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Non-invasive welfare evaluations in horses:
The usefulness of laterality

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"Es gibt immer einen Grund für jede Bewegung, Regung und Handlung des Pferdes. Nur, wer sich Zeit und Mühe nimmt, gut und überlegt zu beobachten, wird im Umgang mit Pferden Erfolg verbuchen."
Jean-Claude Dysli (1935 – 2013)

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ABBREVIATIONS

ACTH	adrenocorticotropic hormone
ADI	average dominance index
ALI	affiliative laterality index
AVP	arginine vasopressin
BAS	behavioural activation system
BIS	behavioural inhibition system
CNS	central nervous system
CRH	corticotropin releasing hormone
FGM	faecal glucocorticoid metabolite
GLM	generalized linear model
GLMM	generalized linear mixed model
GM	glucocorticoid metabolite
HPA	hypothalamic – pituitary – adrenal
IgA	immunoglobulin A
LI	laterality index
ML	motor laterality
SAM	sympathetic – adrenal – medullary
SI	social index
SL	sensory laterality

SUMMARY

Animal welfare is becoming increasingly important, especially for animal owners. In particular, prey animals such as horses (*Equus caballus*) generally suffer in silence. To improve the evaluation of animal welfare in practice and to simplify it in scientific research, new welfare indicators are needed that are easier to assess, less time consuming, and repeatable. Therefore, this thesis aimed to investigate laterality as a welfare indicator that could meet these requirements. Domestic horses were used as model organisms as they display sensory and motor laterality on individual and/or population level; their sensory organs are placed laterally, non-invasive stress hormone analysis is already well-established, as a highly social animal that evolved on open ranges it is prone to suffering from inappropriate human management regimes, and it displays various stress responses.

STUDY 1 investigated whether laterality changes in line with well-established stress parameters. It could be demonstrated that sensory laterality and motor laterality measured while grazing (grazing stance), shifted to the left when natural needs (free movement, social contact) were restricted by a change from group to individual housing. The shift to an enhanced preference for the right brain hemisphere was in line with increased stress hormone concentrations in faeces. Sensory laterality changed immediately after the change of housing conditions, whereas the motor laterality changed with a time delay of one week. This study demonstrated that laterality is a promising welfare indicator that changed in stressful situations.

STUDY 2 investigated whether laterality is an indicator of horses' cognitive bias. Good animal welfare is not only defined as absence of indicators of poor welfare (stress indicators), but also by the presence of positive indicators. Therefore, cognitive bias is a welfare indicator as well. Motor laterality measured as initial forelimb use correlated with the cognitive bias. Right-sided horses were faster to approach an ambiguous stimulus and therefore displayed a positive cognitive bias that was linked to the left brain hemisphere. But neither motor laterality, measured through grazing stance, nor sensory laterality were related to cognitive bias. This study demonstrated that cerebral lateralization is linked to cognitive bias in horses, but further research is needed to improve and standardize the measurement of motor laterality.

STUDY 3 investigated whether sensory laterality is linked to affiliative interactions. Other studies have already demonstrated a link between agonistic interactions and a preference for the left side (right brain hemisphere) in horses. This study demonstrated that a preference for left side sensory organ use is not only evident in negative contexts but also in positive contexts, because the horses also preferred their left side during affiliative interactions that are assumed to induce positive emotions. This study demonstrated that not only the direction of shift in laterality, but also the context of the shift, should be recorded to reliably identify poor or good welfare. Therefore, it is recommended that additional stress parameters be applied to reliably evaluate animal welfare.

STUDY 4 investigated whether the sampling and analysis of faecal stress hormones and immunoglobulin A could be simplified by applying a novel conservation method. Often it is not possible to immediately freeze the faecal samples and/or the transportation to the lab is lengthy. The study demonstrated that faecal samples can be dried a closed system such as an air-tight tube containing silica gel. The samples were dried within 24 hours, as fast as in controlled air-drying conditions at room temperature. The new and simpler drying method prevented the stress hormones (glucocorticoid metabolites) from enzymatic degradation and conserved them, demonstrated by the fact that the detectable concentration remained unchanged. In contrast, immunoglobulin A showed a reduction in the detectable concentration. Therefore, if possible, the conservation of faecal samples should be

avoided when immunoglobulin A is to be analysed, although it would be possible to apply an extrapolation to attain fairly reliable results. This new drying method will simplify research on wild horses into the type of stressors they are confronted with, the impact of natural stressors, and effect of stressors on, for example, their laterality in comparison with domestic horses.

STUDY 5 investigated whether the strength of laterality provides insight into basal physiological and immunological status, stress response, stress reactivity, or cognitive bias. Only a correlation between age and the strength of laterality was found, with strength of laterality increasing with age. However, age could explain only 30 per cent of the inter-individual variation in strength of laterality. The results demonstrated that the strength of laterality is not a reliable indicator of animal welfare. The direction of laterality may be of greater importance.

Altogether, it was demonstrated that laterality is a promising, reliable, repeatable, and objective indictor of animal welfare, which is quick and easy to asses, and inexpensive. Like other well-established welfare and stress indicators, laterality has its limitations. Therefore, it is recommended that other welfare indicator should be simultaneously assessed and changes in laterality recorded, as different traits and personalities result in a high inter-individual variation in base laterality indices. Possible influences and correlations between emotional processing and cerebral lateralization are discussed. Nonetheless, further research is needed to establish a more reliable measurement of motor laterality, and to better understand the relationship between emotional processing and lateralization, as well as possible influencing factors.

ZUSAMMENFASSUNG

Das Tierwohl rückt für Tierbesitzer immer mehr in den Mittelpunkt. Insbesondere Fluchttiere wie Pferde (*Equus caballus*) können mit lediglich subtilen äußere Anzeichen nahezu in Stille leiden. Daher sind Tierwohlindikatoren notwendig, die einfach in ihrer Bestimmung sind, wenig Zeit benötigen und wiederholbar sind, um die Bestimmung des Tierwohls in der Praxis zu verbessern und in der Wissenschaft zu vereinfachen. Deshalb war es das Ziel dieser Arbeit, die Lateralität als potentiellen Tierwohlindikator, der diese Anforderungen erfüllen soll, zu untersuchen. Domestizierte Pferde dienten dabei als Model-Organismen, denn sie zeigen sensorische und motorische Lateralität auf individueller und Populationsebene. Ihre sensorischen Organe sind lateral positioniert, non-invasive Stresshormonanalysen sind bereits etabliert und als sehr soziales Tier, das sich im offenen Grasland entwickelte und spezialisierte, leiden Pferde nicht selten unter den nicht angemessenen Haltungsbedingungen, die ihnen der Mensch bietet. Dies zeigt sich in unterschiedlichen Formen von Stressanzeichen und -verhalten.

STUDY 1 untersuchte, ob sich die Lateralität zeitgleich mit etablierten Stressparametern verändert. Nach Veränderung der Haltungsbedingungen von Gruppenhaltung zur Boxenhaltung, die einige natürliche Bedürfnisse wie Bewegung und Sozialkontakte einschränkt, zeigte sich eine Linksverschiebung in sensorischer und motorischer Lateralität (gemessen als Weideschrittpräferenz). Diese Verschiebung zu einer stärkeren Präferenz der rechten Gehirnhemisphäre geschah im Zusammenhang mit erhöhten fäkalen Stresshormonkonzentrationen. Dabei kam es direkt nach der Veränderung der Haltungsbedingungen zu einer Linksverschiebung in der sensorischen Lateralität, während die Linksverschiebung in der motorischen Lateralität erst nach einer Woche auftrat. Diese Studie zeigte, dass die Lateralität ein vielversprechender Tierwohlindikator ist, der sich in Stresssituationen zur linken Seite hin verschiebt.

STUDY 2 untersuchte, ob die Lateralität einen Hinweis auf den Cognitive Bias gibt. Ein gutes Tierwohl wird nicht nur definiert als ein Nicht-Vorhandensein von Stressindikatoren, sondern auch als ein Vorhandensein von Anzeichen für ein gutes Tierwohl. Daher dient auch der Cognitive Bias als Tierwohlindikator, der jedoch zeitaufwändig in der Bestimmung ist. Die motorische Lateralität, gemessen als bevorzugtes Antrittsbein, korrelierte mit den Cognitive Bias. Pferde, die mit dem rechten Vorderbein bevorzugt aus stehender Position starteten, näherten sich schneller einem neutralen Stimulus und zeigten damit einen positiven Cognitive Bias, der demzufolge in Verbindung mit der linken Gehirnhemisphäre steht. Aber es gab keinen Zusammenhang zwischen der motorischen Lateralität, gemessen als Weideschrittpräferenz, oder der sensorischen Lateralität und dem Cognitive Bias von Pferden. Nichtsdestotrotz zeigt diese Studie, dass es einen Zusammenhang zwischen zerebraler Lateralisierung und dem Cognitive Bias bei Pferden gibt. Dennoch sind zur Verbesserung und Standardisierung der Messungen von motorischer Lateralität weitere Studien notwendig.

STUDY 3 untersuchte, ob es einen Zusammenhang zwischen sensorischer Lateralität und affiliativen Interaktionen gibt. Andere Studien haben bereits gezeigt, dass es einen Link zwischen agonistischen Interaktionen und der Verwendung der linken Körperseite (rechte Hemisphäre) gibt. Nun konnte gezeigt werden, dass die linken sensorischen Organe nicht nur im negativen Kontext agonistischer Interaktionen, sondern auch im positiven Kontext affiliativer Interaktionen bevorzugt wurden. Diese Ergebnisse zeigten, dass nicht nur die Richtung der Lateralitätsverschiebung, sondern auch der Kontext, in dem eine Lateralitätsverschiebung auftrat, von Bedeutung ist, um zuverlässig das Tierwohl beurteilen zu können. Daher wird empfohlen, neben der Lateralität, weitere Parameter zur Beurteilung des Tierwohls heranzuziehen.

STUDY 4 untersuchte eine neue Konservierungsmethode die das Sammeln von Kotproben zur Stresshormon- und Immunglobulin A - Analyse vereinfachen soll. Nach der Kotprobenahme ist es oft

nicht möglich diese für die weitere Analyse unmittelbar einzufrieren. Zudem ist der Transport von gefrorenen Proben aufwändig. Die Studie zeigte, dass Kotproben alternativ in einem geschlossenen System (Zentrifugenröhrchen) mit Silikagel getrocknet werden können. Diese Methode sorgte für eine ebenso schnelle Trocknung innerhalb von 24 Stunden wie unter kontrollierten Bedingungen bei Raumtemperatur. Die neue und einfache Trocknungsmethode schützte Stresshormone (Glucocorticoid Metabolite) vor dem enzymatischen Abbau und konservierte diese, da die nachweisbare Konzentration stabil blieb. Während der Trocknung zeigte hingegen Immunglobulin A eine Reduktion ihrer nachweisbaren Konzentration. Daher wird empfohlen, wenn möglich, auf die Trocknung von Kotproben zu verzichten, wenn Immunglobulin A analysiert werden soll. Ist dies nicht möglich, können die Daten extrapoliert werden, um eine annähernde Konzentration der Probe vor der Trocknung zu berechnen. Die neue Trocknungsmethode kann die Stressforschung bei Wildpferden vereinfachen und bietet somit neue Forschungsmöglichkeiten. So kann zum Beispiel detaillierter untersucht werden, welchen Stressoren Wildpferde ausgesetzt sind, deren Einfluss auf die Pferde und dessen Lateralität. Der Vergleich zu domestizierten Pferden in menschlichen Haltungssystemen könnte weiter Aufschlüsse hinsichtlich Stressbelastung und –bewältigung geben.

In STUDY 5 wurde untersucht, ob die Stärke der Lateralität Aufschluss über den physiologischen und immunologischen Statuts eines Organismus, dessen Stressantworten, Stress Reaktivität oder dem Cognitive Bias gibt. Es zeigte sich nur ein Zusammenhang zwischen Alter der Pferde und der Stärke der Lateralität. Mit zunehmendem Alter nahm die Stärke der Lateralität zu, wobei das Alter nur 30 Prozent der interindividuellen Variationen erklärte. Die Ergebnisse verdeutlichten, dass die Stärke der Lateralität kein zuverlässiger Tierwohlindikator ist. Die Richtung der Lateralität scheint in diesem Zusammenhang von größerer Bedeutung zu sein.

