to a noxious stimuli) which has been described in lame cows (O'CALLAGHAN, 2002) is unlikely to have developed within a 24 hours time span in the present study. Taking all this together, it is possible that the combination of inflammation, pain, and the stressful experimental environment might have resulted in the increase of PSPCs over time. In particular, the inflammatory response to repeated puncture of the vein may have been the most responsible influence for the release of SP.

Therefore, according to the findings of the present study, the presence of a time-of-day-dependent PSPC rhythm cannot be confirmed. Further studies are needed to gain more knowledge about the existence of a circadian rhythm of SP. Blood samples should be collected at specific and especially shorter time intervals over a longer period of time as described by LEFCOURT et al. (1993). For the determination of a circadian rhythm in cortisol secretion, blood sampling was

conducted in intervals of 15 minutes over a period of 48 hours (LEFCOURT et al., 1993). It is additionally recommended to use an intravenous catheter to reduce tissue damage and stress by successive punctation of the vein (MAYER et al., 2020). Furthermore, sample collection could be repeated at weekly intervals as done by LEFCOURT et al. (1993) to assess a consistency to the rhythm.

According to the findings of the present study, it can be recommended to use a jugular vein catheter for repeated blood sample collection to exclude the possible influence of inflammation on PSPCs and to avoid a bias of data for research studies.

3. The term reference values

The term reference range is defined by CONSTABLE et al. (2017) as "the range of values of a test that are expected in a group of healthy animals". Furthermore, it is described that "the reference range for a particular test is usually developed by collecting values from a large number of healthy or normal animals and performing a statistical analysis of the values" (CONSTABLE et al., 2017).

To ensure that only healthy animals were included into the statistical analysis of the present study, results of the clinical examination, laboratory findings, and the preliminary medical report of cows and calves were assessed in combination. Since the laboratory parameters of 85 clinically healthy calves (n = 23 calves of group LEUC, n = 24 calves of group RED, n = 38 calves of group PATH) and 23 clinically healthy cows (group PATH) were not within the reference ranges definend by the Clinic for Ruminants with Ambulatory and Herd Health Services, the reference ranges which were used for the present study were expaned as described above. Mild deviations were accepted up to the specified expanded range as long as the remaining inclusion criteria were accomplished. This expansion in reference ranges of common laboratory parameters is a limitation of the present study but is in accordance with BRAUN et al. (2022), who described that deviations of laboratory parameters from the reference ranges occur in 2 – 24% of healthy animals, depending on the parameter. Therefore it can be concluded that a differentiation between healthy and diseased animals proves to be difficult based on laboratory variables (BRAUN et al., 2022). The inclusion of animals in the present study with e.g. increased leucocyte count (within the defined expaned range of 3 – 15 $x10^3/\mu l$) can be substantiated with the fact that results of BRAUN et al. (2022) also showed leucocyte concentrations within the range of 5 – 10 $x10^9/l$ in

only 78% of cases of healthy control cows.

With the aim to establish “reference values” for PSPCs, the animals of the present study were checked to be healthy, but it cannot be excluded that inflammatory processes which were in an early stage at the time of sampling, were present when blood samples were taken. Consequently, limitations to include or exclude animals were defined to try to make sure that samples were only taken from cows and calves which were judged to be healthy according to the preliminary medical report, the clinical examination, and laboratory findings in defined blood parameters as described and discussed above. A possible limitation to the present study is the size of the data set, which might be insufficient to be considered representative. Originally, a data set of 75 cows, 75 female, and 75 male calves was defined by statistical analysis to be sufficient to be able to establish reference ranges of PSPCs in adult cows and calves. Due to the fact that many animals were excluded because of diseases or pathological deviations in laboratory findings, the SP analysis and the statistical analysis had to be conducted with a smaller number of samples (54 cows, 49 male calves, and 52 female calves). Even in this smaller data set, the results of the present study showed higher PSPCs in adult cattle as well as in calves compared with other studies, and high variances between animals and within individual animals, in single samples taken from the jugular vein, as well as in samples taken at different sampling time points at the tail vein. Another limitation is the analysis of the PSPCs, which was only done with one ELISA kit for the present study. To establish reliable reference ranges, it would have been necessary to analyze samples with different analysis methods and various ELISA kits. However, this would have been associated with a high financial outlay, as ELISA kits for the analysis of PSPCs are expensive (TSCHONER and FEIST, 2022).

Taking all this into account, the term reference range should not be used for the present data. It seems more appropriate to speak of an evaluation of PSPCs in healthy cows and calves. Since PSPCs are influenced by analysis method and sample handling (MOSHER et al., 2014), age (DOCKWEILER et al., 2013), temperament of animals (KASIMANICKAM et al., 2018), inflammatory processes (HUNT and MANTYH, 2001), and psychological and physical stressors (VAN ENGEN et al., 2014), it only makes sense to compare baseline concentrations of SP with concentrations after any procedure or onset of disease within a study, where experimental conditions and influencing factors are as equal as possible.

4. Conclusion

According to our results the following conclusions can be made:

- PSPCs taken from the jugular and the tail vein differ significantly. Researchers should be aware that PSPCs are lower in blood taken from the tail compared with the jugular vein.
- Repeated puncture of the tail vein results in an increase of PSPCs, indicating that a local inflammatory event might have an influence on PSPCs. Therefore, it is recommended to take repeated blood samples for research purposes using a jugular vein catheter to minimize the interference of a possible inflammation.
- Different feeding rations do not seem to influence PSPCs in cows, nor do different housing systems in calves.
- Mild deviations in laboratory blood parameters in cows as well as calves are not associated with changes in PSPCs. However, there is a trend for a difference in PSPCs between calves with physiological and pathological laboratory findings. To provide a more accurate statement a larger study population of animals needs to be investigated.
- PSPCs in female calves are significantly lower compared with male calves, indicating that there is an influence of gender on SP concentrations. Further investigations of SP concentrations in adult bulls and a comparison to adult cows should be done to assess if a difference in PSPCs between genders can also be seen in adult cattle.
- PSPCs in cattle are influenced by the animals' age. Significantly higher concentrations of PSPCs were found in adult cows compared with calves. Further basic research is necessary to assess PSPCs in different age groups other than calves and adult cattle.
- The present study provides an evaluation of PSPCs in healthy cows and calves. Because of the high variance in PSPCs and the multifactorial influence on SP concentrations, a determination of baseline PSPCs in addition to SP concentrations for individual experimental animals after any procedure or onset of disease is recommended. Preferably, researchers should evaluate the course of PSPCs, instead of concentrations assessed from a single blood sample.

VI. SUMMARY

Evaluation of plasma substance P concentrations in healthy German Simmental calves and cows

Background: The evaluation and assessment of pain in cattle is difficult as bovines are stoic patients which strongly mask their pain behavior and discomfort. Several subjective and objective parameters for the assessment of pain have been established in bovine medicine. Substance P (SP) is a neurotransmitter of the tachykinin family, which was first described in 2008 and was found to be a sensitive and objective indicator for pain and nociception in cattle. To our knowledge, no evaluation of SP concentrations in healthy adult cows and calves, which were neither exposed to a painful nor to a stressful event, and which were not subject to a disease or inflammation, have been done to this day. Therefore, the objectives of the present study were 1) to evaluate plasma substance P concentrations (PSPCs) in healthy cows and calves of the breed German Simmental, which were not subject to pain, inflammation, or stress, 2) to compare the PSPCs of blood taken from the jugular and the tail vein in cows, 3) to evaluate PSPCs in the tail vein of cows in blood samples taken every 6 hours over a period of 24 hours, 4) to compare PSPCs between male and female calves, and 5) to assess the influence of age on PSPCs in adult cows and calves.

Material & Methods: A total of 54 adult cows (COW, aged 5.0 ± 1.3 years with a number of 3.2 ± 1.3 lactations), 52 female calves (CALF, aged 17.1 ± 2.3 days), and 49 male calves (CALM, aged 17.2 ± 2.1 days) of the breed German Simmental were included in the present study. Blood samples in cows were taken on day 1 of the trial from both the jugular vein (BJV, 7:45 a.m.) and the tail vein (TV1, 8:00 a.m.). Further blood samples were taken from the tail vein at 2:00 p.m. (TV2) and 8:00 p.m. (TV3) on day 1, and 2:00 a.m. (TV4), and 8:00 a.m. (TV5) on day 2 of the trial. Blood samples in calves were taken at 6:00 a.m. from the jugular vein. A clinical examination was performed both in cows at 8:15 a.m. and in calves at 6:15 a.m. following the blood sampling. Subsequently blood samples were centrifuged and refrigerated until the end of the trial. SP concentrations in the blood plasma were analyzed by using a commercial SP Enzyme-Linked Immunosorbent Assay (ELISA) kit. Statistical analysis was conducted using R.

Results: In adult cattle, the median PSPCs in the jugular and the tail vein were 983.78 pg/ml and 818.31 pg/ml, respectively. There were significant differences between PSPCs of the jugular and the tail vein ($p < 0.01$). PSPCs were significantly different in samples taken from the tail vein between TV1 and TV3 ($p < 0.01$), TV1 and TV4 ($p < 0.01$), TV1 and TV5 ($p < 0.01$), TV2 and TV3 ($p < 0.05$), TV2 and TV4 ($p < 0.05$), TV2 and TV5 ($p < 0.01$), and TV3 and TV4 ($p < 0.05$). PSPCs in the tail vein increased within the 24 hours time span with the highest concentration at TV4 (1023.51 pg/ml). There was no correlation between PSPCs in the blood plasma of the jugular vein and the age of adult cattle.

In calves the median PSPC was 525.92 pg/ml. Median PSPCs in female calves were significantly ($p = 0.01$) lower compared with male calves (491.61 pg/ml and 583.71 pg/ml, respectively). Comparing blood samples taken from the jugular vein, PSPCs of calves were significantly ($p < 0.001$) lower compared with adult cattle.

Discussion: The high variability of PSPCs in cows and calves makes comparison of the present results with previous findings difficult. The results of the present study show that for the evaluation of SP concentrations, it is relevant whether blood is taken from the jugular or the tail vein. PSPCs are lower in blood taken from the tail compared with the jugular vein. The increase of PSPCs within the time span of 24 hours is most likely due to inflammatory changes to the tissue of the sampling site and must be considered when interpreting the present study results. When assessing PSPCs for research purposes, it is recommended to use a jugular vein catheter to minimize data bias by inflammatory processes due to repeated puncture of the same vein. Regarding the laboratory findings in blood parameters in both calves and cattle, the measurement of PSPCs should be possible without any negative effects when there are only mild deviations in laboratory findings. The significant difference in PSPCs between male and female calves might be due to the effect of testosterone, as published for rats. The significant differences in PSPCs between cows and calves and the influence of age on PSPCs cannot be explained so far. Further basic research about the influence of different factors such as gender, age, stress, and inflammation on PSPCs is recommended.