Zusammenfassend konnte gezeigt werden, dass die Lateralität ein vielversprechender, zuverlässiger, wiederholbarer und objektiver Tierwohlindikator sein kann, der mit weniger Zeitaufwand, kostengünstig und einfach zu beurteilen ist. Vergleichbar mit bereits etablierten Stressparametern hat auch die Lateralität als Tierwohlindikator ihre Grenzen. Daher wird empfohlen, zeitgleich andere Tierwohlindikatoren, deren Veränderungen, und die Veränderungen der Lateralität zu erheben. Denn unterschiedliche Eigenschaften und Charakterzüge verursachen eine hohe inter-individuelle Variation in den Basal Lateralitätsindizes. Es werden mögliche Einflüsse und Beziehungen zwischen emotionaler Informationsverarbeitung und zerebraler Lateralisierung diskutiert. Dennoch sind weitere Untersuchungen notwendig um zuverlässigere und standardisierte Messmethoden, insbesondere der motorischen Lateralität, zu etablieren und um den Zusammenhang zwischen emotionaler Informationsverarbeitung und Lateralität, sowie anderen möglichen Faktoren, zu verstehen.

GENERAL INTRODUCTION

Laterality

Laterality may prove to be a useful behavioural welfare indicator because cerebral lateralization is common, fundamental, and a characteristic of not only *Vertebrata* but also *Invertebrata*. Laterality is linked to other welfare indicators such as stress hormones, the immune system, and mental health (cognitive bias) and may be a quick, objective, and repeatable welfare indicator. This will be investigated in the current thesis.

A lateralized brain may have been associated with an evolutionary advantage and an advantage in natural selection. It enables the individual to perform two tasks simultaneously, such as searching for food and detecting predators, and it also may improve memory storage. Therefore, it is assumed that laterality evolved initially on an individual level and afterwards in some species on a population level (Rogers and Vallortigara, 2019). In *Vertebrata* and *Invertebrata* short-term memory formation appears to be controlled by the right hemisphere, whereas long-term memory storage is the responsibility of the left brain hemisphere (Frasnelli et al., 2014; Guo et al., 2016; Robins and Phillips, 2010). Because of this simple and similar division of memory formation between the left and right hemispheres in *Vertebrata* and *Invertebrata* it may have a long evolutionary history (summarized by Vallortigara and Bisazza, 2002). This division of tasks in information processing frees up the right hemisphere for detecting and responding to new stimuli. One example is the lateralization in *Invertebrata*, such as bees, which improves the short term and long term memory storage (Frasnelli et al., 2014). Another example are domestic chicks, which were more effective in discriminating food, and faster in detecting a predator overhead at the same time, when they had a more strongly lateralized brain (Rogers et al., 2004). On a population level, cerebral lateralization could have advantages, for example, in social interactions and coordinated flight responses (Leliveld, 2019). While social animals that flee in the same direction make it more difficult for a predator to select an individual, when a predator learns about the population flight directions, it could then be an advantage to have individual lateralization that is different from the population bias. Therefore, cerebral lateralization can have advantages and disadvantages on population and individual levels depending on the situation (summarized by Vallortigara and Bisazza, 2002). The genetics behind lateralization are not completely understood. Phylogeny and ontogeny may be involved and interact with each other. Influences during embryology and maturation may influence some factors of the lateralization, such as its strength, but the development of the responsibilities of the brain hemispheres may be influenced by genes rather than environmental influences (summarized by (Rogers, 2002).

Cerebral lateralization is displayed as sensory and motor laterality, and has to be distinguished from body asymmetry. Sensory laterality describes the preferred use of the left or right sensory organs such as eyes, ears, and nose. Motor laterality describes the preferred use of the left or right limbs. The body asymmetry is the uneven development of the left and right side of the body that is shown in uneven conformation in muscles, angles of joints, and the skeleton. All three parameters are fundamentally independent from each other, but it cannot be ruled out that they may influence each other to a certain extent. In horses, motor laterality in grazing stance predicted the asymmetry in forelimb load when trotting, with the preferred forelimb taking more load (Rehren, 2018). Furthermore, there is a relationship between the existence of a preferred grazing stance and a movement pattern (including hindquarter angle and lateroflexion) (Rehren, 2018). The development of a preferred grazing stance in

foals will predispose them to uneven hooves and uneven unequal forelimb load. The development of a preferred grazing stance itself may be the result of conformation characteristics such as long limbs and short heads (van Heel et al., 2006), but other parameters are discussed that may also influence laterality, for example emotionality.

Lateralization and emotional processing - Models of cerebral lateralization

Laterality may be a useful welfare indicator across taxa as it is assumed that emotional lateralization is comparable in all vertebrates (Leliveld et al., 2013). Emotional arousal is caused by situations that challenge the organism (stressors) and, consequently, affect the welfare of an individual. It is still unclear how emotions are processed, but they may influence motor and/or sensory laterality. Over the last decades various models of emotional processing have been proposed that all include the lateralization of the brain hemispheres (Table 1):

1. The right hemisphere model assumes that the right hemisphere is predominantly responsible for the processing, perception, and expression of emotions (Borod et al., 1998).

2. The valence model predicts that a negative valence is experienced and expressed by the right hemisphere whereas a positive valence is expressed by the left hemisphere. The balance between both emotions and hemispheres is controlled by the right hemisphere (summarized by Demaree et al., 2005; Silberman and Weingartner, 1986).

3. The approach-withdrawal model is similar to the valence model and proposes that emotions are linked to approach and withdrawal behaviour. It focusses on emotional states, with positive emotions expressed in approach behaviour and controlled by the left hemisphere, and negative emotions expressed in withdrawal behaviour and controlled by the right hemisphere (Davidson et al., 1990).

4. The behavioural activation (BAS) and behavioural inhibition system (BIS) model focus on emotional states as relative stable characteristics. The BAS is sensitive to non-punishment, reward, and escape from punishment, and it induces activity. The BIS is sensitive to novelty, non-reward, and punishment, and it is more likely to inhibit behaviour (Carver and White, 1994). Therefore, positive feelings are associated with the activation of BAS which is controlled by the left hemisphere. Negative feelings are associated with the activation of BIS which is controlled by the right hemisphere (summarized by Demaree et al., 2005). Enhanced sensitivity of the BAS may be associated with enthusiasm and enhanced sensitivity of BIS may be associated with increased anxiety (Carver and White, 1994).

5. The circumplex-dominance model includes three factors of an emotion: the valence (positive – negative), the arousal (low – high), and the dominance (submissive – dominant). It is linked to the approach-withdrawal and BIS/BAS models, but includes the feelings of dominance (left frontal arousal) and submission (right frontal arousal) (Demaree et al., 2005).

All five models have been supported by various studies, but there are also studies that are contradictory towards one or more models (for summary see Demaree et al., 2005). The truth may lie

somewhere in between all models. Nonetheless, it is known that the different hemispheres are responsible for different tasks. The left hemisphere is responsible for approach behaviour, proactive behaviour, routine situations, and detailed attention. The right hemisphere is responsible for withdrawal behaviour, reactive behaviour, global attention, control of novel situations, physiological, and behavioural stress responses (summarised by Rogers, 2010). The brain hemispheres are connected to the contralateral sides of the body, with the exception of the olfactory organs which are ipsilaterally connected (Brooks et al., 1999). Therefore, cerebral lateralization is displayed in both motor and sensory laterality.

Table 1: The division of tasks between the left and the right brain hemisphere according to the five models of cerebral lateralization of emotional processing.
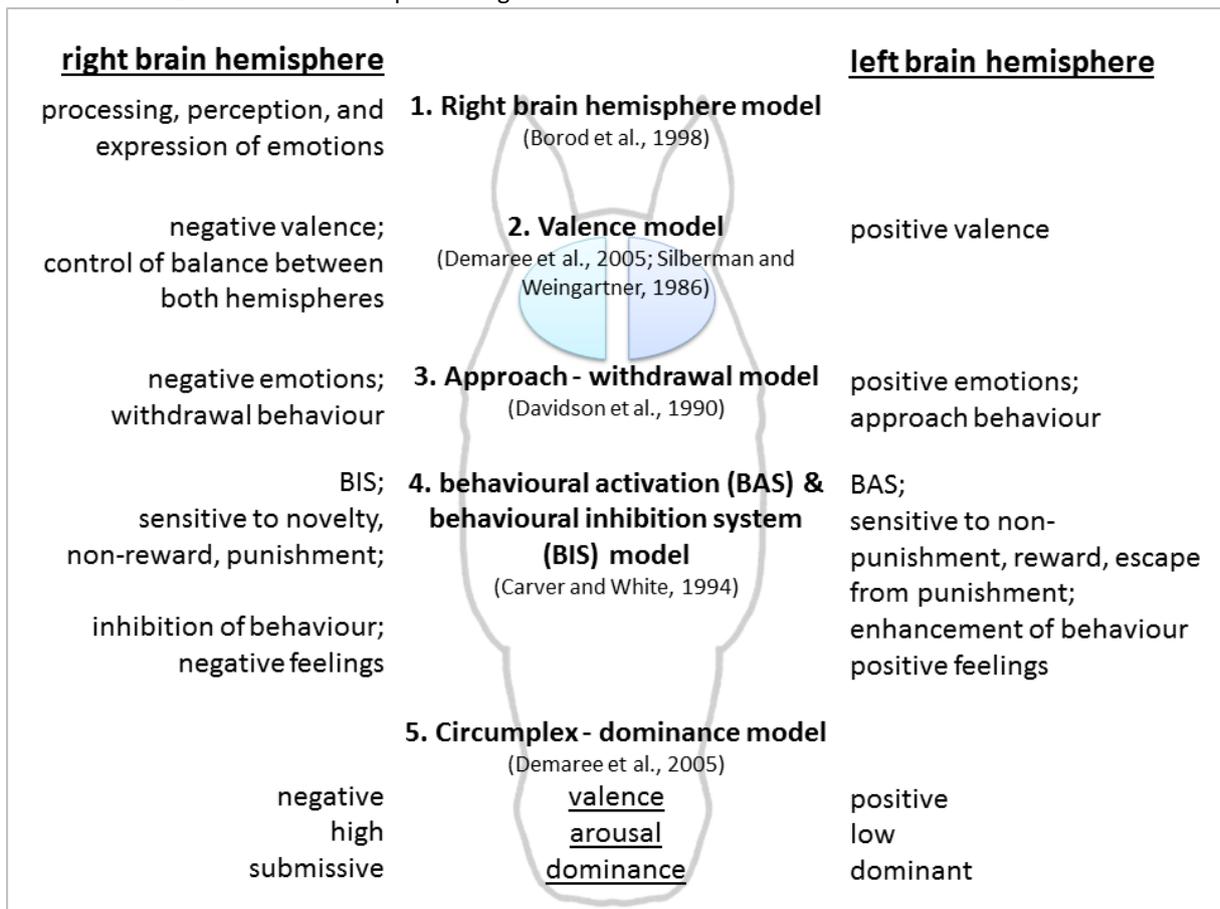
Sensory laterality

Sensory laterality indicates the specialization of the brain hemispheres for perception (A. Hook-Costigan and J. Rogers, 1998), and the use of sensory organs on a specific side (eyes and ears) indicates the hemisphere in which the information is processed. Knowledge of how an individual perceives its environment (with emotionality, arousal, reactivity, fearfulness etc.) can help in the evaluation of welfare. It is assumed that sensory laterality evolved before motor laterality, as sensory laterality does not correlate with motor laterality and lateralization of eye preference is stronger than paw preference (Chapelain and Blois-Heulin, 2009).

Lateralization may have evolved first on an individual level and afterwards on population level. Based on observed left-eye preferences it seems that threats and arousing interactions between individuals of the same species, such as agonistic interactions, are processed by the right hemisphere,

independent of the social organisation. For example, an enhanced left eye preference was observed during agonistic interactions with conspecifics in non-social living animals such as lizards (Deckel, 1995; Hews et al., 2004). In social animals, such as wild horses (minimum of two generation of wild living) and Przewalski horses, a population left-side preference for sensory organ use was displayed in agonistic interactions, vigilance and reactivity (Austin and Rogers, 2014, 2012). However, not all mammals show a population bias in laterality during social interactions, for example, domestic pigs (Camerlink et al., 2018). This suggests that other variables and circumstances may influence laterality in social interactions. Lateralized sensory organ use may also be a function of emotionality. For example, increased emotionality in domestic horses was associated with a higher usage of the left sensory organs (Larose et al., 2006) and horses with a left-side preference in sensory laterality displayed increased fearfulness and reactivity (Austin and Rogers, 2007). Furthermore, a potential threatening object that caused arousal indicated by vocalization behaviour in marmosets resulted in a shift to the left in eye preference, but viewing familiar objects was associated with a right eye preference on population level (A. Hook-Costigan and J. Rogers, 1998). Cattle and dogs also showed a left eye preference on a population level when viewing an unfamiliar, potentially arousing object, but shifted to a right eye preference when the object became familiar (Phillips et al., 2015; Robins and Phillips, 2010; Siniscalchi et al., 2010). It is argued that this kind of familiarisation learning, that involves emotionality, may be lateralized. It shifts from left to right side sensory organs and frees up the left eye to scan the environment for new potential threats (Robins and Phillips, 2010; Siniscalchi et al., 2010). Therefore, the left eye preference may not only depend on emotionality, but also on the preference for one side when observing the environment (Farmer et al., 2010). The emotional valence may also be important because in domestic horses the left eye has been shown to be used for negative objects, a binocular view for a positive object, and the right eye for a novel neutral object (De Boyer Des Roches et al., 2008). Different factors seem to influence the sensory laterality and it is unclear how stress may influence sensory laterality.

Motor laterality

Motor laterality indicates the tendency to process information in the left or right hemisphere (Gordon and Rogers, 2015). Thus, the predominant use of the left or right forelimb indicates a predisposition to rely on the left or right hemisphere. Knowledge of the individuals' predisposition to perceive its environment more emotionally, fearfully, curiously etc. can help in the evaluation of welfare, as it may indicate stressors, stress sensitivity, coping strategies and the wellbeing of an individual. For example, dogs with right-sided motor laterality and, consequently, the tendency to process information in the left hemisphere, were calmer in unfamiliar situations that were potentially arousing (Batt et al., 2009).Left-sided motor laterality indicates that the individual may rely on the right hemisphere, and therefore show more reactive behaviour, as well as physiological and behavioural stress responses. For example, left-pawed mice showed a higher hypothalamic-pituitary-adrenal axis activity measured by plasma corticosterone than mice that were right-pawed or showed no paw preference (Neveu and Moya, 1997). It is argued that such inter-individual differences in physiological stress responses may enhance the survival of the species (Neveu, 1996). Individual differences in stress responses are displayed in various behaviour patterns and, consequently, with different indices of motor laterality. Motor laterality can be observed on an individual as well as on a population level. Horses that have lived wild for a minimum of two generations and Przewalski horses showed no population bias in motor laterality during grazing (grazing stance, Austin and Rogers, 2014, 2012). But domesticated breeds like Thoroughbred and Standardbred horses showed a left-sided population bias

in advanced forelimb position during grazing on pasture (grazing stance, McGreevy and Rogers, 2005; McGreevy and Thomson, 2006), whereas Quarter Horses showed no preference (McGreevy and Thomson, 2006). These differences in laterality between the breeds may be caused by selective breeding and/or training (McGreevy and Thomson, 2006). In contrast, donkeys displayed a population bias to the right side in motor laterality that changed to no forelimb preference on population level when their living space was reduced in size (Zucca et al., 2011). It seems that motor laterality can develop from an individual level to a population level and from a population level to an individual level. This may be caused either by human (artificial) selection for specific traits that may influence/be displayed in motor laterality, by natural selection for adaptation to environment, or by changing environmental factors. Motor laterality may change with age, as shown in Thoroughbreds, in which the strength of laterality increased with age irrespective of the initial direction (McGreevy and Rogers, 2005). Different experiences may be the reason for this. In contrast, no relationship between age and motor laterality was observed in common marmosets (A. Hook-Costigan and J. Rogers, 1998). In unstressed donkeys, motor laterality was biased by age and sex, but the bias disappeared when space availability was reduced and the animals potentially stressed (Zucca et al., 2011). There are some conflicting studies (summarized by Leliveld, 2019), indicating that there is a complex relationship between such factors as age/maturation and environment that influence the direction as well as the strength of laterality.

Strength of laterality

It is debated whether the direction or the strength of laterality may be of greater importance. More strongly lateralized individuals may have some advantages. Individuals with a more strongly lateralized brain are more effective in performing two tasks simultaneously, in memory formation, and may have enhanced cognitive capacity (Magat and Brown, 2009; Reddon and Hurd, 2009), and these abilities may enable the individual to better cope with challenging situations (stressors). Therefore, knowledge of the individual's strength of laterality may help the understanding of how it copes in stressful situations and allow improvements can be made to the environment to enhance the individual's welfare.

Strongly lateralized parrots (motor or sensory laterality) performed better in a discrimination task, and in a task where a problem had to be solved by using foot and beak, irrespective of the direction of lateralization (Magat and Brown, 2009). Also, strongly motor lateralized marmosets (irrespective of direction) performed better in multitasking than weakly lateralized conspecifics, but this difference could not be observed when the animals were confronted with only one task at a time (summarized by Rogers, 2017). Although it seems that the direction of lateralization may be linked to some behavioural traits as outlined above, the strength of cerebral lateralization is thought to be linked to different personality traits. Strongly lateralized animals may be able to make behavioural decisions more quickly, as the dominant brain hemisphere may inhibit the other hemisphere, freeing it up for other tasks resulting in a fast information processing. Those animals appear to be more risk-prone, less cautious, and to show more extreme behaviour. Weakly lateralized animals may take more time to make behavioural decisions because of the consensus-making process of the two non-lateralized hemispheres. These animals may show more hesitation, may appear more fearful, and be less impulsive (Reddon and Hurd, 2009). For example, in domestic dogs a weaker paw preference was associated with an increased reactivity towards sounds (Branson and Rogers, 2006). In unfamiliar or fear inducing situations dogs that displayed a weaker motor lateralization were more restless and reactive (Batt et al., 2009; Branson and Rogers, 2006). In contrast ewes and lambs with weaker

laterality showed lower arousal during a separation test (Barnard et al., 2016) and in Port Jackson sharks there was a link between weakly lateralized individuals and decreased stress reactivity during rotational swimming (Barnard et al., 2016).

The strength of cerebral lateralization may also influence social life. The duration of conflicts in strong lateralized domestic pigs was shorter, but laterality had no influence on the contest outcome (Camerlink et al., 2018). Although some results appear to be conflicting, in general, weakly motor lateralized animals seem to be more fearful, more excitable, show poorer performance in multitasking and inhibited responses to novel objects; their social life is affected and the inter-hemispheric communication is less effective (summarized by Rogers, 2017).

The strength of lateralization may depend on the task the animals are confronted with. In a T-maze task, Port Jackson Sharks displayed no correlation between stress reactivity and strength of lateralization (Byrnes et al., 2016). It is assumed that the strength of lateralization is a result of ontogeny and experiences (Robins and Phillips, 2010). When the predisposition to use the left or right hemisphere more often (displayed as direction of motor laterality) and strength of that laterality are affected by life experiences, it should be possible to measure an individual's welfare by observing its lateral behaviour. The lateral behaviour should then be compared with already established stress parameters that indicate welfare, such as non-invasive stress hormone analysis. This is a well-established and a common method for horses, which are the model organisms in this thesis.

The horse (*Equus caballus*) as model organism

The domestic horse displays both motor and sensory laterality, and these are easy to observe because their eyes and ears are laterally positioned and they usually display a grazing stance with one foreleg advanced. Furthermore, non-invasive stress hormone analysis is well-established for horses. Therefore, domestic horses are good model organisms for investigation of laterality as a welfare indicator. To explain the natural needs of domestic horses and the factors in their welfare, the following section summarizes equine evolution, and describes the natural environment in which they evolved in comparison with the usual housing conditions of domestic horses.

Evolution of limbs, sensory organs and social behaviour

The evolution of the domestic horse (*Equus caballus*) has been widely described and illustrated in the literature (for example in Molen, 2009). Therefore, the evolution of structures and behaviours which are of relevance to this study will be briefly described, starting 60 million years ago with *Hyracotherium*, a dog-like mammal.

Hyracotherium, that is better known as *Eohippus*, was characterized by a tetradactyl (four-toed) forelimb, a tridactyl (three-toed) hind limb and lived 50 to 60 million years ago in the Eocene Epoch (MacFadden, 2005; Molen, 2009). The bone morphology of the fore- and hind limbs led researchers to conclude that *Hyracotherium* was already specialized for running (Radinsky, 1966). Some species of *Hyracotherium* may have lived in harems, as the sex ratio was 1 male to 1.5 – 2 females according to archaeological findings (Waring, 2003). The horses' natural need for free movement/locomotion and social interactions seems to have evolved already at a very early evolutionary stage. In the Oligocene epoch, 30 million years ago, the ancestral tridactylic horse species firstly developed a more pronounced middle toe, and then a brain comparable that of modern horses (Waring, 2003). Therefore, it is assumed that the characteristic equine intelligence evolved in this epoch and according

to their morphology they seemed to be well adapted to open country with grassland (MacFadden, 2005; Waring, 2003). Besides social interaction and locomotion, in this epoch horses seem to have evolved their natural need for long grazing/eating periods every day. During the Miocene (5 to 23 million years ago), equine ancestral genera became monodactylic and the metapodials had no further function and disappeared. According to the bone morphology of limbs, which were a perfect 'spring foot type', the genera of this epoch were well adapted to running on open grassland (Sondaar, 1969; Waring, 2003). Also, social living was widespread among the dominant genera like *Merychippus, Parahippus* and *Dinohippus* (Waring, 2003). From these dominant genera, *Equus* evolved 3.9 million years ago in the Pliocene. Within this genus three lineages, asses, zebras and caballine horses (including domestic and Przewalski horses), evolved independently (George and Ryder, 1986). During the evolution from *Hyracotherium* to *Equus* the cheek teeth shifted to an anterior position, whereas the eyeballs increasingly moved to more posterior and lateral positions (Radinsky, 1983).

It took still some million years until humans started to use horses. The first evidence of horseback riding dates back 6,000 years and was found in the region that is today's Ukraine. But it is unclear whether the horses were already domesticated or were living in wild herds (Anthony et al., 1991). The first evidence for horse domestication was found in the same region, and dates from 4,000 to 3,500 years ago (Anthony et al., 1991). The evolution of the horse, with its natural needs and responses, took more than 50 million years, whereas the domestication and breeding selection began only some thousand years ago. Therefore, the natural needs and responses of modern horses are still essentially the same as for wild horses: to stay physically and mentally healthy through locomotion, having food available for a long period every day, having a social life, and having fresh air.

Housing of domestic horses

Today, there are various different housing concepts that affect the horses' welfare, either positively or negatively, by enabling or restricting their natural needs. In Germany, single stable housing is still the most prevalent, followed by open stabling, then 24 hour pasture, box with access to a paddock, and other free stabling concepts (Hölker, 2016). As outlined in the previous chapter, the evolution of the horse, its morphological appearance, and its physiological and mental needs, took millions of years, whereas domestication started only some thousand years ago. Therefore, the physiological and mental needs of the modern horse are essentially the same as for wild horses: locomotion, long feeding periods, conspecifics for social interactions, and fresh air and light, and these should not be restricted by housing conditions and management.