VII. ZUSAMMENFASSUNG

Bestimmung von Plasma Substanz P Konzentrationen bei gesunden Fleckviehkühen und -kälbern

Hintergrund: Die Beurteilung von Schmerzen bei Rindern ist schwierig, da Rinder stoische Patienten sind und Anzeichen von Schmerzverhalten und Unbehagen verbergen. In der Rindermedizin haben sich mehrere subjektive und objektive Parameter zur Schmerzbeurteilung etabliert. Substanz P (SP) ist ein Neurotransmitter der Familie der Tachykinine, der 2008 erstmals als sensibler und objektiver Indikator für Schmerzen und Nozizeption bei Rindern beschrieben wurde. Nach derzeitiger Kenntnis wurde bisher keine Untersuchung der SP Konzentrationen bei gesunden Kühen und Kälbern, die weder einem Schmerzreiz noch einer Entzündung oder Stress ausgesetzt waren, durchgeführt. Die Ziele der vorliegenden Studie waren es daher 1.) die Plasma Substanz P Konzentrationen (PSPCs) im Blut der Halsvene bei gesunden, unbehandelten Fleckviehkühen und -kälbern zu untersuchen 2.) die PSPCs im Blut von Hals- und Schwanzvene bei Kühen zu vergleichen 3.) die PSPCs der Schwanzvene alle 6 Stunden über einen Zeitraum von 24 Stunden zu bestimmen 4.) die PSPCs zwischen männlichen und weiblichen Kälbern zu vergleichen und 5.) den Einfluss des Alters zu beurteilen.

Material und Methoden: Die Daten von 54 Fleckviehkühen (COW, 5,0 ± 1,3 Jahre alt, 3,2 ± 1,3 Laktationen), 52 weiblichen Fleckviehkälbern (CALF, 17,1 ± 2,3 Tage alt) und 49 männlichen Fleckviehkälbern (CALM, 17,2 ± 2,1 Tage alt) wurden in der vorliegenden Studie ausgewertet. Bei den Kühen erfolgte die Blutentnahme an Tag 1 des Versuchs um 07:45 Uhr aus der Halsvene (BJV) und um 8:00 Uhr aus der Schwanzvene (TV1). Weitere Blutproben an der Schwanzvene wurden um 14:00 Uhr (TV2) und 20:00 Uhr (TV3) und am Folgetag um 2:00 Uhr (TV4) und um 8:00 Uhr (TV5) entnommen. Bei den Kälbern wurde eine Blutprobe zur Bestimmung des PSPCs Wertes um 6:00 Uhr morgens aus der Jugularvene entnommen. Eine klinische Untersuchung der Kühe und der Kälber wurde jeweils anschließend an die Blutprobenentnahme um 8:15 Uhr bzw. 6:15 Uhr durchgeführt. Die Blutproben wurden zentrifugiert und eingefroren. Die PSPCs Werte wurden mittels eines kommerziellen SP Enzyme-Linked Immunosorbent Assay (ELISA) Kits bestimmt. Die statistische Analyse wurde mit dem Programm R durchgeführt.

Ergebnisse: Bei den Kühen betrugen der Median der PSPCs Werte in der Hals- und Schwanzvene jeweils 983,78 pg/ml bzw. 818,31 pg/ml. Es wurde ein signifikanter Unterschied zwischen den PSPCs Werten im Plasma der unterschiedlichen Venen festgestellt ($p < 0,01$). Es konnten signifikante Unterschiede in den PSPCs zwischen TV1 und TV3 ($p < 0,01$), TV1 und TV4 ($p < 0,01$), TV1 und TV5 ($p < 0,01$), TV2 und TV3 ($p < 0,05$), TV2 und TV4 ($p < 0,05$), TV2 und TV5 ($p < 0,01$) sowie TV3 und TV4 ($p < 0,05$) nachgewiesen werden. Die PSPCs Werte stiegen innerhalb der 24- stündigen Zeitspanne an, mit der höchsten gemessenen Konzentration zum Zeitpunkt TV4 (1023,51 pg/ml). Es konnte keine Korrelation zwischen den PSPCs Werten der Halsvene und dem Alter der Kühe festgestellt werden.

Bei den Kälbern betrug der Median der PSPCs Werte 525,92 pg/ml. Der Median der PSPCs der weiblichen Kälber (491,61 pg/ml) war signifikant ($p = 0,01$) niedriger als jener der männlichen Kälbern (583,71 pg/ml). Vergleicht man die PSPCs Werte von erwachsenen Kühen mit den Werten von Kälbern, so wurden bei erwachsenen Rindern statistisch höhere Werte ($p < 0,001$) erreicht.

Diskussion: Es ist bekannt, dass die Variabilität der PSPCs Werte bei Kühen und Kälbern hoch ist, daher ist es schwierig, die Ergebnisse der vorliegenden Studie mit früheren Erkenntnissen zu vergleichen. Die vorliegenden Ergebnisse zeigen, dass es für die Bewertung der SP-Konzentrationen von Bedeutung ist, ob das Blut aus der Jugularvene oder aus der Schwanzvene entnommen wird. Die PSPCs sind im Blut aus der Schwanzvene niedriger als im Blut aus der Jugularvene. Für Forschungszwecke wird bei wiederholter Blutentnahme die Verwendung eines Venenverweilkatheters empfohlen. Dadurch kann eine Datenverzerrung durch den Einfluss von Entzündungsprozessen, welche durch wiederholte Punktion derselben Vene ausgelöst werden, verhindert werden. Hinsichtlich der Laborergebnisse bei den untersuchten Blutparametern der Kälber zeigen die Ergebnisse, dass die Messung der SP Werte durch leicht abweichende Laborparameter nicht beeinflusst wird. Ein Einfluss des Geschlechtes auf die PSPCs Werte könnte durch das Vorhandensein von Testosteron bei männlichen Kälbern zu erklären sein. Die signifikanten Unterschiede der PSPCs Werte zwischen Kühen und Kälbern und der Einfluss des Alters auf die PSPCs Werte lassen sich bisher nicht erklären. Weitere Grundlagenforschung zum Einfluss von verschiedenen Faktoren wie Geschlecht, Alter, Stress oder Entzündungsgeschehen auf die PSPCs Werte wird empfohlen.

VIII. BIBLIOGRAPHY

Allen K, Coetzee J, Edwards-Callaway L, Glynn H, Dockweiler J, KuKanich B, Lin H, Wang C, Fraccaro E, Jones M. The effect of timing of oral meloxicam administration on physiological responses in calves after cautery dehorning with local anesthesia. Journal of Dairy Science 2013; 96: 5194-5205.

Alsemgeest S, Kalsbeek H, Wensing T, Koeman J, Van Ederen A, Gruys E. Concentrations of serum Amyloid-a (SAA) and haptoglobin (HP) as parameters of inflammatory diseases in cattle. Veterinary Quarterly 1994; 16: 21-23.

Anand KJ, Craig KD. New perspectives on the definition of pain. Pain-Journal of the International Association for the Study of Pain 1996; 67: 3-6.

Anderson DE, Muir WW. Pain management in cattle. Veterinary Clinics: Food Animal Practice 2005a; 21: 623-635.

Anderson DE, Muir WW. Pain management in ruminants. Veterinary Clinics: Food Animal Practice 2005b; 21: 19-31.

Anderson DE, Edmondson MA. Prevention and management of surgical pain in cattle. Veterinary Clinics: Food Animal Practice 2013; 29: 157-184.

Barragan A, Piñeiro J, Schuenemann G, Rajala-Schultz P, Sanders D, Lakritz J, Bas S. Assessment of daily activity patterns and biomarkers of pain, inflammation, and stress in lactating dairy cows diagnosed with clinical metritis. Journal of Dairy Science 2018; 101: 8248-8258.

Barragan AA, Bauman LM, Schuenemann GM, Velez J, Lakritz J, Coetzee JF, Gonzalez JDR, Piñeiro JM, Menichetti B, Bas S. Administration of acetylsalicylic acid after parturition in lactating dairy cows under certified organic management: Part II. Biomarkers of nociception, inflammation, and stress. Journal of Dairy Science 2020; 103: 11713-11722.

Baumgartner W. Allgemeiner klinischer Untersuchungsgang. In: Klinische Propädeutik der Haus- und Heimtiere, 8. edn. Baumgartner W, ed. Stuttgart: Enke Verlag 2014: 43-195.

Berghen P, Hilderson H, Vercruysse J, Dorny P. Evaluation of pepsinogen, gastrin and antibody response in diagnosing ostertagiasis. Veterinary Parasitology 1993; 46: 175-195.

Boissy A, Le Neindre P. Behavioral, Cardiac and Cortisol Responses to Brief Peer Separation and Reunion in Cattle. Physiology & Behavior 1997; 61: 693-699.

Boivin X, Le Neindre P, Garel J, Chupin J. Influence of breed and rearing management on cattle reactions during human handling. Applied Animal Behaviour Science 1994; 39: 115-122.

Bonnet X, El Hassani M, Lecq S, Michel C, El Mouden E, Michaud B, Slimani T. Blood mixtures: impact of puncture site on blood parameters. Journal of Comparative Physiology B 2016; 186: 787-800.

Braun U, Zürcher S, Hässig M. Evaluation of eating and rumination behaviour in 300 cows of three different breeds using a noseband pressure sensor. BMC Veterinary Research 2015; 11: 1-6.

Braun U, Nuss K, Oschlies C, Reif C, Warislohner S, Gerspach C. Häufigkeit und diagnostische Zuverlässigkeit von Laborbefunden bei Kühen mit Reticuloperitonitis traumatica und Labmagengeschwüren. Tierärztliche Praxis Ausgabe G: Großtiere/Nutztiere 2022; 50: 91-100.

Bristow DJ, Holmes DS. Cortisol levels and anxiety-related behaviors in cattle. Physiology & Behavior 2007; 90: 626-628.

Bustamante HA, Rodríguez AR, Herzberg DE, Werner MP. Stress and pain response after oligofructose induced-lameness in dairy heifers. Journal of

Veterinary Science 2015; 16: 405.

Cardoso CL, King A, Chapwanya A, Esposito G. Ante-Natal and Post-Natal Influences on Neonatal Immunity, Growth and Puberty of Calves—A Review. Animals 2021; 11: 1212.

Castro N, Capote J, Morales-Delanuez A, Rodríguez C, Argüello A. Effects of newborn characteristics and length of colostrum feeding period on passive immune transfer in goat kids. Journal of Dairy Science 2009; 92: 1616-1619.