The normal time budgets in the daily routine vary between the four seasons. The most time (50 to 70 per cent) per day is spent foraging, 15 to 20 per cent is spent standing at rest, 3 to 10 per cent lying down, 4 to 10 per cent standing vigilantly, and 6 to 10 per cent walking, trotting or galloping around, as observed in Camargue and Przewalski horses (Boyd et al., 1988; Duncan, 1985, 1980). To ensure a high standard of welfare these natural needs should also be covered in domestic horses. Restrictions in one or more of the natural needs will challenge the horses' coping strategies. For example, Dutch Warmblood horses showed similar time budgets to Camargue and Przewalski horses after they adapted to individual and pairwise housing (housing with two horses being kept in the same area). Foraging took 50 to 60 per cent of the day, but locomotion was restricted to walk, and took only 5 per cent of the day, while standing vigilantly and sleeping each took about 20 per cent (Visser et al., 2008). Compared with group housing, single stabling reduced locomotion and lying down in German Warmbloods (Wille, 2010). The natural time budget evolved over millions of years, and shifts in it caused by the restrictions of domestication can lead to abnormal and/or stereotypic behaviour if

horses fail to adapt, and the welfare of these horses is compromised (summarized by Veissier and Boissy, 2007). A link between stereotypic behaviour and different management factors, such as the time spent in the stable, foraging time, forage type, type of bedding, and box design has been shown in Thoroughbreds (McGreevy et al., 1995a), and in eventing and dressage horses, stereotypic behaviour was related to the time spent in boxes (McGreevy et al., 1995b). Within 12 weeks, 67 per cent of young Dutch Warmbloods developed stereotypic behaviour after moving from group housing to individual housing. However, no individual among pairwise housed horses showed stereotypic behaviour, although they displayed a similar time budgets to individually housed horses (Visser et al., 2008), indicating the importance of the contact to conspecifics (Hennessy et al., 2009). This was also supported by Yarnell et al. (2015), which showed that even minimal social contact reduced the stress hormone concentration.

Nonetheless, even in group housing, stress levels can be increased by the type of management. For example, limited space allowance can result in a higher aggression level and cause stress in domestic horses (Flauger and Krueger, 2013). Therefore, stress analysis can help to detect stress factors in diverse housing conditions, and eliminating these factors can prevent horses from developing abnormal or stereotypic behaviour, and thus improve welfare.

Stress analysis in animals – neuroendocrinology, immune system, behaviour

The simplest definition of good welfare is the absence of stress-indicating stress parameters, as well as the presence of positive indicators (summarized by Staley et al., 2018). Stress modulates a variety of measurable physiological, immunological, and behavioural parameters, which are summarized in the term stress parameters. The determination of most stress parameters is complex and time consuming. Consequently, the question arose whether laterality can serve as stress parameter that is quick and objective to assess and may therefore be a useful welfare indicator.

Stress is often defined by, and related to, an activation of the hypothalamic-pituitary-adrenal axis (HPA) and the sympathetic-adrenal-medullary axis (SAM) resulting in changes of neuroendocrine parameters (e.g. glucocorticoids, adrenaline, noradrenaline), cardiovascular parameters (e.g. heart rate, heart rate variability), behavioural parameters (e.g. abnormal behaviour, stereotypies, pattern of behaviour), body/eye temperature (König v. Borstel et al., 2017), immunological parameters (e.g. immunoglobulin A, different immune cells), and parameters of perception (e.g. laterality, cognitive bias) (Figure 1). Neuroendocrine and immunological parameters are commonly analysed from invasive blood sampling that can itself cause stress responses in animals and falsify the results (Balcombe et al., 2004; Morrow et al., 2002; Palme et al., 1996; Säkkinen et al., 2004; Sheriff et al., 2011). Therefore, to determine stress in horses in the present study only non-invasive faeces sampling was used to analyse glucocorticoid metabolites that served as neuroendocrine parameter, and immunoglobulin A that served as immunological parameter. Furthermore, behavioural parameters (sensory and motor laterality) were applied.

Stress responses can vary between individuals and between different species because the stress response depends on how an animal perceives a situation (aversive/pleasant), the level of emotion triggered (arousal), and the animal's evaluation of the environment (predictability and controllability) (Borell, 2009; Miller et al., 2007; Romero, 2004). Therefore, stressors can result in different behavioural and physiological responses (summarized by Veissier and Boissy, 2007). Consequently, the measurement of only one physiological or behavioural stress parameter is not sufficient to reliably evaluate stress responses and welfare in animals, and a combination of different stress parameters is needed (König v. Borstel et al., 2017).
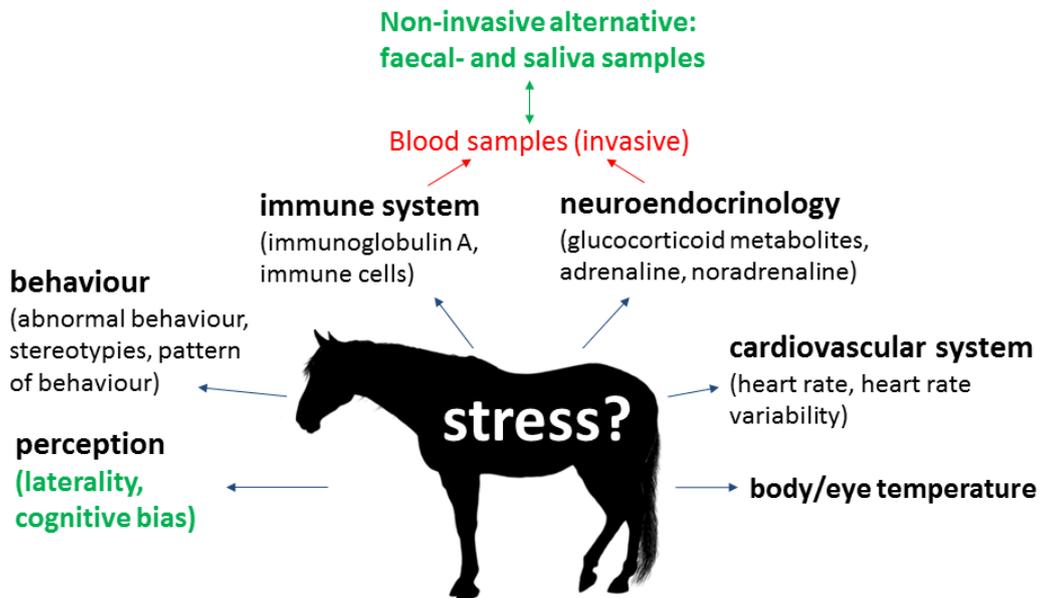
Figure 1: Stress parameter in animals. Animals suffering from illness, pain, poor housing or training conditions show only subtle signs of stress. Therefore, stress should be determined by using different parameters in parallel, and not by using only one parameter. Invasive blood sampling can give false results because the process of taking the sample itself can cause stress responses. Non-invasive sampling using faeces or saliva allows samples to be taken without further stress for the animal.

Stress physiology and stress hormones

When an organism is challenged by situations that may affect its welfare, a stress response is initiated. First, a stress response involves the sensory systems, which perceive the physiological or psychological stressor (Borell, 2009). As outlined under the chapter "Sensory laterality", the sensory system is subject to cerebral lateralization and therefore stress responses may be similarly lateralized. A stress response consists of transmitters and neuroendocrine systems that ensure communication of the organism's brain, including the limbic system, with the peripheral system (Smith and Vale, 2006) in the evaluation of the situation, coordination of behavioural responses, and formation of memories (Sapolsky et al., 2000; Smith and Vale, 2006). Different kinds of stressors (physical or psychological) will trigger different signal ways and stress responses. With reference to early experiences in similar situations the signals are evaluated by the central nervous system, and this may occur consciously or unconsciously. The arousal level increases (vigilance and fight or flight) and related emotions arise if the situation is assessed to be dangerous (Borell, 2009). The stress response can take two different signal ways which both start in the brain and use neurons and the vascular system to reach the organs and tissues. Both signal ways result in physiological stress responses such as increased stress hormones, enhanced respiration and cardiovascular system, and increased availability of energy (Borell, 2009). The first and most quickly triggered signal way is the sympathetic-adrenal-medullary axis (SAM) (Sapolsky et al., 2000). Therefore, in acute stress situations the SAM axis is the most important, and it releases the neurotransmitters/hormones catecholamines, adrenalin, and noradrenalin and the neuropeptides that regulate the cardio-vascular system, respiration, skeletal muscles, and the gastrointestinal tract. It also provides energy for a possible fight or flight response (Borell, 2009; Sapolsky et al., 2000). The second signal way is called the hypothalamic-pituitary-adrenal

axis (HPA) and it is slower to react in a stress situation (Sapolsky et al., 2000). Therefore, this system is important in long term stress situations. The HPA axis releases glucocorticoids (stress hormones) and provides further energy by enhancing the effect of the catecholamines, but it inhibits the immune system and inflammatory reactions (Borell, 2009; Smith and Vale, 2006).

Sympathetic-adrenal-medullary axis (SAM)

The SAM axis is activated by psycho-social, physical, and psycho-emotional stressors (Padgett and Glaser, 2003; Sapolsky et al., 2000; Stefanski, 2000). The central nervous system receives the stress stimuli from the environment and stimulates the brain stem that then releases neuronal signals. These signals activate the limbic system, especially the hypothalamus, resulting in a release of the corticotropin releasing hormone (CRH) (Smith and Vale, 2006) (Figure 2). CRH is a neurotransmitter, and it stimulates a release of noradrenalin in *Locus coeruleus* that activates the sympathetic nervous system (Borell, 2009; Smith and Vale, 2006) (Figure 2). The neuronal signals are transmitted to the organs via the sympathetic nervous system. The adrenal medulla, in particular, starts to release catecholamines (Figure 2) and neuropeptides to regulate the cardio-vascular system, respiration, skeletal muscles, and gastrointestinal tract (Borell, 2009; Sapolsky et al., 2000), and immune functions (Padgett and Glaser, 2003). The adrenal medulla releases opioids to help the organism to cope with the stress. The response of the SAM axis is regulated by many negative feedback mechanisms. Some regulatory mechanisms start immediately the transmitters are released, for example noradrenalin that shows a negative feedback on its own release. Catecholamines also have a negative feedback mechanism towards the CRH and adrenocorticotropic hormone (ACTH) release from the hypothalamus and hypophysis (Engelhardt and Breves, 2005) (Figure 2). Furthermore, an enzymatic degradation of the catecholamines starts and helps to terminate the SAM activation.

Hypothalamic–pituitary–adrenal axis (HPA)

The HPA axis shows delayed activation in comparison with the SAM axis (Sapolsky et al., 2000). The HPA axis is activated by similar stressors as the SAM axis: psycho-social, physical, and psycho-emotional stressors (Padgett and Glaser, 2003; Sapolsky et al., 2000; Stefanski, 2000). The activation also starts at the central nervous system (CNS), which receives stress stimuli from the environment (Borell, 2009; Padgett and Glaser, 2003). The CNS stimulates the brain stem, which releases neuronal signals that activate the limbic system, especially the hypothalamus, resulting in a release of the synthesised corticotropin releasing hormone (CRH) and Arginine Vasopressin (AVP) (Borell, 2009; Miller et al., 2007; Smith and Vale, 2006). Both CRH and AVP activate the secretion of adrenocorticotropic hormone (ACTH) in the hypophysis (Borell, 2009; Miller et al., 2007; Smith and Vale, 2006) (Figure 2). Furthermore, CRH activates the SAM axis and affects emotions and behaviour (Borell, 2009; Sapolsky et al., 2000; Smith and Vale, 2006). During the stress response, emotions serve as a kind of motivation that leads the organism to find a solution to the situation that initiated the stress responses. ACTH is transported via the vascular system to the adrenal cortex to stimulate the release of glucocorticoids (Miller et al., 2007; Padgett and Glaser, 2003; Smith and Vale, 2006) (Figure 2). The released glucocorticoids are transported via the vascular system to the organs and cells. There, glucocorticoids stimulate, for example, gluconeogenesis, lipolysis, heart rate, inflammation, and/or inhibit various immune cells and the absorption of glucose in different tissues such as muscles (Miller et al., 2007; Padgett and Glaser, 2003; Sapolsky et al., 2000). Similar to the SAM axis, the HPA axis has many negative feedback mechanisms, for example cortisol shows a negative feedback mechanism towards ACTH and CRH release (Miller et al., 2007; Smith and Vale, 2006) (Figure 2). However, during chronic stress situations these negative feedback mechanisms are disturbed and a chronic elevation

of stress hormones may cause pathological diseases (Miller et al., 2007; Padgett and Glaser, 2003; Sheriff et al., 2011; Smith and Vale, 2006). It is known that the intensity of a stressor modulates the HPA response, but it is not yet fully understood how. It has been demonstrated that enhanced ACTH concentration results in extended cortisol production. Chronic elevation of stress hormones results in different expressions of various proteins (Padgett and Glaser, 2003; Smith and Vale, 2006). Predictable and controllable stressors activate cognition and behaviour, whereas unpredictable and uncontrollable stress leads to a depression of behaviour unless the individual finds coping strategies which activate the SAM axis and result in a fight or flight response (Borell, 2009; Sapolsky et al., 2000; Stefanski, 1998).
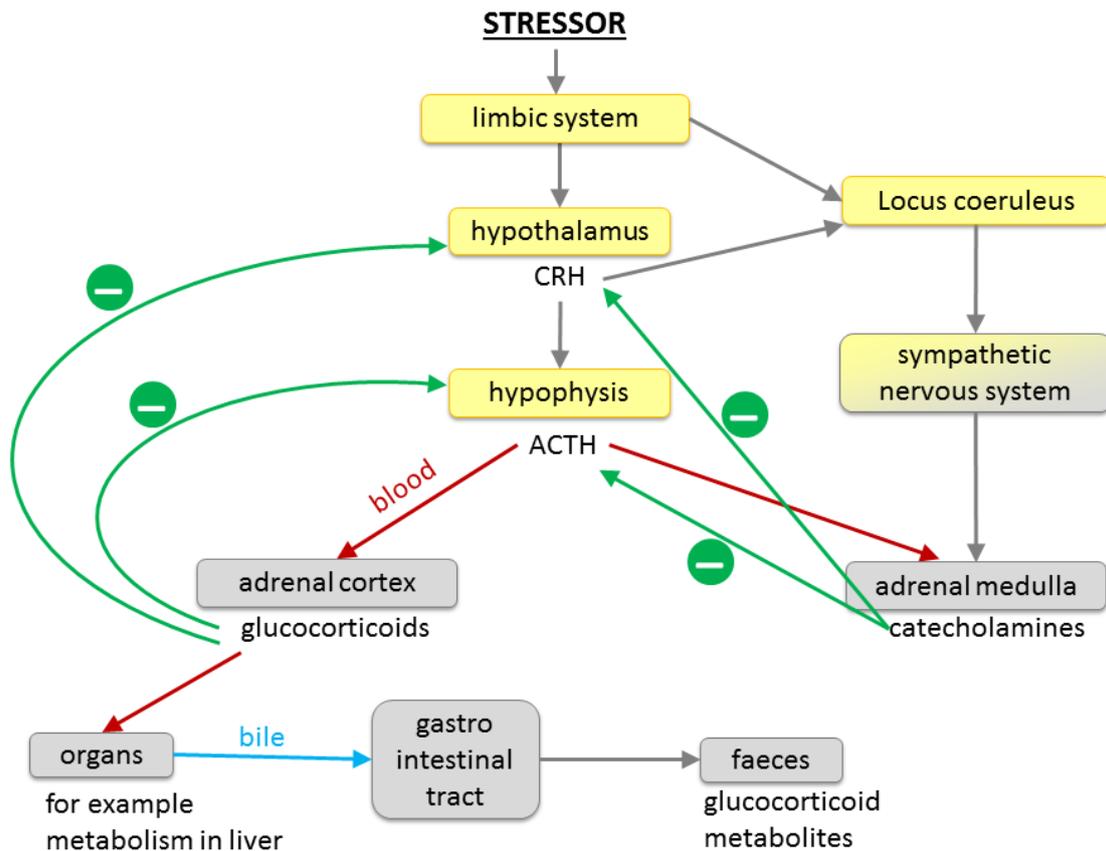
Figure 2: Schematic overview of hypothalamic–pituitary–adrenal (HPA) and sympathetic-adrenal-medullary axis (SAM) and the excretion of glucocorticoid metabolites via faeces (according to Engelhardt and Breves, 2005; Miller et al., 2007; Möstl and Palme, 2002; Padgett and Glaser, 2003; Smith and Vale, 2006). ACTH (adrenocorticotropic hormone) and glucocorticoids are transported via the vascular system (red). There are several negative feedback mechanisms of glucocorticoids and catecholamines back to the hypothalamus and hypophysis affecting corticotropin releasing hormone (CRH) and ACTH (green).

The stress response seems to be a complex network (Figure 2) of neurotransmitters, hormones and organs which are precisely adjusted to each other and result in diverse effects on gene expression and protein production on cellular basis. This is shown in diverse effects on physiology and behaviour of an organism. Besides the sensory system, the HPA axis, the cortical arousal, and the cortisol release may also be related to cerebral lateralization, as they are under primary control of the right hemisphere (Silberman and Weingartner, 1986; Westergaard et al., 2000; Wittling and Pflüger, 1990), but it is not clear how stress may influence sensory laterality.

Physiological stress responses in horses measured by glucocorticoids

To evaluate laterality as welfare indicator, an established stress indicator is needed. Measuring glucocorticoids is a well-established method in many species to evaluate stress. Glucocorticoids can be measured in body fluids such as blood, saliva, and faeces. Salivary cortisol in horses is influenced by a diurnal rhythm showing high cortisol levels in the morning and reduced concentrations in the evening (Aurich et al., 2015; Erber et al., 2013), and additional changes in salivary cortisol can indicate stress. For example, in three-year old mares, salivary cortisol significantly increased after they were transferred from group housing to a management programme that included individual stabling with visual contact, no turnout, and regular training for 30 minutes per day. After the change of housing the diurnal rhythm in salivary cortisol was re-established within one day, but the cortisol concentration remained elevated even five days after the change of housing conditions (Erber et al., 2013). In horses, HPA-axis activation is also indicated by an increase of plasma cortisol levels. This can be caused, for example, by the stress of competition in dressage or jumping, with novice horses showing a greater increase in plasma cortisol than more experienced horses (Cayado et al., 2006). Social stress in horses can also activate their HPA-axis (Alexander, 1998), and this can be measured in faecal samples as demonstrated in an alpha male horse by Wolter et al. (2014). The equine digestion process takes about 24 hours (Palme et al., 1996), therefore changes in faecal stress hormone concentration due to a stress response can be measured 24 hours after the onset of the stressor. If the increases in stress hormones are caused by acute stress that terminates during the digestion process, stress hormone levels may be diluted by the time defecation occurs, as has been demonstrated in 2-year-old mares. Initial training does not appear to be a strong stressor, as no changes were detected in faecal glucocorticoid metabolites (Gorgasser et al., 2007). While salivary cortisol increased significantly during training in young horses, the stress hormone level returned to normal after only about one hour, indicating that initial training is only a short-term stressor (Schmidt et al., 2010). Consequently, the measurement of faecal glucocorticoid metabolites is only useful for the detection of strong and long-lasting stressors. However, extended chronic stress that constitutes compromised welfare results in lowered faecal glucocorticoid concentrations, as shown in horses with behaviour and postures that clearly indicated a compromised welfare (Pawluski et al., 2017). Therefore, the measurement of stress that is supported only by physiological parameters such as stress hormone levels is not reliable. This has also been demonstrated in young Dutch Warmbloods, which showed clear behavioural differences between individual and pairwise housing, but no differences were detected in physiological parameters (Visser et al., 2008). Additionally, the determination of HPA activity via glucocorticoid concentration from different body fluids on its own is not a reliable indicator of animal welfare, as it reflects only the emotional arousal of the organism and not the valence, i.e. whether the current situation is perceived positive or negative (summarized by Staley et al., 2018). Therefore, further stress parameters are needed and, as outlined above (Stress physiology and stress hormones), stress also affects the immune system, so immune parameters may contribute to a more reliable stress evaluation.

Immunologic stress responses in general and in horses

The immune system is affected by stress responses and also seems to be related to cerebral lateralization. Therefore, laterality may be a helpful welfare indicator. It has been demonstrated that lesions of the left cortex resulted in a reduction of some immune functions including number and function of T-cells, proliferation of B- and T-lymphocytes, Interleukin-1 production, and immunoglobulin G synthesis, whereas lesions of the right cortex enhanced the same immune functions (Barneoud et al., 1988; Neveu et al., 1986; Qiu-shi and Gui-zhen, 1987; Renoux et al., 1983). Therefore, it can be assumed that brain lateralization may not only be related to the HPA axis, but may also

influence sympathetic activity and the immune system (Neveu, 1996). The immune system is related to HPA and SAM axis activity, and some immune parameters are activated, while others are depressed, by stress (Ader et al., 1995; Padgett and Glaser, 2003) as mediated by glucocorticoids and catecholamines, respectively (Staley et al., 2018). The duration and intensity of a stressor activates, suppresses or dysregulates different immune functions (Dhabhar, 2009; Stefanski et al., 2013). As summarized by Dhabhar (2009), physiological concentrations of glucocorticoids activate immune functions such as immunoglobulin production, but the negative feedback loop from the HPA axis during a stronger stress response suppresses the immune response to prevent the organism from overreaction and self-damage. Acute and chronic stress affect natural killer cells and their cytotoxicity, and B- and T-lymphocytes and their proliferation (Grün et al., 2013; Padgett and Glaser, 2003; Stefanski, 2000; Stefanski et al., 2013; Stefanski and Engler, 1999, 1998). Consequently, cytokine and immunoglobulin production are also affected, for example immunoglobulin A (IgA), that is synthesized by differentiated B-lymphocytes (Woof, 2013).

IgA is a common surface antibody that is found in many mammalian species, especially in the gastrointestinal tract (Newby and Stokes, 1984). It can be detected in different body fluids such as saliva, tears, and intestinal fluids. It prevents the formation of bacterial colonies on the mucosa, reduces inflammatory effects, neutralises bacterial toxins and enzymes, inhibits the penetration of viruses in epithelial cells, and protects against allergens and carcinogens (Newby and Stokes, 1984; Tsujita and Morimoto, 1999). In horses, IgA is a common immunoglobulin and may be affected by stress responses (May, 2007; Vaerman et al., 1971), and the duration and intensity of a stressor also activates, suppresses or dysregulates IgA production (Campos-Rodríguez et al., 2013). Therefore, the analysis of IgA concentration, in addition to physiological stress parameters, can help to improve the assessment of animal welfare because it provides information about the valence of the stressor (Staley et al., 2018). However, little is known about IgA in horses and how it is influenced by stress.

Cognitive Bias as behavioural stress parameter

As outlined previously (Stress analysis in animals – neuroendocrinology, immune system, behaviour), welfare is not only defined by the absence of stress-indicating stress parameters, but also by the presence of positive indicators, for example a good mood/positive cognitive bias.

Laterality could not only suggest poor welfare but also good welfare when it is related to cognitive bias and, therefore, it may serve as welfare indicator. Changes in the activity of HPA- and SAM-axis, which seem to be related to cerebral lateralization, also affects the behaviour of an organism. Processing a stress signal involves the sensory system, and this system is related to cerebral lateralization. After the stress situation is evaluated, decisions are made in different areas of the central nervous system. Firstly, the present situation is compared to past emotional experiences which are stored in the limbic system. The limbic system activates the hypothalamus to start a stress response (SAM and HPA) according to the decision the prefrontal cortex made about the situation. As outlined above, the induced emotions help the organism to cope with the stress situation, find solutions, and remember the experiences to avoid similar unpleasant situations in the future (LeDoux, 2003). Therefore, emotions seem to be important parameters for the organism in stress situations. Furthermore emotions can influence cognition, and cognition can initiate emotions, resulting in a cognitive bias (Roelofs et al., 2016).

In human research, cognitive bias is used to describe the effects of emotions on cognitive processes, but in animal research the term is often used as a synonym for attention, memory and judgment bias (Mendl et al., 2009). In this thesis it is used to describe the judgment bias that is defined by behaviour

towards neutral or ambiguous stimuli, depending on the anticipation of either positive or negative events (Mendl et al., 2009). This perception may not only be influenced by the personality, but also by the animal's present mood, stress level, and/or past experiences, all of which are associated with different emotions. Therefore, cognitive bias can serve as a behavioural stress parameter, as has been demonstrated in rats that were living in unpredictable housing, which resulted in a negative cognitive bias that was shown as a reduced anticipation of positive events (Harding et al., 2004). Similarly, a relationship between environmental enrichment and a more positive cognitive bias was found in horses (Löckener et al., 2016). It is assumed that acute short-term stressors may enhance dopaminergic activity, which increases reward valuation, and such animals may respond to an ambiguous stimulus optimistically. Conversely, uncontrollable long-term stressors may reduce the dopaminergic activity, resulting a more negative or pessimistic mood (Mendl et al., 2009). Furthermore, cognitive bias seems to be closely associated with motor laterality, because right-pawed dogs were shown to be more optimistic than left-pawed dogs (Wells et al., 2017). Left-pawed dogs took the same time to approach an ambiguous stimulus as to approach a near positive and near negative stimulus, whereas right-pawed and ambilateral dogs were significant faster in approaching the stimulus the closer it was to the positive location (Wells et al., 2017).