Chang MM, Leeman SE, Niall HD. Amino-acid sequence of substance P. Nature New Biology 1971; 232: 86-87.

Coetzee JF, Lubbers BV, Toerber SE, Gehring R, Thomson DU, White BJ, Apley MD. Plasma concentrations of substance P and cortisol in beef calves after castration or simulated castration. American Journal of Veterinary Research 2008; 69: 751-762.

Coetzee JF, Mosher RA, KuKanich B, Gehring R, Robert B, Reinbold JB, White BJ. Pharmacokinetics and effect of intravenous meloxicam in weaned Holstein calves following scoop dehorning without local anesthesia. BMC Veterinary Research 2012; 8: 1-15.

Constable PD, Hinchcliff KW, Stanley HD, Grünberg W. Clinical Examination and Making a Diagnosis. In: Veterinary Medicine, 11. edn. Constable PD, Hinchcliff KW, Stanley HD, Grünberg W, eds. Elsevier 2017: 1-28.

Costa J, Von Keyserlingk M, Weary D. Invited review: Effects of group housing of dairy calves on behavior, cognition, performance, and health. Journal of Dairy Science 2016; 99: 2453-2467.

Dirksen G, Gründer H-D, Stöber M (1979) Die klinische Untersuchung des Rindes. Verlag Paul Parey, Berlin und Hamburg. 718.

Dirksen G. Krankheiten von Gekröse, Bauchfell und Bauchwand. In: Innere Medizin und Chirurgie des Rindes, 5 edn. Dirksen G, Gründer H-D, Stöber M, eds. Parey 2006: 667-695.

Dockweiler J, Coetzee J, Edwards-Callaway L, Bello N, Glynn H, Allen K, Theurer M, Jones M, Miller K, Bergamasco L. Effect of castration method on neurohormonal and electroencephalographic stress indicators in Holstein calves of different ages. Journal of Dairy Science 2013; 96: 4340-4354.

Duggan A, Hendry I, Morton C, Hutchinson W, Zhao Z. Cutaneous stimuli releasing immunoreactive substance P in the dorsal horn of the cat. Brain Research 1988; 451: 261-273.

Ebner K, Rupniak NM, Saria A, Singewald N. Substance P in the medial amygdala: emotional stress-sensitive release and modulation of anxiety-related behavior in rats. Proceedings of the National Academy of Sciences 2004; 101: 4280-4285.

Ebner K, Muigg P, Singewald G, Singewald N. Substance P in stress and anxiety: NK-1 receptor antagonism interacts with key brain areas of the stress circuitry. Annals of the New York Academy of Sciences 2008; 1144: 61-73.

Ettle T, Obermeier A, Schuster H, Hammerl G (2020) Einfluss einer gestaffelten ruminalen N-Bilanz auf die Futteraufnahme und Milchleistung von Braunvieh- und Fleckviehkühen. 58. Jahrestagung der BAT e.V., Online. 100-105.

Ettle T, Obermeier A, Edelmann G, Hammerl G (2021a) Einfluss einer Wasserzugabe zur Totalen Mischration auf Futteraufnahme und Leistung von Fleck- und Braunviehkühen. 59. Jahrestagung der BAT e.V., Online. 196-201.

Ettle T, Riepl F, Obermaier A, Bauer L, Windisch W (2021b) Effects of supplementation of diets for high-producing dairy cows in mid lactation with rumen protected methionine. Proceedings of the Society of Nutrition Physiology. 63.

Fraser A, Broom D. Describing, recording and measuring behaviour. In: Farm Animal Behaviour and Welfare, 3. edn. Fraser A, Broom D, eds. CAB International 1990: 7-16.

Gaddum J, Schild H. Depressor substances in extracts of intestine. The Journal of Physiology 1934; 83: 1-14.

Geracioti TD, Carpenter LL, Owens MJ, Baker DG, Ekhator NN, Horn PS, Strawn JR, Sanacora G, Kinkead B, Price LH. Elevated cerebrospinal fluid substance p concentrations in posttraumatic stress disorder and major depression. American Journal of Psychiatry 2006; 163: 637-643.

Gleerup KB, Andersen PH, Munksgaard L, Forkman B. Pain evaluation in dairy cattle. Applied Animal Behaviour Science 2015; 171: 25-32.

Guatteo R, Levionnois O, Fournier D, Guemene D, Latouche K, Leterrier C, Mormède P, Prunier A, Serviere J, Terlouw C. Minimising pain in farm animals: the 3S approach-'Suppress, Substitute, Soothe'. Animal 2012; 6: 1261-1274.

Harrison S, Geppetti P. Substance p. The International Journal of Biochemistry & Cell Biology 2001; 33: 555-576.

Heinrich A, Duffield T, Lissemore K, Millman S. The effect of meloxicam on behavior and pain sensitivity of dairy calves following cautery dehorning with a local anesthetic. Journal of Dairy Science 2010; 93: 2450-2457.

Henke J, Erhardt W. Schmerzmanagement bei Klein-und Heimtieren. Henke J, Erhardt W, eds. Stuttgart: Enke Verlag 2001: 135.

Herbut P, Angrecka S, Godyń D, Hoffmann G. The physiological and productivity effects of heat stress in cattle–a review. Annals of Animal Science 2019; 19: 579-593.

Hökfelt T, Kellerth JO, Nilsson G, Pernow B. Substance P: localization in the central nervous system and in some primary sensory neurons. Science 1975; 190: 889-890.

Hökfelt T, Pernow B, Wahren J. Substance P: a pioneer amongst neuropeptides. Journal of Internal Medicine 2001; 249: 27-40.

Holzer P, Holzer-Petsche U. Tachykinins in the gut. Part I. Expression, release and motor function. Pharmacology & Therapeutics 1997a; 73: 173-217.

Holzer P, Holzer-Petsche U. Tachykinins in the gut. Part II. Roles in neural excitation, secretion and inflammation. Pharmacology & Therapeutics 1997b; 73: 219-263.

Honore P, Rogers S, Schwei M, Salak-Johnson J, Luger N, Sabino M, Clohisy DR, Mantyh P. Murine models of inflammatory, neuropathic and cancer pain each generates a unique set of neurochemical changes in the spinal cord and sensory neurons. Neuroscience 2000; 98: 585-598.

Hopster H, van der Werf JT, Erkens JH, Blokhuis HJ. Effects of repeated jugular puncture on plasma cortisol concentrations in loose-housed dairy cows. Journal of Animal Science 1999; 77: 708-714.

Horadagoda N, Knox K, Gibbs H, Reid S, Horadagoda A, Edwards S, Eckersall P. Acute phase proteins in cattle: discrimination between acute and chronic inflammation. Veterinary Record 1999; 144: 437-441.

Hudson C, Whay H, Huxley J. Recognition and management of pain in cattle. In Practice 2008; 30: 126-134.

Hunt SP, Mantyh PW. The molecular dynamics of pain control. Nature Reviews Neuroscience 2001; 2: 83-91.

Huxley J, Whay H. Current attitudes of cattle practitioners to pain and the use of analgesics in cattle. Veterinary Record 2006; 159: 662-668.

Johnson C, Gibson T, Flint P, Wilson P, Mellor D (2008) New techniques for pain recognition: What are the applications, where are the limits? Proceedings of the Australian Animal Welfare Strategy International Conference, Conrad Jupiters, Gold Coast, Queensland, Australia.

Karlen KJ, Baier FS, Odegard SL, Baumann RM, Coetzee JF, Kehoe SI, Vogel KD. Efficacy of oral meloxicam as primary pain mitigation following caustic paste disbudding of three day old Holstein calves. Translational Animal Science 2019.

Kasimanickam R, Hall J, Estill C, Kastelic J, Joseph C, Aziz RA, Nak D. Flunixin meglumine improves pregnancy rate in embryo recipient beef cows with an excitable temperament. Theriogenology 2018; 107: 70-77.

Kasimanickam R, Kasimanickam V, Gold J, Moore D, Kastelic J, Pyrdek D, Ratzburg K. Injectable or transdermal flunixin meglumine improves pregnancy rates in embryo transfer recipient beef cows without altering returns to estrus. Theriogenology 2019; 140: 8-17.

Kirker-Head CA, Chandna V, Agarwal R, Morris EA, Tidwell A, O'Callaghan MW, Rand W, Kumar M. Concentrations of substance P and prostaglandin E2 in synovial fluid of normal and abnormal joints of horses. American Journal of Veterinary Research 2000; 61: 714-718.

Kleinhenz MD, Van Engen NK, Gorden PJ, Coetzee JF. Topical Flunixin Meglumine Effects on Pain Associated Biomarkers after Dehorning. Animal Industry Report 2016; 662: 48.

Kleinhenz MD, Van Engen NK, Smith J, Gorden PJ, Ji J, Wang C, Perkins S, Coetzee JF. The impact of transdermal flunixin meglumine on biomarkers of pain in calves when administered at the time of surgical castration without local anesthesia. Livestock Science 2018; 212: 1-6.

Kovács L, Jurkovich V, Bakony M, Szenci O, Póti P, Tőzsér J. Welfare implication of measuring heart rate and heart rate variability in dairy cattle: literature review and conclusions for future research. Animal 2014; 8: 316-330.

Kovács L, Kézér FL, Ruff F, Jurkovich V, Szenci O. Assessment of heat stress in 7-week old dairy calves with non-invasive physiological parameters in different thermal environments. PLOS ONE 2018; 13: e0200622.

Lauder JK, Marti S, Schwartzkopf-Genswein KS, Jelinski MD, Janzen ED. Measuring behavioral and physiological responses to pain mitigation for ovariectomy in Bos taurus yearling beef heifers. Journal of Animal Science 2020; 98: 1-12.

Lefcourt A, Bitman J, Kahl S, Wood D. Circadian and ultradian rhythms of peripheral cortisol concentrations in lactating dairy cows. Journal of Dairy Science 1993; 76: 2607-2612.

Lembeck F. Zur Frage der zentralen Übertragung afferenter Impulse. Naunyn-Schmiedebergs Archiv für experimentelle Pathologie und Pharmakologie 1953; 219: 197-213.

Lombard J, Garry F, Tomlinson S, Garber L. Impacts of dystocia on health and survival of dairy calves. Journal of Dairy Science 2007; 90: 1751-1760.

Mantyh CR, Gates TS, Zimmerman RP, Welton ML, Passaro EP, Vigna SR, Maggio JE, Kruger L, Mantyh PW. Receptor binding sites for substance P, but not substance K or neuromedin K, are expressed in high concentrations by arterioles, venules, and lymph nodules in surgical specimens obtained from patients with ulcerative colitis and Crohn disease. Proceedings of the National Academy of Sciences 1988; 85: 3235-3239.