Compared to a judgement bias test, motor laterality is easy to determine, more repeatable, less time consuming and may also serve as a parameter to determine cognitive bias in horses. The assessment of equine stress level and welfare using only behavioural observation is not easy and is limited (Lesimple and Hausberger, 2014), therefore behavioural stress parameters should also be supported by other stress parameters.

Objectives of the thesis - laterality as welfare indicator

A knowledge of the use and predisposition to use the left or right hemisphere and its possible connection to positive or negative emotions, may not only help the understanding of behaviour, but may also provide insight into the mental health of an animal. The absence of stress parameters may not reliably indicate the absence of stress or poor welfare (Harewood and McGowan, 2005). The presence of a good mental state (e.g. positive cognitive bias) indicates good animal welfare. The measurement of the direction and strength of motor and sensory laterality may give insight into emotional arousal and cognitive bias and may potentially be an objective measure of animal welfare. Furthermore, both these parameters are easy to determine and repeatable, as there is no learning effect.

The right brain hemisphere seems to be linked both to emotions, and to the neuroendocrine system, such as HPA axis activity measured as plasma cortisol level (Ocklenburg et al., 2016). Additionally, emotions are linked to higher HPA axis activity as demonstrated in various studies summarized by Sullivan (2004). Increased dominance of the right hemisphere increases stress sensitivity and may impair the individual's coping strategy, and so is a welfare issue (Sullivan, 2004). However, HPA axis activity is not only linked to cerebral asymmetry, but also to the immune system, with an immunosuppressive effect being linked to the right hemisphere and immune activation linked to the left hemisphere (Sullivan, 2004). In donkeys, the population bias of right forelimb preferences declined after a reduction in space availability that is assumed to be a stressor. Stress seemed to be linked to the right hemisphere, leading to enhanced use of the left side compared with the control situation (Zucca et al., 2011), but it has not yet been investigated whether sensory laterality is linked to enhanced stress hormones.

To summarize, laterality seems to be connected with different stress parameters, stress responses, and the consequences of stress, such as HPA axis activity and the ensuing glucocorticoid release, the immune system, emotional arousal, and cognitive bias. Consequently, the key objective of the thesis was to evaluate laterality as a potential welfare indicator that could be reliable, repeatable, objective, and easy to determine. In order to attain this key objective, the aims of the individual studies were to investigate first whether laterality correlates with well-established stress parameters such as the measurement of stress hormones and serves as stress parameter (STUDY 1). Second, it was investigated whether laterality correlates with the mental mood of an animal and serves as parameter of the cognitive bias (STUDY 2). Third, it was investigated whether sensory laterality is correlated with affiliative behaviour (STUDY 3) and therefore may serve as parameter of well-being. Fourth, to improve the methodology of non-invasive stress hormone and immunoglobulin A analysis from faeces, the conservation of faecal samples by drying in silica gel was investigated. Sometimes when studying animals, especially free-ranging animals, the transportation of the samples to the lab or to a freezing facility takes too long, and the stress hormones and immunoglobulin A may decay (STUDY 4). The last, unpublished, part of the thesis investigates and discusses whether the strength of laterality may indicate stress sensitivity or cognitive bias, and so may serve as an animal welfare indicator (STUDY 5). As welfare is linked to a healthy mental state and, therefore, is also linked to emotionality, the five models of emotional processing are discussed in support of the investigation into the link between welfare, emotionality, and laterality.

STUDY 1

Non-invasive stress evaluation in domestic horses (Equus caballus): Impact of housing conditions on sensory laterality and immunoglobulin A

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1.   Summary

The study aimed to evaluate sensory laterality and concentration of faecal immunoglobulin A (IgA) as non-invasive measures of stress in horses by comparing them with the already established measures of motor laterality and faecal glucocorticoid metabolites (FGMs). Eleven three-year-old horses were exposed to known stressful situations (change of housing, initial training) to assess the two new parameters. Sensory laterality initially shifted significantly to the left and faecal FGMs were significantly increased on the change from group to individual housing and remained high through initial training. Motor laterality shifted significantly to the left after one week of individual stabling. Faecal IgA remained unchanged throughout the experiment. We therefore suggest that sensory laterality may be helpful in assessing acute stress in horses, especially on an individual level, as it proved to be an objective behavioural parameter that is easy to observe. Comparably, motor laterality may be helpful in assessing long-lasting stress. The results indicate that stress changes sensory laterality in horses, but further research is needed on a larger sample to evaluate elevated chronic stress, as it was not clear whether the horses of the present study experienced compromised welfare, which it has been proposed may affect faecal IgA.

Keywords: glucocorticoid metabolite; horse; immunoglobulin A; motor; sensory; laterality

2.   Introduction

It has been suggested that some horse management regimes, such as individual stabling without contact with conspecifics, may compromise the animal's natural needs and result in various types of stress responses, for example, increased stress hormone concentration, eye temperature and heart rate, and the display of stereotypic behaviour [1,2].

Stressors that last for a few minutes or hours may cause acute stress responses, while stressors that last for several hours per day over weeks or months may cause chronic stress responses [3]. Some consider acute stress responses to be "positive", beneficial physiological responses and chronic stress responses to be "negative", maladaptive responses, although the differentiation between them remains vague [4]. The duration and extent of a physiological stress response has been shown to depend on the individual's stress sensitivity, perception, and processing of the stressor, in humans [5], rats [6,7], mice [8], pigs [9], and dogs [10]. Animals' stress levels are commonly assessed by analysing the levels of glucocorticoid metabolites (GMs) in different body fluids. Minimally to non-invasive methods, including the analysis of stress hormones such as glucocorticoids in saliva [11] or their metabolites in faeces [12], allow samples to be taken with little or no stress to the animals. Blood sampling for the measurement of stress hormones is invasive and may cause a stress response in wild, domestic and laboratory animals [13–17]. Furthermore, the analysis of glucocorticoids may not be suitable for differentiating between short-term and long-term stress because glucocorticoid production can be increased by both [5], and in the case of chronic compromised welfare, may decrease to baseline levels and below after an initial increase [18].

Acute stress situations, which activate the sympathetic adrenal medullary system, elevate the number and activity of certain immune cells, i.e. blood neutrophils, providing an enhanced first line immune defence [3,6,19]. Furthermore, cortisol is released when stress activates the hypothalamic-pituitary-adrenal axis causing a redistribution of lymphocytes from the blood to different organs for immune defence [3,6,19]. The number and activity of leucocytes may influence immunoglobulin A (IgA) production and concentration in various different ways, including the number and activity of B-lymphocytes, plasma cell activity, and the production of secretory component [7,20]. Additionally, the

intensity and duration of a stressor modulates the production of secretory IgA [21]. In many species, IgA is the most commonly secreted immunoglobulin in the gastrointestinal tract and has an important role in mucosal immune defence [22]. In general, it has been shown that secretory IgA increases under acute stress but decreases under chronic stress [23] in rats [7], mice [8], pigs [9], and horses [24]. Therefore, non-invasive IgA analysis may help to determine stress in animals reliably.

The analysis of physiological stress responses may be supplemented with observations of behaviour [18], but as the interpretation of animal behaviour is complex and time consuming, a simple, quickly determinable, objective behavioural parameter is needed. It has been proposed that lateral limb, paw, claw or hoof use, i.e. motor laterality [25–29], is such an indicator. As motor functions are controlled by the contralateral brain hemisphere [30], a left shift in limb use indicates increased processing by the right hemisphere, and a right shift indicates increased processing in the left hemisphere. Stress leads to increased information processing by the right brain hemisphere, which has been shown to control responses to stress, novelty, social interactions, and predators [25]. The right hemisphere also controls the sympathetic nervous system activity, indicated by increased cardiac activity in horses under stress [31]. At the physiological level, increased information processing by the right hemisphere has been linked to changes in immune reactivity such as higher blood lymphocyte numbers, lower IFN-γ production and reduced antibody response [27,28], and has been shown in mice [32] and dogs [33,34]. The left hemisphere has been shown to be responsible for categorization of stimuli and routine situations [25,26,35–41].

As with motor laterality, sensory laterality is also associated with one sided hemispheric information processing [42,43]. Social information, both in agonistic and affiliative contexts, is processed by the left hemisphere and this reflects the need to respond quickly and appropriately to emotional information [43]. The use of sensory organs on the left side has been shown to correspond with increased emotionality in horses [31,42,44–46], red-capped mangabeys [47], lizards [48], and dogs [49]. However, it remains unclear how stress affects visual and auditory laterality. Sensory laterality has been said to be a flexible parameter [50], and so may change faster and be more situation-related than motor laterality.

Horses are good model organisms for comparing the established stress indicators of GMs and motor laterality with the potential stress parameters of IgA and sensory laterality because there has already been substantial work in this field. Previous studies have demonstrated that stressors such as changes from social housing to individual housing (which restricts movement and social interaction), increase the concentration of cortisol both in horses' saliva and of GMs in the faeces [1,2]. The initial training increased cortisol concentrations in horses' saliva [51] but not the GM concentrations in faeces [52]. It has also been demonstrated that stress associated with road transport increased, and physiological responses associated with anaesthesia decreased, faecal IgA concentration [24]. Motor laterality has been shown to be evenly distributed at population level under normal circumstances in free-roaming Przewalski and domestic horses [37,53]. However, it has been suggested that stress causes a population preference for left forelimb use in Quarter Horses [40], and it is possible that sensory laterality is also affected by stress. However, it has been proposed that enhanced lateral forelimb use [35,37,53] and enhanced sensory laterality [54] may partially be caused by one-sided training in horses. Any left or right preference in sensory organ use is easy to assess in horses, as horses' eyes and ears are laterally positioned, and they generally shift their heads when switching between the use of the sensory organs of one side and the other.

The current study aimed to evaluate acute and long-term stress responses in horses to answer the question of whether faecal IgA and sensory laterality change comparably with the established stress parameters of GMs and motor laterality in known stress situations as suggested by researchers

[1,2,24,42,44,51]. It was conducted in three-year-old castrated male riding horses and addressed the change from social to individual housing and initial training, which are known to cause stress responses in horses, followed by regular training and continuing individual stabling for two months to analyse possible effects of such challenging situations.

3. Materials and Methods

3.1. Animals and location

Data were collected between November 2015 and January 2016 at the state stud farm Marbach, Gomadingen, Germany. The state stud farm provided 12 three-year-old (foaled in 2013), castrated male German Warmblood horses (Equus caballus) which were raised together in one social group. The horses were identified through their coat colours and white markings on their heads and legs. All animals were in good health and feeding condition and were checked by the stud's veterinarian on a regular basis. The horses were used to being led on a halter and being tied up to eat grain from wall mounted feeders.

At the start of the experiment, all 12 horses were kept in open housing at Marbach and had no previous experience of single boxes. They were kept in one group on a single 5.9-hectare pasture with a barn for shelter and handling. They had permanent access to grass and water, and received hay in addition. When they were in the barn for routine handling and veterinary treatment, they received about 500 grams of grain each. After they had been housed in a stable group composition at this facility for 5 weeks, they were led to the stabling complex where they were housed in 12 individual boxes (sized 3.2 m x 3.5 m). The boxes allowed visual and physical contact between the horses, but only through the metal bars which separated the boxes. In the boxes, they received hay and water ad libitum, and grain three times a day (the amount depended on individual needs). Straw bedding was used in the barn and in the individual boxes. This experiment was restricted to 12 horses, as the stabling complex only had 12 suitable individual boxes.

3.2. Experimental procedure

The horses were exposed to four test situations (for an overview of the time schedule see Table S1):

a) A change from social housing on pasture to individual housing in single boxes.

This test situation was expected to cause acute stress responses as a result of a changed environment, reduced physical contact to conspecifics, and movement restriction [1,2]. Faecal GMs (FGMs) were expected to be elevated, motor laterality to be unchanged, faecal IgA to be elevated, and sensory laterality to be changed. During the first week, the horses received exercise in groups of 6, in the form of free movement in an indoor arena, for 30 minutes per day. During the first week of individual housing, one horse was injured by another during free exercise and had to be excluded. Therefore, 11 horses were studied.

b) After 1 week of individual housing.