Marshall W, Chiu B, Inman RD. Substance P and arthritis: analysis of plasma and synovial fluid levels. Arthritis & Rheumatism: Official Journal of the American College of Rheumatology 1990; 33: 87-90.

Mashaghi A, Marmalidou A, Tehrani M, Grace PM, Pothoulakis C, Dana R. Neuropeptide substance P and the immune response. Cellular and Molecular Life Sciences 2016; 73: 4249-4264.

Mayer C, Zablotski Y, Rieger A, Knubben-Schweizer G, Feist M. Verhaltensassoziierte und physiologische Indikatoren für Schmerz und Unwohlsein bei Kälbern nach Schwanzamputation. Tierärztliche Praxis Ausgabe G: Großtiere/Nutztiere 2020; 48: 328-337.

Mayer M. missRanger: Fast Imputation of Missing Values. R package version 2.1.3. 2021. https://CRAN.R-project.org/package=missRanger. 28.06.2022.

McGuirk SM. Disease management of dairy calves and heifers. Veterinary Clinics of North America: Food Animal Practice 2008; 24: 139-153.

McLennan KM. Why pain is still a welfare issue for farm animals, and how facial expression could be the answer. Agriculture 2018; 8: 127.

Meléndez D, Marti S, Pajor E, Moya D, Heuston C, Gellatly D, Janzen E, Schwartzkopf-Genswein K. Effect of band and knife castration of beef calves on welfare indicators of pain at three relevant industry ages: I. Acute pain. Journal of Animal Science 2017; 95: 4352-4366.

Meléndez D, Marti S, Pajor E, Moya D, Gellatly D, Janzen E, Schwartzkopf-Genswein K. Effect of a single dose of meloxicam prior to band or knife castration in 1-wk-old beef calves: I. Acute pain. Journal of Animal Science 2018; 96: 1268-1280.

Merighi A. Costorage and coexistence of neuropeptides in the mammalian CNS. Progress in Neurobiology 2002; 66: 161-190.

Merskey H. Pain terms: a list with definitions and notes on usage. Pain 1979; 6: 247-252.

Michelgård Å, Appel L, Pissiota A, Frans Ö, Långström B, Bergström M, Fredrikson M. Symptom provocation in specific phobia affects the substance P neurokinin-1 receptor system. Biological Psychiatry 2007; 61: 1002-1006.

Mogil JS. Animal models of pain: progress and challenges. Nature Reviews Neuroscience 2009; 10: 283-294.

Molony V, Kent J. Assessment of acute pain in farm animals using behavioral and physiological measurements. Journal of Animal Science 1997; 75: 266-272.

Moniello G, Bovera F, Solinas IL, Piccolo G, Pinna W, Nizza A. Effect of age and blood collection site on the metabolic profile of ostriches. South African Journal of Animal Science 2005; 35: 268-272.

Mosher RA, Coetzee JF, Allen PS, Havel JA, Griffith GR, Wang C. Effects of sample handling methods on substance P concentrations and immunoreactivity in bovine blood samples. American Journal of Veterinary Research 2014; 75: 109-116.

Muir WW, Woolf CJ. Mechanisms of pain and their therapeutic implications. Journal of the American Veterinary Medical Association 2001; 219: 1346-1356.

O'Callaghan K. Lamneness and associated pain in cattle-challenging traditional perceptions. In Practice 2002; 24: 212-219.

O'Connor TM, O'Connell J, O'Brien DI, Bennett MW, Goode T, Burke L, Bredin CP, Shanahan F. Upregulation of neurokinin-1 receptor expression in the lungs of patients with sarcoidosis. Journal of Clinical Immunology 2003; 23: 425-435.

Ogino M, Matsuura A, Yamazaki A, Irimajiri M, Suzuki Y, Kushibiki S, Singu H, Kasuya E, Hasegawa Y, Hodate K. Plasma cortisol and prolactin secretion rhythms in cattle under varying external environments and management techniques. Animal Science Journal 2014; 85: 58-68.

Oku R, Satoh M, Takagi H. Release of substance P from the spinal dorsal horn is enhanced in polyarthritic rats. Neuroscience Letters 1987; 74: 315-319.

Ollerenshaw S, Jarvis D, Sullivan C, Woolcock A. Substance P immunoreactive nerves in airways from asthmatics and nonasthmatics. European Respiratory Journal 1991; 4: 673-682.

Olson M, Ralston B, Burwash L, Matheson-Bird H, Allan ND. Efficacy of oral meloxicam suspension for prevention of pain and inflammation following band and surgical castration in calves. BMC Veterinary Research 2016; 12: 1-11.

Park SJ, Piao M, Kim H, Kang HJ, Seo J, Lee S, Baik M. Effects of castration and a lidocaine-plus-flunixin treatment on growth and indicators of pain, inflammation, and liver function in Korean cattle bull calves. Livestock Science 2018; 216: 61-66.

Pearson JM, Pajor EA, Campbell JR, Caulkett NA, Levy M, Dorin C, Windeyer MC. Clinical impacts of administering a nonsteroidal anti-inflammatory drug to beef calves after assisted calving on pain and inflammation, passive immunity, health, and growth. Journal of Animal Science 2019; 97: 1996-2008.

Pernow B. Studies on substance P: Purification, occurrence and biological actions. Acta Physiologica Scandinavia 1953; 29: 1-90.

Polidoro G, Giancola F, Fracassi F, Pietra M, Bettini G, Asti M, Chiocchetti R. Substance P and the neurokinin-1 receptor expression in dog ileum with and without inflammation. Research in Veterinary Science 2017; 114: 297-307.

Powell D, Leemann S, Tregear GW, Nial HD, Potts JT. Radioimmunoassay for substance P. Nature New Biology 1973; 241: 252-254.

Prunier A, Mounier L, Le Neindre P, Leterrier C, Mormède P, Paulmier V, Prunet P, Terlouw C, Guatteo R. Identifying and monitoring pain in farm animals: a

review. Animal 2012; 7: 998.

Rääf A, Olsen SR. The effect of meloxicam treatment after disbudding on pain-related behaviours and weight gain in Danish Holstein calves. A comparative study between one day and four days of treatment. Master`s Thesis 2017; University of Copenhagen.

Remnant JG, Tremlett A, Huxley JN, Hudson CD. Clinician attitudes to pain and use of analgesia in cattle: where are we 10 years on? Veterinary Record 2017; 181: 400-400.

Repenning P, Ahola J, Callan R, French J, Giles R, Bigler B, Coetzee J, Wulf L, Peel R, Whittier J. Impact of oral meloxicam administration before and after band castration on feedlot performance and behavioral response in weanling beef bulls. Journal of Animal Science 2013; 91: 4965-4974.

Ribeiro-da-Silva A, Hökfelt T. Neuroanatomical localisation of substance P in the CNS and sensory neurons. Neuropeptides 2000; 34: 256-271.

Riepl F, Windisch W, Obermaier A, Hovenjürgen M, Ettle T (2021) Einfluss einer Ergänzung der Milchviehration mit pansengeschütztem Methionin und Lysin bei abgesenkter RNB auf Milchleistung und Futteraufnahme. 132 VDLUFA-Kongress.

Rodriguez AR, Herzberg DE, Werner MP, Müller HY, Bustamante HA. Plasma concentration of norepinephrine, β-endorphin, and substance P in lame dairy cows. Journal of Veterinary Research 2018; 62: 193-197.

Šamanc H, Kirovski D, Lakić N, Celeska I, Bojković-Kovačević S, Sladojević Ž, Ivanov I. A comparison of the concentrations of energy-balance-related variables in jugular and mammary vein blood of dairy cows with different milk yield. Acta Veterinaria Hungarica 2014; 62: 52-63.

Schaible H-G, Jarrott B, Hope P, Duggan A. Release of immunoreactive substance

P in the spinal cord during development of acute arthritis in the knee joint of the cat: a study with antibody microprobes. Brain Research 1990; 529: 214-223.

Schwendenwein I. Untersuchung des Blutes. In: Klinische Propädeutik der Haus- und Heimtiere, 8. edn. Baumgartner W, ed. Stuttgart: Enke Verlag 2014: 432-461.

Scott I, Stear M, McKellar Q. Comparison of four methods for the determination of plasma pepsinogen concentration. Research in Veterinary Science 1995; 59: 234-237.

Sears P, Paape M, Pearson R, Gwazdauskas F. Comparison between tail vein and jugular vein cannulation in cattle. Journal of Dairy Science 1978; 61: 974-979.

Sickinger M, Leiser R, Failing K, Doll K. Evaluation of differences between breeds for substance P, vasoactive intestinal polypeptide, and neurofilament 200 in the abomasal wall of cattle. American Journal of Veterinary Research 2008; 69: 1247-1253.

Sickinger M, Roth J, Failing K, Wehrend A. Serum Levels of Neuropeptides in Cows with Left Abomasal Displacement. Veterinary Sciences 2018a; 5: 103.

Sickinger M, Roth J, Failing K, Wehrend A. Serum neuropeptide concentrations in cows with intrapartum uterine torsion. Animal Reproduction Science 2018b; 196: 193-196.

Snijdelaar DG, Dirksen R, Slappendel R, Crul BJ. Substance P. European Journal of Pain 2000; 4: 121-135.

Stilwell G, Lima MS, Broom DM. Effects of nonsteroidal anti-inflammatory drugs on long-term pain in calves castrated by use of an external clamping technique following epidural anesthesia. American Journal of Veterinary Research 2008a; 69: 744-750.

Stilwell G, De Carvalho C, Lima M, Broom D. The effect of duration of manual restraint during blood sampling on plasma cortisol levels in calves. Animal Welfare 2008b; 17: 383-385.

Stock ML, Millman ST, Barth LA, Van Engen NK, Hsu WH, Wang C, Gehring R, Parsons RL, Coetzee JF. The effects of firocoxib on cautery disbudding pain and stress responses in preweaned dairy calves. Journal of Dairy Science 2015; 98: 6058-6069.

Stojkov J, Von Keyserlingk M, Marchant-Forde J, Weary D. Assessment of visceral pain associated with metritis in dairy cows. Journal of Dairy Science 2015; 98: 5352-5361.

Sutherland M, Lowe G, Huddart F, Waas J, Stewart M. Measurement of dairy calf behavior prior to onset of clinical disease and in response to disbudding using automated calf feeders and accelerometers. Journal of Dairy Science 2018; 101: 8208-8216.

Swann JM, Newman SW. Testosterone regulates substance P within neurons of the medial nucleus of the amygdala, the bed nucleus of the stria terminalis and the medial preoptic area of the male golden hamster. Brain Research 1992; 590: 18-28.