After one week of individual stabling as described above, the horses were expected to show different coping strategies. Some horses might be quicker to adapt to the novel situation than others. Different stress responses were expected including still elevated FGMs, changed motor laterality, decreased faecal IgA (in the case of reactive coping strategies), FGMs at baseline level, unchanged motor laterality, and baseline faecal IgA (in the case of proactive coping strategies). Sensory laterality was expected to return to baseline. The group average would depend on how many animals cope actively or passively.

c) Initial training.

This commenced after one week of individual stabling and was expected to elicit acute stress responses again [51], with increased FGMs but unchanged motor laterality, as each training session lasted for less than 1 hour [3]. The initial training consisted of 20 min lunging in an indoor arena on two consecutive days. Two horses were lunged in the arena at the same time to reduce isolation stress.

d) Regular training and individual stabling with no access to pasture or paddock.

The horses were stabled in single boxes and received regular training (5 times a week) for two months, as is the usual practice in Germany (most horses are stabled in single boxes, and training practice is in accordance with the guidelines of the FN) [55]. This situation was assessed for possible long-term influences on the investigated parameters. It was possible that these parameters could still be slightly altered, depending on the individuals' coping strategies (FGMs elevated or reduced in the case of compromised welfare, motor laterality changed, IgA reduced in the case of compromised welfare, sensory laterality changed).

3.3. Sampling and behavioural observation

GM and IgA analysis, as well as observations of motor laterality and sensory laterality were conducted in all test situations. Faecal samples for the baseline values were collected in the open barn while the horses were tied up for feeding, and samples for the test situations were collected from the individual boxes. All horses served as their own controls.

To calculate stable mean baseline values for each horse, three samples were taken for FGM and faecal IgA seven days before, 6 days before, and one the day before the first test situation (a). As horses' intestinal passage takes about 24 hours [56] faecal samples were taken during each test situation as follows: (a) 24 h and 48 h after the change of housing conditions, (b) one week after the change of housing conditions, (c) 24 h and 48 h after the first training session, (d) after two month of individual stabling (for an overview on the sampling schedule see Table S1). Motor and sensory laterality were observed once to establish baseline values and for each test situation with observations spread over three consecutive days, as detailed below under 3.7.

3.4. Glucocorticoid metabolites (GMs)

To determine GMs, faecal samples were taken between 8.00 and 10.00 to control for diurnal variations (IgA [57], GM [58]). Fresh faecal samples were collected from the centre of the pile with a freezer bag between 1 and 2 min after defecation and kneaded for 1–2 min to ensure an equal distribution of IgA and GMs. They were then kept on ice in a cool box until they were frozen in the laboratory at −20°C to avoid a decline in FGM concentration through bacterial decay, as has been demonstrated elsewhere [59]. The samples were processed as described elsewhere [60]. GMs were extracted from faecal samples by adding 5 ml of 80% methanol [61] to 0.5 g wet faeces. The suspension was vortexed for 2 min, incubated at room temperature for 15 min and vortexed again. After the centrifugation at 2500g for 15 min, the supernatant was aliquoted and frozen at −20°C until further analysis [62]. As validated and described for horses elsewhere [60], the diluted supernatant (in assay buffer) was analysed by an 11-oxoaeticholanolone enzyme immunoassay measuring 11,17-dioxoandrostanes.

Several enzyme immunoassay plates were needed. Therefore, samples at the plates were pipetted always in the same order, starting with horse number 1. In addition, samples from the same two control values were used on all plates, always at the end and in the same wells to calculate an inter-assay coefficient of variation (CV). Control 1 had a CV of 0.18 and control 2 of 0.20, which is slightly

above the elsewhere described CVs [60] which were between 0.13 and 0.16. To allow statistical comparison between samples from different plates, all samples were multiplied by a correction factor, which was calculated as follows for each plate: the mean value of the control samples from all plates divided by the actual control value of each plate.

3.5. Immunoglobulin A (IgA)

Faecal samples were collected as described for GMs in §2.4. The protocol for the extraction of IgA from faecal samples was adapted from elsewhere described protocols [57,63–65]. Faecal samples were thawed at room temperature (21°C). Ten millilitres of phosphate-buffered saline were added to 1 g wet faeces in a tube. The suspensions were shaken (with the top of the tube downwards), vortexed for 3 min and incubated for 15 min at room temperature. This process was then repeated. The samples were then centrifuged for 20 min at 1600g. The supernatant (1.2 ml) of the suspension was transferred into a new tube and centrifuged for 15 min at 3260g (21°C). The supernatant was aliquoted and frozen at −20°C until further analysis. IgA concentration was measured by enzyme immune assay (EIA) according to the manufacturer's protocol of the Horse IgA enzyme-linked immunosorbent assay (ELISA) Quantification Set (E70-116, Bethyl Laboratories, Inc., Montgomery, USA).

As for GM analysis, on all plates, the same two control values were used to calculate an inter-assay CV for IgA. The CV for IgA was 0.12 and 0.13. A correction factor was used as described for GMs.

3.6. Motor laterality

Motor laterality was observed by scan sampling, recording how often the left or right forelimb of the horse was placed in front while grazing on pasture and while eating hay from the floor in the box [37]. Sixty observations were made at 30 s intervals, at different times of day, spread over up to 3 days, for each horse in each test situation.

A motor laterality index (MLI) was calculated for each horse as described elsewhere [37]: LI = (R − L)/(R + L), where R describes the number of observations when the right forelimb was in front and L describes the number of observations when the left forelimb was in front. A negative MLI indicates a left preference and a positive MLI indicates a right preference.

3.7. Sensory laterality

Sensory laterality was observed using ad libitum sampling. When a horse raised its head to observe the environment, the direction in which the head was turned was recorded if it was 30 degrees or more to the left or right, as described elsewhere [53]. The observations were made for 2 h for each test situation. Additionally, sensory laterality was assessed using a novel-object test which was conducted either on the pasture or in the individual boxes, according to the test situation. On the pasture, the test horse was separated from the others with the help of a person who prevented the other horses from approaching. In the stabled situations, the novel-object test was done in the horses' boxes. In both cases, the novel-object was placed 1–2 m in front of each horse and we recorded which side of the head (left, right or frontal) was used for the initial investigation of the object. In each situation, six unfamiliar objects from the following list were presented to each horse: cones of various colours, balls of various colours, a rubber boot, buckets in different colours, an unfilled air mattress, a blanket, pieces of pipes in different colours, cartons of various shapes and colours, half a swimming noodle and bags of various colours filled with straw.

A sensory laterality index (SLI) was calculated for each horse as described for motor laterality in 3.6.

3.8. Experimenters

The two experimenters were PhD and Master students from Nuertingen-Geislingen University and University of Hohenheim. Before the experiment started, the inter-observer agreement between the two experimenters was tested for the assessment of sensory and motor laterality and a Cohen's Kappa coefficient was calculated (sensory laterality: $\kappa = 0.75$, motor laterality: $\kappa = 0.93$). Behavioural parameters (sensory laterality, motor laterality) were recorded on paper. One experimenter collected and kneaded the faecal samples, while the other observed the motor laterality and the head lifts for the sensory laterality. Both experimenters conducted the novel-object test to determine sensory laterality. The horses were handled and trained by employees of the state stud farm Marbach.

3.9. Statistical analysis

The R Studio (v. 0.99.484, Boston MA, USA) and R commander (2.2-1) were used for the statistical analysis. Figures were constructed with BP-tool (Add-In) by Microsoft Excel 2010 (Microsoft Corporation, Washington, USA). Three baseline measurements were taken for GM and IgA each to calculate stable mean baseline values for each horse during group housing (without experimental stressors; for all data see electronic supplementary material, table S2). The baseline values served as controls for comparison with values from the different test situations for each individual horse. As data were not normally distributed at all (Shapiro–Wilk test $W < 0.83$, $N = 11$, $p < 0.03$ for FGM 48 h training, IgA 24 h, 48 h, one week individual stabling, IgA 24 h, 48 h after initial training, motor laterality basal values, sensory laterality after initial training; for all others $W > 0.86$, $N = 11$, $p > 0.07$), we applied a generalized linear model (GLM) to analyse whether deviations between the baseline values and the test situations differed from 0 (formula = parameter [difference between baseline and test situation for each horse]~test situations, family = Gaussian (identity)). All tests were two tailed. The significance level was set at 0.05.

4.   Results

(a) Change from social to individual stabling

FGMs increased significantly from the baseline value taken at group housing to the value taken 24 h by 22.9 ng g^{-1} (Study 1 - Figure 1: GLM: FGM.difference $\sim$ test situation, $N = 11$, $t = 3.5$, $p = 0.001$) and 48 h by 15.6 ng g^{-1} (Study 1 - Figure 1: GLM: FGM.difference $\sim$ test situation, $N = 11$, $t = 2.4$, $p = 0.02$) after the change to individual stabling. IgA and motor laterality (ML) remained unchanged (Study 1 - Figure 2: GLM: IgA.difference $\sim$ test situation, $N = 11$, both $p > 0.05$; Study 1 - Figure 2: GLM: ML.difference $\sim$ test situation, $N = 11$, $t = -1.1$, $p = 0.26$). Sensory laterality (SL) shifted significantly to the left by LI -0.46 (Study 1 - Figure 1: GLM: SL.difference $\sim$ test situation, $N = 11$, $t = -2.4$, $p = 0.02$).

(b) 1 week of individual stabling

After one week of individual stabling, FGMs returned to baseline values (Study 1 - Figure 1: GLM: FGM.difference $\sim$ test situation, $N = 11$, $t = 0.9$, $p = 0.38$) and IgA remained unchanged (Study 1 - Figure 1: GLM: IgA.difference $\sim$ test situation, $N = 11$, $t = -0.04$, $p = 0.97$). Motor laterality significantly shifted to the left by LI -0.25 after one week of individual stabling compared with baseline values (Study 1 - Figure 2: GLM: ML.difference $\sim$ test situation, $N = 11$, $t = -2.5$, $p = 0.01$). Sensory laterality showed a trend to shift to the left by LI -0.33 (Study 1 - Figure 2: GLM: SL.difference $\sim$ test situation, $N = 11$, $t = -1.7$, $p = 0.096$).

(c) Initial training

FGMs showed a trend to be elevated 24 h after the first training session (initial training) by 12.1 ng g^{-1} (Study 1 - Figure 1: GLM: FGM.difference $\sim$ test situation, N = 11, t = 1.8, p = 0.07), and 48 h after the first training session FGMs had significantly increased by 14.3 ng g^{-1} (Study 1 - Figure 1: GLM: FGM.difference $\sim$ test situation, N = 11, t = 2.2, p = 0.03). IgA remained unchanged (Study 1 - Figure 1: GLM: IgA.difference $\sim$ test situation, N = 11, both p > 0.05). Motor laterality and sensory laterality returned to baseline values after the first training session (Study 1 - Figure 2: GLM: ML.difference $\sim$ test situation, N = 11, t = −1.6, p = 0.11; GLM: SL.difference $\sim$ test situation, N = 11, t = −1.6, p = 0.13).