Theurer M, Anderson D, White B, Miesner M, Mosier D, Coetzee J, Lakritz J, Amrine D. Effect of Mannheimia haemolytica pneumonia on behavior and physiologic responses of calves during high ambient environmental temperatures. Journal of Animal Science 2013; 91: 3917-3929.

Tometten M, Klapp BF, Joachim R, Fest S, Zenclussen AC, Peters EM, Hertwig K, Arck PC. Nerve growth factor and its functional receptor TrkA are up-regulated in murine decidual tissue of stress-triggered and substance P-mediated abortion. American Journal of Reproductive Immunology 2004; 51: 86-93.

Tregear GW, Nial HD, Potts JT, Leemann SE, Chang MM. Synthesis of substance P. Nature New Biology 1971; 232: 87-89.

Tschoner T, Behrendt-Wippermann M, Rieger A, Metzner M, Knubben-Schweizer G, Reichmann F, Feist M. Course of plasma substance P concentrations during umbilical surgery in calves. Berliner und Münchener Tierärztliche Wochenschrift 2018: 522-528.

Tschoner T, Sauter-Louis C, Peinhofer V, Feist M. Attitudes of Bavarian bovine veterinarians towards pain and pain management in cattle. Veterinary Record 2020a; 187: 90.

Tschoner T, Zablotski Y, Knubben-Schweizer G, Feist M. Effect of xylazine administration before laparoscopic abomasopexy to correct left displaced abomasum on markers of stress in dairy cows. Journal of Dairy Science 2020b; 103: 9318-9331.

Tschoner T. Methods for Pain Assessment in Calves and Their Use for the Evaluation of Pain during Different Procedures—A Review. Animals 2021; 11: 1235.

Tschoner T, Feist M. Substance P concentrations in the blood plasma and serum of adult cattle and calves during different painful procedures and conditions–a systematic review. BMC Veterinary Research 2022; 18: 1-21.

Tyler JW, Hancock DD, Thorne JG, Gay CC, Gay JM. Partitioning the mortality risk associated with inadequate passive transfer of colostral immunoglobulins in dairy calves. Journal of Veterinary Internal Medicine 1999; 13: 335-337.

Van Engen NK, Stock ML, Engelken T, Vann R, Wulf LW, Karriker LA, Busby W, Lakritz J, Carpenter A, Bradford B. Impact of oral meloxicam on circulating physiological biomarkers of stress and inflammation in beef steers after long-distance transportation. Journal of Animal Science 2014; 92: 498-510.

Von Euler U, Gaddum J. An unidentified depressor substance in certain tissue extracts. The Journal of Physiology 1931; 72: 74-87.

Weary DM, Niel L, Flower FC, Fraser D. Identifying and preventing pain in animals. Applied Animal Behaviour Science 2006; 100: 64-76.

Wen-Zhe H, Jian-Ping L, Xian-Hua Z, Uvaydova M, Douglas SD. Human monocytes and macrophages express substance P and neurokinin-1 receptor. The Journal of Immunology 1997; 159: 5654-5660.

Whitlock B, Coffman E, Coetzee J, Daniel J. Electroejaculation increased vocalization and plasma concentrations of cortisol and progesterone, but not substance P, in beef bulls. Theriogenology 2012; 78: 737-746.

Williams ACC, Craig KD. Updating the definition of pain. Pain 2016; 157: 2420-2423.

Wong SS, Sun NN, Keith I, Kweon C-B, Foster DE, Schauer JJ, Witten ML. Tachykinin substance P signaling involved in diesel exhaust-induced bronchopulmonary neurogenic inflammation in rats. Archives of Toxicology 2003; 77: 638-650.

Wong SS, Sun NN, Lantz RC, Witten ML. Substance P and neutral endopeptidase in development of acute respiratory distress syndrome following fire smoke inhalation. American Journal of Physiology-Lung Cellular and Molecular Physiology 2004; 287: L859-L866.

Woolf CJ. Pain: moving from symptom control toward mechanism-specific pharmacologic management. Annals of Internal Medicine 2004; 140: 441-451.

Yoshikawa K, Hong J-S. Sex-related difference in substance P level in rat anterior pituatary: a model of neonatal imprinting by testosterone. Brain Research 1983; 273: 362-365.

IX. APPENDIX

1. List of figures

2. List of tables

3. Supplementals

Supplemental 1: General findings (age in years, number of lactations, days post-partum, days pregnant, and milk yield), findings of clinical examination according to DIRKSEN et al. (1979), and group assignment in 54 individual adult cows sampled for the evaluation of substance P concentrations in healthy German Simmental cattle. Cows were assigned to one of two feeding groups, group 1 (n = 19, 35.2% of cows) or group 3 (n = 35, 64.8% of cows). The experimental number (Nr.) which was assigned to the cows for the trial is given in the first column.

Nr. Cow	Age in years	Number of lactations	Days post-partum (d)	Days pregnant (d)	Milk yield (kg)	Temperature (°C)	Heart Rate (beats/minute)	Respiratory Rate (breaths/minute)	Rumen Cycle (in 2 minutes)	Feeding group
1	6.4	4	186	84	34.1	38.2	84	36	2	3
2	4.5	3	141	53	37.3	38.2	84	24	3	3
3	3.7	3	178	54	34.4	38.3	84	28	2	3
4	6.1	4	192	89	31.4	38.5	84	36	3	1
5	5.5	4	132	45	45.6	38.0	80	28	3	3
6	3.8	2	153	43	39.1	38.1	76	24	2	3
8	4	2	239	166	35.1	38.1	84	32	3	1
9	4.9	3	191	119	31.8	38.3	80	32	2	1
10	3.7	2	157	43	36.8	38.6	80	32	3	1
11	6.1	4	238	166	31.9	38.0	76	36	2	1
14	6.9	5	188	74	41.2	38.1	72	36	3	3
15	4.9	3	205	102	42.4	38.0	84	32	3	3
16	4.9	3	200	76	41	38.2	80	32	2	3
17	4.8	3	194	50	48.5	38.0	80	28	3	3
18	3.9	2	186	43	40	38.0	76	28	3	3

Continuing Supplemental 1:

Nr. Cow	Age in years	Number of lactations	Days post-partum (d)	Days pregnant (d)	Milk yield (kg)	Temperature (°C)	Heart Rate (beats/minute)	Respiratory Rate (breaths/minute)	Rumen Cycle (in 2 minutes)	Feeding group
19	4.1	2	189	105	36.6	38.3	84	24	2	3
20	5.5	4	129	51	42.9	38.2	80	20	3	3
21	5.5	4	184	60	45.4	38.1	84	32	2	3
24	6.2	4	210	75	31.9	38.0	80	40	3	1
25	7.8	6	159	47	39.4	38.1	88	44	2	3
28	5.3	3	180	40	45.6	38.2	84	40	3	3
29	4.9	3	137	63	51.6	38.3	72	44	2	1
30	3.5	2	141	46	37.5	38.9	88	44	3	3
32	7.6	6	126	48	36.5	39.0	80	40	3	1
34	5	3	120	39	38.4	38.9	84	48	2	1
35	4.9	3	216	74	38	39.1	80	40	3	3
36	4.5	2	233	40	40.5	39.0	88	48	3	1
38	3.6	2	177	48	33.3	38.2	84	48	3	3
41	3.9	2	202	126	35.1	38.3	80	40	3	3
42	4.7	3	168	52	39.3	38.5	68	40	3	3
43	5.8	4	142	68	41.1	38.0	88	44	3	3
44	3.6	2	177	91	34	38.7	80	32	2	3
45	9.1	7	233	68	37.5	38.0	92	28	2	3
46	3.4	2	117	46	40.8	38.7	88	32	2	1
47	5.6	4	164	47	32.7	38.6	80	24	2	1

Continuing Supplemental 1:

Nr. Cow	Age in years	Number of lactations	Days post-partum (d)	Days pregnant (d)	Milk yield (kg)	Temperature (°C)	Heart Rate (beats/minute)	Respiratory Rate (breaths/minute)	Rumen Cycle (in 2 minutes)	Feeding group
48	5.3	3	149	41	38.6	38.5	80	36	2	1
49	3.6	2	133	60	37.5	38.2	80	32	2	1
51	3.9	2	128	58	33.2	38.6	80	32	3	3
52	4.8	3	173	50	35.1	38.3	76	32	2	3
54	5.9	4	197	52	36.1	38.8	96	24	2	3
55	5.2	3	205	41	29.5	38.4	80	28	1	3
56	4.8	3	119	43	34.4	38.8	76	28	2	3
57	5.7	4	170	46	31.7	38.5	80	32	1	1
59	5	3	228	54	31.7	38.3	80	32	3	3
60	3.8	2	186	47	35.2	38.1	100	24	1	3
61	5.1	3	241	50	29.3	39.4	68	36	2	3
62	4.9	3	216	99	32.5	38.2	84	40	1	3
63	5.5	4	156	65	38	38.5	76	40	2	3
64	4	2	230	47	29.6	38.1	80	36	1	3
66	9.1	7	143	62	32.6	38.0	76	36	2	1
72	3.7	2	163	73	45.7	38.5	88	36	2	1
73	3.6	2	150	42	39	38.5	96	44	1	3
74	3.9	2	227	77	38.4	38.6	88	32	0	1
76	3.8	2	169	97	38.3	38.6	86	24	1	1

Supplemental 2: Study protocol for the analysis of substance P concentrations in healthy German Simmental cows. The study protocol included the documentation of the general animal data and the results of the clinical examination.

Anlage 7.2:
Aufzeichnungsmuster zur Tierversuchsanzeige
„Erstellen von Referenzwerten für Substanz P bei Fleckviehkühen“

Datum: Ohrmarkennummer Versuchsdurchführende Person:	□ Kuh Herkunft des Tieres: Versuchstiernummer:

Geboren am:

Laktation/Anzahl Kälber:

letzte Kalbung:

Probleme bei Kalbung:

Besamt am:

Laktationstag:

Gewicht:

Vorangegangene Erkrankungen und Behandlungen abfragen (ggf. Datum und Präparat):

Tag	Zeit	Probe		Notiz
1	07:45	Basalwert (Hals)	- Blutprobe ziehen	
	08:00		- Blutprobe ziehen (Schwanzvene)	
	08:15	1(SZ)	- Klinische Untersuchung	
	14:00	2(SZ)	- Blutprobe ziehen (Schwanzvene)	
	20:00	3(SZ)	- Blutprobe ziehen (Schwanzvene)	
2	02:00	4(SZ)	- Blutprobe ziehen (Schwanzvene)	
	08:00	5(SZ)	- Blutprobe ziehen (Schwanzvene)	

Continuing Supplemental 2:

Fortführung Anlage 7.2:
Aufzeichnungsmuster zur Tierversuchsanzeige
„Erstellen von Referenzwerten für Substanz P bei Fleckviehkühen“

Protokoll Klinische Untersuchung:

Allgemeinverhalten:

Haltung:

Futteraufnahme:

Temperatur:

Lymphknoten:

Herzfrequenz (Frequenz, Intensität, Rhythmus, abgesetzt, Nebengeräusche)**:**

Atemfrequenz (Typ, pathologische Geräusche)**:**

Pansenauskultation (Frequenz, Intensität, Füllung, Schichtung)**:**

Schwing-, Perkussionsauskultation:

Darmperistaltik, Bauchdecke:

Kot (Menge, Farbe, Konsistenz, Beimengungen)**:**

Harn (Ketonkörper)**:**

Supplemental 3: General findings (age in days (d), birth weight in kilogram (kg), and liters (l) of colostrum intake), housing, and findings of clinical examination according to DIRKSEN et al. (1979) in 101 individual calves (n = 49 for male (CALM) and n = 52 for female calves (CALF)) sampled for the evaluation of substance P concentrations in healthy German Simmental calves. Data for birth weight is missing in n = 2 (2.0%) of total calves. Information about the amount and if a calf has received colostrum is missing in n = 11 (10.9%) of total calves. Calves were either housed in individual (n = 21, 20.8% of calves) or group (n = 80, 79.2% of calves) igloos. Number of animals in group housing is given in brackets. The experimental number (Nr.) which was assigned to the calves is given in the first column.

Nr. CALM	Age (d)	Birth Weight (kg)	Colostrum intake (l)	Housing	Temperature (°C)	Heart Rate (beats/minute)	Respiratory Rate (breaths/minute)	Auscultatory Findings	Consistency of Faeces	Umbilical Palpation
1	14	58	–	group (7)	39.3	120	36	mild	physiologic	physiologic
3	19	44	3.5	group (4)	39.2	149	49	mild	physiologic	physiologic
4	19	47	4	group (8)	39.2	140	40	mild	physiologic	physiologic
5	17	43	3	group (7)	39.2	108	44	mild	physiologic	physiologic
6	15	52	3	group (7)	39.2	120	48	mild	loose	physiologic
7	17	41	2.5	group (7)	38.1	160	40	mild	physiologic	physiologic
8	18	39	3	group (7)	39.3	120	36	mild	physiologic	physiologic
9	18	37	3	group (7)	39.1	120	36	mild	physiologic	physiologic
11	16	46	3	group (5)	39.1	116	36	physiologic	physiologic	physiologic
13	16	44	1.5	group (9)	38.7	108	36	mild	physiologic	physiologic
14	17	50	2	group (9)	39.0	160	40	mild	physiologic	physiologic
15	16	49	1.5	group (5)	38.7	120	36	physiologic	physiologic	physiologic
16	16	36	–	group (2)	39.4	160	44	physiologic	physiologic	physiologic
18	18	47.5	2.5	single	39.3	120	36	physiologic	physiologic	physiologic
23	14	45	3	group (8)	39.1	140	40	mild	physiologic	physiologic
24	16	45	2.5	group (5)	39.1	140	40	physiologic	physiologic	physiologic

Continuing Supplemental 3:

Nr. CALM	Age (d)	Birth Weight (kg)	Colostrum intake (l)	Housing	Temperature (°C)	Heart Rate (beats/minute)	Respiratory Rate (breaths/minute)	Auscultatory Findings	Consistency of Faeces	Umbilical Palpation
26	15	41	2	single	39.1	140	36	physiologic	physiologic	physiologic
28	16	41	2	single	39.5	160	48	mild	physiologic	physiologic
29	19	–	2	group (7)	39.2	160	48	physiologic	physiologic	physiologic
31	19	41.2	–	group (5)	39.4	148	48	physiologic	physiologic	physiologic
32	18	53	3	group (5)	38.7	152	44	physiologic	physiologic	physiologic
33	17	47	3	group (5)	39.2	160	44	physiologic	physiologic	physiologic
34	20	32	2	group (6)	38.7	128	40	physiologic	physiologic	physiologic
35	16	37	–	group (3)	38.8	116	44	mild - moderate	physiologic	physiologic
36	14	42	2	single	38.8	168	48	mild - moderate	loose	physiologic
37	17	47	–	group (3)	39.0	120	44	mild - moderate	physiologic	physiologic
38	17	41	2	group (3)	38.9	160	48	mild	thickly	physiologic
39	15	43	2	group (3)	39.1	164	48	mild - moderate	physiologic	physiologic
41	16	48	2	group (6)	38.3	160	44	physiologic	physiologic	physiologic
42	21	50	–	single	37.9	160	44	mild	physiologic	physiologic
45	14	44	2	group (3)	38.9	128	28	mild	thickly	physiologic
47	21	48	0.5	group (6)	38.9	160	44	physiologic	physiologic	physiologic
50	14	54	2	single	38.0	152	44	physiologic	physiologic	physiologic
51	20	46	3	group (8)	39.2	152	40	physiologic	physiologic	physiologic
54	21	42	7	single	38.5	124	40	physiologic	physiologic	physiologic
55	16	44	3	group (8)	38.4	148	40	mild	physiologic	physiologic

Continuing Supplemental 3:

Nr. CALM	Age (d)	Birth Weight (kg)	Colostrum intake (l)	Housing	Temperature (°C)	Heart Rate (beats/minute)	Respiratory Rate (breaths/minute)	Auscultatory Findings	Consistency of Faeces	Umbilical Palpation
56	18	46	2.5	group (6)	39.2	140	44	moderate	physiologic	physiologic
57	18	49	3.5	group (8)	39.3	116	36	moderate	physiologic	physiologic
58	16	41	2	group (8)	39.4	120	40	physiologic	physiologic	physiologic
60	21	42	4	group (6)	38.9	124	24	mild	physiologic	physiologic
61	21	50	3.5	group (6)	38.9	124	20	physiologic	physiologic	physiologic
63	16	40	2	group (3)	39.3	136	32	physiologic	physiologic	Umbilical Hernia
64	18	50	3	group (3)	39.3	144	40	physiologic	physiologic	physiologic
65	19	42	3	group (6)	38.9	160	40	moderate	physiologic	physiologic
67	16	45	3.5	group (3)	39.2	120	28	physiologic	physiologic	physiologic
70	19	43	3	group (6)	38,8	120	32	physiologic	physiologic	physiologic
71	16	49	0,5	group (8)	38,9	120	28	physiologic	physiologic	physiologic
72	19	41	2,5	single	38,8	120	32	physiologic	loose	physiologic
73	14	45	3	group (3)	39,2	124	32	physiologic	physiologic	Umbilical Hernia
Nr. CALF	**Age (d)**	**Birth Weight (kg)**	**Colostrum intake (l)**	**Housing**	**Temperature (°C)**	**Heart Rate (beats/minute)**	**Respiratory Rate (breaths/minute)**	**Auscultatory Findings**	**Consistency of Faeces**	**Umbilical Palpation**
1	15	28	2	single	38.7	100	24	physiologic	physiologic	physiologic
4	17	45	2.5	group (7)	39.5	120	44	mild	physiologic	Umbilical Hernia
6	18	51	–	group (8)	39.3	120	40	physiologic	loose	physiologic
9	14	37	2	group (8)	39.1	116	44	mild	loose	physiologic

Continuing Supplemental 3:

Nr. CALF	Age (d)	Birth Weight (kg)	Colostrum intake (l)	Housing	Temperature (°C)	Heart Rate (beats/minute)	Respiratory Rate (breaths/minute)	Auscultatory Findings	Consistency of Faeces	Umbilical Palpation
10	19	36	3	group (4)	38.8	120	36	physiologic	physiologic	physiologic
11	18	41	2.5	group (4)	39.4	160	36	mild - moderate	loose	physiologic
12	17	42	3	group (4)	39.2	116	28	physiologic	physiologic	physiologic
13	14	–	3	single	39.4	120	36	mild	physiologic	physiologic
14	17	44	3	group (7)	39.1	160	44	mild	physiologic	Umbilical Hernia
15	16	43	3	group (7)	39.3	160	44	physiologic	physiologic	Umbilical Hernia
16	21	51	2.5	group (4)	39.0	140	40	mild	loose	physiologic
18	19	42	2.5	group (5)	38.7	120	32	physiologic	physiologic	physiologic
19	19	34	3	group (5)	39.4	160	40	physiologic	physiologic	physiologic
21	19	43	1.9	group (8)	39.0	120	40	mild	thinly	Umbilical Hernia
23	16	34	3	single	38.1	160	40	mild	physiologic	physiologic
25	16	49	3	group (9)	39.1	140	40	mild	physiologic	physiologic
26	15	33	1.5	group (9)	38.9	108	36	mild	physiologic	physiologic
27	15	44	3	single	39.2	160	40	physiologic	physiologic	Umbilical Hernia
28	14	44	2.5	group (9)	39.2	160	40	mild	physiologic	physiologic
30	14	45	0.5	single	38.8	160	32	physiologic	thickly	physiologic
31	14	50	1.5	group (8)	38.2	160	44	mild	loose	physiologic
32	19	50	–	group (8)	39.1	140	40	mild	loose	physiologic
37	15	38	3	group (7)	39.1	120	36	physiologic	loose	physiologic
40	15	38	–	group (8)	39.4	140	40	mild	physiologic	physiologic

Continuing Supplemental 3:

Nr. CALF	Age (d)	Birth Weight (kg)	Colostrum intake (l)	Housing	Temperature (°C)	Heart Rate (beats/minute)	Respiratory Rate (breaths/minute)	Auscultatory Findings	Consistency of Faeces	Umbilical Palpation
45	20	40	2	group (6)	38.7	140	44	physiologic	physiologic	physiologic
46	14	43	2	group (7)	38.9	160	48	physiologic	physiologic	physiologic
47	18	44	2	group (4)	38.1	160	44	physiologic	physiologic	physiologic
48	17	44	3	group (6)	39.0	160	36	mild	physiologic	physiologic
49	17	43	2	group (6)	39.4	160	36	physiologic	physiologic	physiologic
52	21	30	2.5	group (6)	38.7	160	44	physiologic	physiologic	physiologic
53	20	30	2	group (6)	39.3	156	40	physiologic	physiologic	physiologic
54	19	32	2	group (6)	39.2	160	44	mild	physiologic	physiologic
57	20	30	2	single	38.6	148	32	moderate	thickly	physiologic
61	17	38	3	single	38.8	160	40	physiologic	physiologic	physiologic
62	15	36	1.5	single	38.4	160	40	physiologic	physiologic	physiologic
63	14	48	–	single	39.2	140	32	mild	physiologic	physiologic
64	21	50	2	single	38.9	120	24	physiologic	thickly	physiologic
66	20	40	2	single	39.2	176	32	mild	physiologic	physiologic
67	20	45	2	single	38.9	160	40	mild	physiologic	physiologic
68	17	41	3	group (6)	38.8	120	40	physiologic	physiologic	physiologic
70	15	38	1.5	single	38.6	104	44	physiologic	loose	Umbilical Hernia
72	21	42	2.5	group (6)	38.6	124	40	physiologic	physiologic	physiologic
75	16	43	1.5	group (3)	38.7	100	32	physiologic	physiologic	physiologic
77	19	42	3.5	group (6)	39.0	120	40	physiologic	physiologic	physiologic

Continuing Supplemental 3:

Nr. CALF	Age (d)	Birth Weight (kg)	Colostrum intake (l)	Housing	Temperature (°C)	Heart Rate (beats/minute)	Respiratory Rate (breaths/minute)	Auscultatory Findings	Consistency of Faeces	Umbilical Palpation
78	19	34	1	group (6)	39.2	120	36	physiologic	physiologic	physiologic
80	16	41	2	group (8)	39.5	120	40	mild	physiologic	physiologic
81	16	39	2.5	group (3)	39.2	140	28	physiologic	physiologic	Umbilical Hernia
82	19	54	4	group (4)	39.2	144	44	moderate	physiologic	physiologic
83	17	44	1	group (4)	38.8	140	36	mild	physiologic	physiologic
84	14	54	2	group (8)	38.8	124	28	physiologic	physiologic	physiologic
85	15	44	4	group (3)	39.2	120	28	physiologic	physiologic	physiologic
88	14	39	–	group (3)	38.4	140	32	physiologic	physiologic	physiologic

Supplemental 4: Study protocol for the analysis of substance P concentrations in healthy German Simmental calves. The study protocol included documentation of the general animal data and the results of the clinical examination.

Anlage 7.1:
Aufzeichnungsmuster zur Tierversuchsanzeige
„Erstellen von Referenzwerten für Substanz P bei Fleckviehkälbern“

Datum: Ohrmarkennummer Versuchsdurchführende Person:	□ Kalb Geschlecht: Herkunft des Tieres: Versuchstiernummer:

Geboren am:

Zughilfe, Probleme bei der Kalbung?

Kolostrumaufnahme:

Fütterung:

Gewicht:

Vorangegangene Erkrankungen und Behandlungen abfragen (ggf. Datum und Präparat):

Impfungen:

Tag	Zeit	Probe		Notiz
1	06:00	Basalwert (Hals)	- Blutprobe ziehen	
	06:15		- Klinische Untersuchung	

Continuing Supplemental 4:

Fortführung Anlage 7.1:
Aufzeichnungsmuster zur Tierversuchsanzeige
„Erstellen von Referenzwerten für Substanz P bei Fleckviehkälbern“

Protokoll Klinische Untersuchung:

Allgemeinverhalten:

Haltung:

Saugreflex:

Temperatur:

Lymphknoten:

Herzfrequenz (Frequenz, Intensität, Rhythmus, abgesetzt, Nebengeräusche)**:**

Atemfrequenz (Typ, pathologische Geräusche)**:**

Schwing-, Perkussionsauskultation:

Darmperistaltik, Bauchdecke:

Kot (Menge, Farbe, Konsistenz, Beimengungen)**:**

Supplemental 5: Plasma substance P concentrations (PSPCs) in pg/ml in the blood taken from the Vena jugularis externa (jugular vein, BVJ) and Vena caudalis mediana (tail vein, TV1 – TV5) of 54 individual adult cows of the German Simmental breed. Blood samples were taken at the interval of 6 hours over the course of 24 hours (8:00 a.m. (TV1), 2:00 p.m. (TV2), 8:00 p.m. (TV3), 2:00 a.m. (TV4) and 8:00 a.m. (TV5). Missing values are presented as na (not applicable). Samples were missing in 3.7% (n = 2) of cows for TV2, in 7.4% (n = 4) of cows for TV3, and in 9.3% (n = 5) of cows for TV4 and 1.9% (n = 5) of cows for TV5, respectively. The experimental number (Nr.) which was assigned to the cows is given in the first column.

Nr. Cow	BJV	TV1	TV2	TV3	TV4	TV5
1	1313.72	986.82	1250.19	167.78	1406.74	1239.07
2	762.16	753.87	612.24	810.98	529.24	694.01
3	1285.62	850.28	815.55	928.12	851.28	841.66
4	1000.83	791.70	777.89	910.71	921.77	na
5	807.39	686.29	775.81	na	na	na
6	822.53	712.21	636.49	167.78	na	762.35
7	863.76	830.89	799.03	1117.30	774.92	826.70
8	810.20	835.11	853.96	594.84	na	961.56
9	886.64	1150.54	1013.85	1057.94	1001.67	883.20
10	653.79	636.17	591.08	999.20	1135.86	457.73
11	1770.43	1798.15	1540.96	1543.52	1411.48	2244.76
12	618.17	635.42	713.66	728.97	748.09	465.26
13	1883.12	2009.85	2511.62	2292.84	2973.53	3359.53
14	958.23	985.08	1049.53	na	1440.29	1280.48
15	866.28	753.90	945.20	1122.96	1314.43	1219.41
16	1476.57	1390.16	na	na	1694.94	1497.31
17	782.90	655.34	738.42	1314.43	1472.47	1201.00
18	822.24	779.60	953.86	945.20	1162.48	1125.72
19	842.02	792.91	789.55	877.80	1238.23	860.59
20	2337.25	1083.71	1295.84	1227.09	1364.55	na
21	2201.71	2531.00	2197.66	2277.71	2378.63	2094.69
22	960.87	995.17	1037.33	1452.43	1175.03	na
23	865.47	779.59	713.94	900.11	1019.92	983.86
24	1005.72	725.89	660.38	867.26	842.84	849.30
25	1001.05	856.07	947.77	559.10	940.31	962.64
26	699.87	515.75	579.92	780.67	1074.47	811.51
27	757.22	736.50	803.82	692.85	808.48	928.78
28	1060.56	952.84	1252.19	1305.87	1301.57	1334.02
29	2092.05	765.73	969.83	769.37	1653.73	1248.10
30	1072.58	940.07	756.71	980.33	1052.39	1148.55
31	855.57	805.73	762.11	960.13	937.47	1672.67
32	2018.64	1560.13	1429.13	1297.05	839.36	1812.53
33	1079.64	797.11	819.39	793.58	860.28	843.88

Continuing Supplemental 5:

Nr. Cow	BJV	TV1	TV2	TV3	TV4	TV5
34	1180.33	978.67	1151.69	1354.44	1174.76	1352.45
35	697.49	714.19	660.43	852.98	1023.21	659.28
36	1303.34	1174.20	1243.83	1430.55	1345.20	1485.81
37	1215.62	1157.68	1085.18	1242.64	1407.37	1185.94
38	973.77	409.19	890.30	564.14	793.16	948.16
39	1166.90	1417.35	1180.29	1204.74	1274.24	1230.59
40	1191.53	849.20	1390.57	1400.42	na	952.43
41	999.95	764.34	na	1111.78	1023.81	866.59
42	738.73	303.49	2195.97	419.04	414.85	648.44
43	1075.10	1039.20	888.74	1274.24	980.45	858.39
44	958.89	192.34	621.24	720.04	480.62	538.84
45	1271.44	1085.77	1026.87	1713.36	1986.10	1384.60
46	600.52	582.19	879.74	500.31	1025.89	167.78
47	1986.10	1384.60	600.52	582.19	879.74	500.31
48	508.19	610.20	600.02	483.97	973.45	566.37
49	1051.22	890.24	921.45	991.94	1448.74	771.49
50	727.18	833.24	686.82	na	1080.52	935.61
51	501.95	723.96	496.87	743.31	811.35	760.42
52	993.80	594.94	791.21	969.40	830.53	937.36
53	998.49	838.96	839.35	896.93	870.41	923.51
54	761.46	650.97	739.93	570.76	na	na

Supplemental 6: Laboratory findings of selected blood parameters in 54 individual adult cows sampled for the evaluation of substance P concentrations in healthy German Simmental cattle. Parameters assessed were leucocyte count, packed cell volume (PCV), hemoglobin concentration (Hb), total protein (TP), glutaraldehyde test, beta-hydroxybutyrate (BHBA), non-esterified fatty acids (NEFA) and glutathione peroxidase (GSHPX). Defined reference ranges are given below each parameter. Clinically healthy cows were either assigned to group PHYS (n = 30, 55.6% of cows) or to group PATH (n = 24, 44.4% of cows) according to their laboratory findings. The experimental number (Nr.) which was assigned to the cows is given in the first column.

Nr. Cow	Leukocytes (4-10 x10³/µl)	Hb (10-13 g/dl)	PCV (30-36%)	GSHPX (>250)	TP (40-80 g/l)	NEFA (< 0,57 mmol/l)	BHBA (< 1,2 mmol/l)	Glutaraldehyde Test (>15 Min)
1	6.82	11.40	35.32	600	77.10	0.15	0.56	14
2	6.23	8.60	28.94	611	75.20	0.10	0.55	16
3	10.29	10.00	31.42	443	76.20	0.12	0.62	16
4	5.21	10.00	32.96	482	75.30	0.10	0.59	16
5	5.81	10.30	32.86	559	83.60	0.11	0.57	14
6	8.99	10.90	34.95	622	71.90	0.10	0.66	16
8	7.08	10.10	32.32	696	74.50	0.09	0.51	16
9	6.52	12.20	36.77	557	70.00	0.10	0.84	16
10	5.68	9.00	27.93	598	72.30	0.10	0.83	16
11	6.75	10.00	30.54	647	71.80	0.12	0.54	16
14	5.92	10.90	35.61	423	81.00	0.09	0.69	16
15	6.03	10.60	33.36	547	74.90	0.10	0.42	16
16	6.51	9.70	30.24	510	68.00	0.10	0.42	16
17	6.21	10.00	33.53	664	75.40	0.16	0.64	16
18	5.70	9.60	31.25	821	71.30	0.30	0.46	16

Continuing Supplemental 6:

Nr. Cow	Leukocytes (4-10 x10³/µl)	Hb (10-13 g/dl)	PCV (30-36%)	GSHPX (>250)	TP (40-80 g/l)	NEFA (< 0,57 mmol/l)	BHBA (< 1,2 mmol/l)	Glutaraldehyde Test (>15 Min)
19	7.51	10.60	33.11	696	73.50	0.15	0.47	16
20	6.71	10.00	33.59	367	78.30	0.11	0.36	16
21	5.78	11.40	35.53	477	72.50	0.15	0.61	16
24	7.82	11.30	37.16	530	67.30	0.10	0.87	16
25	4.00	10.50	36.02	496	75.80	0.15	0.83	16
28	6.7	8.4	27.85	587	72.80	0.08	0.78	16
29	4.49	8.30	27.52	719	73.10	0.06	0.98	16
30	7.95	9.90	30.19	623	71.10	0.14	0.70	16
32	6.13	9.60	28.96	478	78.80	0.11	0.77	16
34	6.90	9.50	29.57	514	78.80	0.12	0.78	16
35	8.65	10.20	30.71	555	71.40	0.13	0.75	16
36	4.84	9.90	30.33	667	76.40	0.15	0.73	16
38	8.19	12.50	36.16	684	80.80	0.15	0.35	16
41	5.52	12.00	35.76	598	67.50	0.11	0.34	16
42	10.25	11.50	35.45	651	73.50	0.14	0.40	16
43	7.31	10.80	33.61	512	77.70	0.10	0.52	16
44	5.39	10.10	30.12	573	77.60	0.07	0.67	16
45	8.36	11.20	34.93	661	74.30	0.10	0.78	16
46	7.52	8.70	26.53	559	67.00	0.07	0.62	16
47	3.96	9.40	29.10	521	67.10	0.03	0.84	16

Continuing Supplemental 6:

Nr. Cow	Leukocytes (4-10 x10³/µl)	Hb (10-13 g/dl)	PCV (30-36%)	GSHPX (>250)	TP (40-80 g/l)	NEFA (< 0,57 mmol/l)	BHBA (< 1,2 mmol/l)	Glutaraldehyde Test (>15 Min)
48	7.90	9.40	29.72	526	73.30	0.09	0.57	16
49	8.75	10.00	30.15	766	78.90	0.07	0.46	16
51	6.71	10.80	33.53	559	74.80	0.06	0.82	16
52	9.87	10.10	31.75	602	77.20	0.07	0.53	16
54	12.71	12.00	36.78	643	79.80	0.10	0.56	16
55	7.56	10.80	34.20	766	76.30	0.14	0.60	16
56	4.50	10.60	34.40	747	75.50	0.10	0.55	16
57	9.96	12.00	37.02	703	82.30	0.11	0.43	16
59	7.60	13.60	41.61	571	75.50	0.10	0.82	16
60	7.50	9.50	29.65	614	79.60	0.13	0.79	16
61	6.56	11.40	35.57	781	73.70	0.14	0.45	16
62	6.26	10.60	33.91	828	72.30	0.17	0.47	16
63	5.38	10.60	32.06	802	73.90	0.15	0.85	16
64	8.46	8.90	27.78	813	76.40	0.11	0.60	16
66	6.67	10.80	31.94	501	81.00	0.10	0.51	16
72	7.38	9.5	32.58	641	73.8	0.12	0.41	16
73	6.97	9.3	29.96	799	75	0.1	0.59	16
74	7.71	10.3	32.37	283	71.8	0.08	0.89	16
76	6.35	9.2	29.81	590	68.5	0.07	0.45	16

Supplemental 7: Plasma substance P concentrations (PSPCs) in pg/ml in the blood taken from the Vena jugularis externa (jugular vein), and laboratory findings of selected blood parameters in 101 individual calves (n = 49 for male (CALM) and n = 52 for female calves (CALF)) sampled for the evaluation of substance P concentrations in healthy German Simmental calves. Parameters assessed were leucocyte count, packed cell volume (PCV), hemoglobin concentration (Hb), total protein (TP) and glutathione peroxidase (GSHPX). Defined reference ranges are given below each parameter. Clinically healthy calves were either assigned to group PHYS (n = 16, 15.8% of calves), group LEUC (n = 23, 22.8% of calves), group RED (n = 24, 23.8% of calves), or group PATH (n = 38, 37.6% of calves) according to their laboratory findings. The experimental number (Nr.) which was assigned to the calves is given in the first column.

Nr. CALM	PSPCs (pg/ml)	Leukocytes (4-10 x10³/µl)	Hb (10-13 g/dl)	PCV (30-36%)	GSHPX (>250)	TP (40-80 g/l)
1	451.40	12.20	11.70	35.66	591	57.80
3	301.56	5.28	10.90	34.12	436	60.80
4	319.11	9.75	12.20	38.55	490	65.20
5	421.94	9.33	11.30	35.54	464	56.70
6	436.41	7.88	10.10	31.58	571	55.80
7	423.03	9.20	10.80	35.54	438	52.50
8	417.95	6.96	11.20	36.14	578	55.60
9	394.80	5.28	10.40	33.26	525	52.60
11	489.91	12.60	11.90	37.75	613	50.60
13	366.52	6.38	12.40	35.49	534	50.40
14	456.00	7.18	11.50	36.26	411	58.80
15	415.55	14.29	11.20	34.02	706	64.20
16	558.39	11.39	11.20	36.34	426	52.00
18	453.23	14.20	11.60	36.26	330	48.80
23	422.06	10.21	10.70	35.82	406	47.50
24	500.76	9.68	10.70	34.62	580	62.40
26	3869.37	7.98	12.10	38.64	491	55.50
28	524.69	13.02	13.90	39.70	570	59.20
29	586.01	10.86	14.20	40.81	532	56.50
31	573.11	12.56	11.00	32.31	527	47.80
32	932.45	13.88	12.80	36.28	412	54.90
33	743.07	10.99	11.20	33.44	451	60.50
34	778.58	7.09	9.90	29.61	456	52.50
35	676.42	8.80	12.30	36.25	401	62.60
36	550.13	10.28	10.50	32.33	580	60.70
37	742.34	7.82	9.60	28.54	583	63.20
38	651.77	14.89	12.10	34.46	377	61.00
39	1129.07	14.62	10.40	32.04	678	54.10
41	1057.04	10.95	11.40	35.46	705	59.80

Continuing Supplemental 7:

Nr. CALM	PSPCs (pg/ml)	Leukocytes (4-10 x10³/µl)	Hb (10-13 g/dl)	PCV (30-36%)	GSHPX (>250)	TP (40-80 g/l)
42	509.64	10.10	13.30	36.75	602	56.10
45	796.83	5.51	10.90	32.23	624	56.90
47	455.21	8.19	11.90	34.77	656	49.30
50	974.16	13.52	12.90	39.74	619	57.20
51	1085.49	7.57	11.90	36.94	572	71.40
54	583.71	7.68	12.00	38.98	666	57.80
55	525.15	5.38	13.10	39.95	618	56.80
56	549.16	12.16	11.90	37.03	616	59.30
57	451.40	9.18	11.80	37.57	715	57.00
58	301.56	11.70	12.20	36.73	617	60.10
60	319.11	11.87	12.80	39.27	717	59.60
61	421.94	10.84	12.60	38.35	749	62.00
63	436.41	11.02	12.5	38.55	669	56.40
64	423.03	12.39	11.80	37.07	576	58.10
65	417.95	13.10	12.40	40.61	799	55.20
67	394.80	9.15	12.30	39.36	823	51.20
70	489.91	11.57	11.30	35.55	638	71.80
71	366.52	13.65	11.00	35.99	905	54.80
72	456.00	9.40	10.00	32.20	715	60.90
73	415.55	13.03	10.00	32.32	995	56.20
Nr. CALF	**PSPCs (pg/ml)**	**Leukocytes (4-10 x10³/µl)**	**Hb (10-13 g/dl)**	**PCV (30-36%)**	**GSHPX (>250)**	**TP (40-80 g/l)**
1	508.03	9.48	12.00	36.51	367	49.50
4	366.37	11.91	11.00	34.04	650	57.00
6	245.52	12.27	12.20	37.73	571	53.30
9	272.31	12.39	12.3	37.64	401	61.80
10	386.10	8.95	9.4	30.42	425	58.40
11	236.10	3.20	10.9	35.07	432	63.70
12	486.21	11.87	12.9	38.71	372	67.10
13	499.22	12.27	12.9	38.21	340	62.80
14	475.02	13.44	10.4	31.49	582	58.00
15	385.20	9.88	11.2	34.75	618	50.60
16	526.94	8.48	11.7	35.36	474	50.60
18	271.19	7.46	11.2	35.55	602	66.40
19	273.09	12.59	9.9	33.23	615	56.90
21	359.67	10.45	10.7	34.87	529	53.90
23	471.90	9.82	11.3	35.17	668	58.60
25	461.57	9.90	12.0	36.32	370	55.20
26	288.64	5.42	11.8	36.52	450	47.70
27	475.02	8.99	11.9	38.06	430	52.60

Continuing Supplemental 7:

Nr. CALF	PSPCs (pg/ml)	Leukocytes (4-10 x10³/µl)	Hb (10-13 g/dl)	PCV (30-36%)	GSHPX (>250)	TP (40-80 g/l)
28	667.08	11.73	10.6	33.9	482	60.10
30	432.94	7.33	11.8	37.30	507	47.60
31	839.09	9.73	12.8	39.72	491	59.40
32	357.55	11.43	11.3	35.09	297	62.20
37	407.35	6.14	9.5	29.78	458	55.20
40	469.95	3.92	10.5	36.68	408	53.20
45	229.27	12.39	10.8	31.41	642	57.40
46	499.12	13.45	11.7	34.07	572	56.60
47	540.42	8.83	10.1	29.13	519	53.50
48	488.81	6.36	10.1	30.39	498	54.20
49	354.67	12.44	12.6	34.87	602	69.40
52	787.18	4.20	10.5	31.59	549	54.50
53	515.52	5.64	9.0	27.85	527	54.50
54	494.41	5.81	11.4	31.96	396	61.30
57	536.56	9.27	11.9	36.44	448	55.20
61	236.71	5.97	12.3	37.69	586	65.70
62	604.60	8.59	9.9	30.47	643	57.10
63	330.67	9.15	11.1	33.58	870	61.00
64	639.51	10.19	12.4	39.70	667	54.10
66	755.13	9.64	11.5	34.46	473	55.80
67	528.95	10.33	11.3	36.13	542	55.60
68	486.96	12.63	14.0	39.97	694	65.70
70	408.90	14.67	12.3	37.31	637	52.50
72	754.00	10.85	11.5	35.54	828	56.8
75	591.35	3.34	12.2	38.31	772	55.70
77	704.17	13.28	12.5	38.05	753	56.80
78	876.38	8.68	12.4	37.23	658	60.00
80	650.48	9.43	12.6	38.88	640	57.30
81	945.51	11.04	12.30	38.13	677	56.90
82	1026.68	8.35	13.0	40.81	767	58.80
83	789.16	9.09	11.5	38.24	701	57.30
84	1068.62	13.65	11.0	35.99	984	53.90
85	1262.70	13.03	10.0	34.70	882	64.10
88	1170.66	10.84	12.3	41.35	533	55.50

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