a. change from group to individual housing - **b**. 1 week individual stabling
c. initial training - **d**. two month regular training & individual stabling
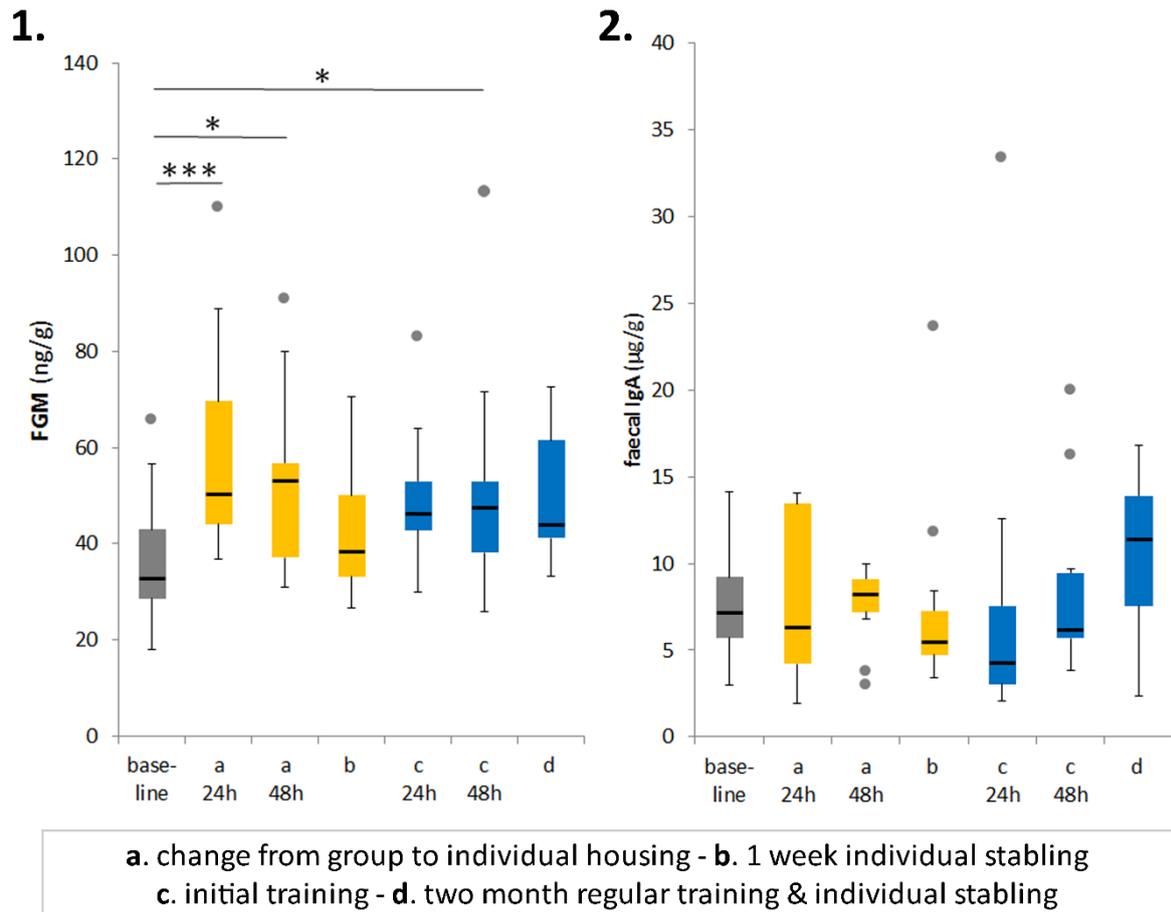
Study 1 - Figure 1: Physiological and immunological stress parameters. FGM concentrations (1) and faecal immunoglobulin A concentrations (2) in test situation without stressors (base, during group housing) and 24 h (a 24 h) and 48 h (a 48 h) after the change of housing condition, one week of changed housing condition (b), 24 h (c 24 h) and 48 h (c 48 h) after initial training, and two months of regular training and individual stabling (d). Yellow: changed housing conditions, blue: combination of individual housing and initial/regular training. Box plots display the medians, interquartile ranges from 25% to 75%, whiskers (minimum and maximum values) and outliers (dots) for values higher or lower than 1.5 interquartile range. Outlier at 89 µg g^{-1} faecal IgA 24 h after the change from group to individual housing is not shown by the figure. ***p < 0.001, *p < 0.05.

(d) Two months of regular training and individual stabling

Compared with baseline values FGMs tended to increase by 13.1 ng g^{-1} (Study 1 - Figure 1: GLM: FGM.difference $\sim$ test situation, N = 11, t = 2.0, p = 0.05) after two months of individual stabling and regular training. IgA remained unchanged (Study 1 - Figure 1: GLM: IgA.difference $\sim$ test situation, N = 11, t = 0.6 p = 0.54). Motor laterality significantly shifted to the left by LI −0.24 (Study 1 - Figure 2: GLM: ML.difference $\sim$ test situation, N = 11, t = −2.4, p = 0.02). Sensory laterality remained unchanged (Study 1 - Figure 2: GLM: SL.difference $\sim$ test situation, N = 11, t = −1.5, p = 0.14).

a. change from group to individual housing - b. 1 week individual stabling
c. initial training - d. two month regular training & individual stabling
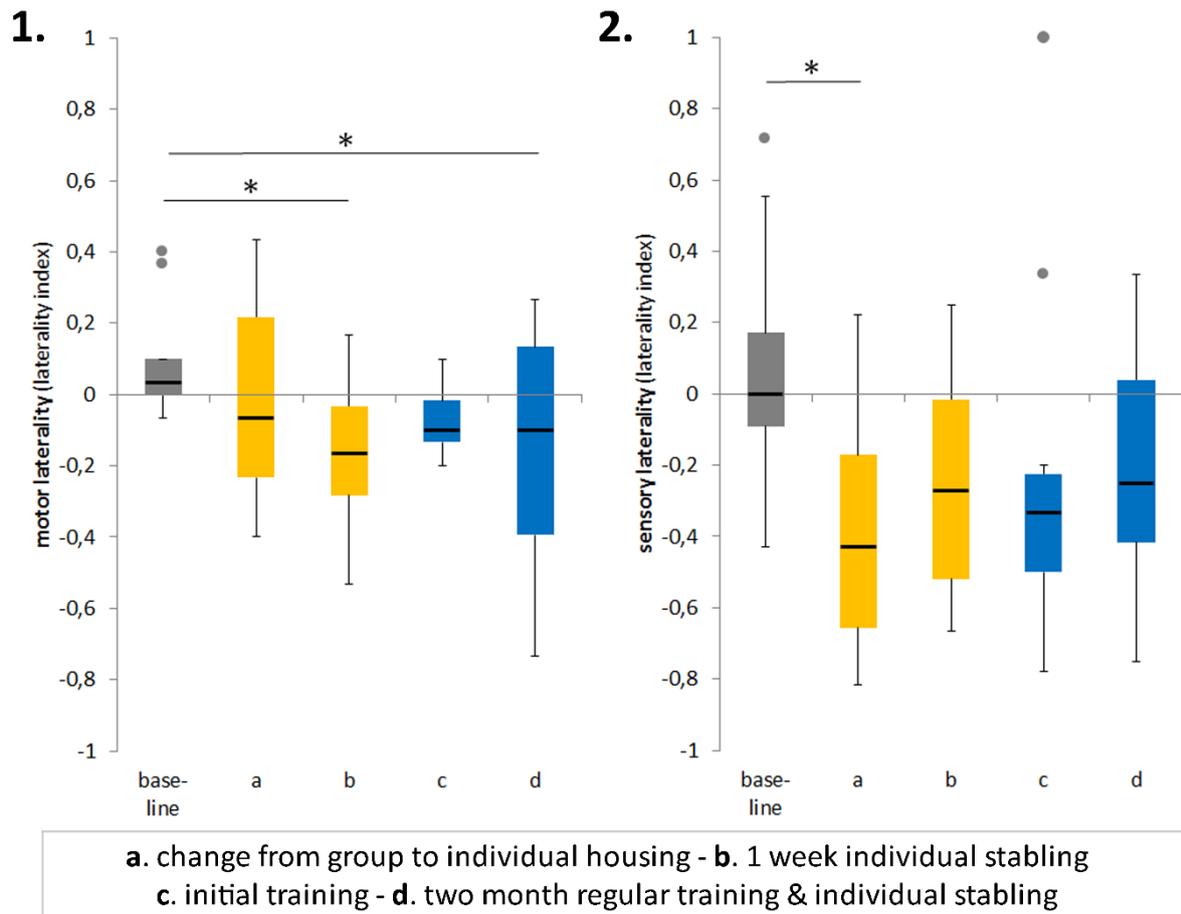
Study 1 - Figure 2: Motor and sensory laterality indices. Motor laterality (1) and sensory laterality (2) in test situation without stressors (base, during group housing) and after the change of housing condition (a), one week of changed housing condition (b), after initial training (c) and two months of regular training and individual stabling (d). Yellow: changed housing conditions, blue: combination of individual housing and initial/regular training. Box plots display the medians, interquartile ranges from 25% to 75%, whiskers (minimum and maximum values) and outliers (dots) for values higher or lower than 1.5 interquartile range. *$p < 0.05$.

5.   Discussion

The change from group to individual housing and the combination of individual housing with initial/regular training caused related stress responses indicated by changes in FGMs and motor laterality. After two months, FGMs continued to show an elevated trend, and the motor laterality changes persisted. Significant left shifts in sensory laterality occurred in parallel with increases in the established stress parameter FGMs, and this provides more insight into acute stress responses resulting from changed housing conditions. IgA concentrations did not change significantly. The change from group to individual housing is considered to be a long-term stressor [1,2]. In the present study, significantly increased FGMs suggested an acute stress response that declined after one week. Afterwards FGMs tended to remain elevated compared with baseline. Therefore, it is presumed that the horses did not experience severely and permanently compromised welfare which may reduce GMs as described elsewhere [18]. In addition, motor laterality shifted to the left as predicted by other researchers [36]. Prior to the initial training the observed left shift in the present study was not caused by one-sided training [35,37,53], as the horses were already accustomed to being handled from the left in their previous social housing. Therefore, the results support the proposal that motor laterality

is a suitable behavioural parameter to supplement physiological stress assessment for long-lasting stress conditions [25,26].

Sensory laterality may also be a suitable behavioural parameter for assessing acute stress responses as it shifted significantly to the left with the change from group to individual housing. As a left shift in sensory and motor laterality was found in parallel with the increase of FGMs, and as the right brain hemisphere controls cortisol secretion in emotional situations [66], it is suggested that the dominance of the right hemisphere in the observed stressful situations caused the left shift in sensory and motor laterality [25,26].

As long-term stress has been repeatedly reported to have immuno-suppressive effects [3,19,21,67], a decreased IgA concentration was expected. However, IgA concentrations remained unchanged. As indicated by the other stress parameters, the stress in the specific test situations may not have been strong and long enough to trigger decreases in faecal IgA concentration [8,21]. Obviously, further research is required, especially on the effect of chronic stress on IgA in horses.

All the parameters showed large inter-individual variation. This may have been due to individual stress sensitivity, perception and the processing of the stressor [3,5,6]. For some horses, the ongoing training and the novel housing may have become routine more quickly and led to a decline in stress responses [68]. Therefore, further research on individual responses is required.

This study shows that the investigated acute and long-lasting challenging situations caused changes in the investigated stress parameters. Sensory laterality appears to be a good behavioural parameter for the non-invasive evaluation of acute stress responses, such as the change from social to individual housing, that involve a change in environment as well as a restriction in movement and reduced contact with conspecifics. Sensory laterality changed more quickly and was more situation related than motor laterality. However, it remains unclear whether the left shift in motor laterality in these maturing horses would persist in continued individual housing and training, as the experimental period lasted only for two months. This requires further research. Besides implicating animal welfare issues [25], a left shift in sensory and motor laterality indicating an increased information processing by the right hemisphere could indicate training and handling issues. A higher left eye preference in animals has been reported to show enhanced emotionality or increased fearful behaviour [36,44], and so may indicate an increased likelihood of unpredictable and dangerous reactions during handling by humans. Furthermore, a significant increase in the horse's emotionality would have disadvantages for training, as emotionality has been shown to be negatively correlated with trainability in horses [69]. Apart from this, it has been demonstrated that left-sided horses are more likely to treat an ambiguous stimulus as negative [29]. While low or moderate stress can enhance trainability, prolonged and/or elevated stress hormone concentration can cause memory disruption [70]. Moreover, less stressed, pasture-kept horses have been shown to reach training criteria more quickly than stabled horses [71] and to be easier to handle and to train [72]. In addition, stress may lead to a higher prevalence of abnormal behaviour, stereotypies and depressive-type behaviour [73,74]. Therefore, the easy and objective evaluation of sensory and motor laterality may help to improve animal welfare, and future research on laterality and stress should focus on stress levels, degree of left shifts [75], emotionality, mental health and consequences for handling and training. In stress situations similar to those in the present study behavioural parameters have been shown to be potentially more indicative in situations where stress hormones did not show any change, for example, after changed housing conditions [76]. Nonetheless, in addition to changes in laterality, the evaluation of other stress parameters is also recommended for a reliable assessment of stress responses [77]. Although the sample size of our study is low, based on the results it is expected that the observation of left shifts in motor and sensory

laterality may be helpful objective parameters for stress analysis in horses, especially on an individual level.

Faecal IgA was expected to be a sensitive, non-invasive parameter for detecting activity of sympathetic adrenal medullary system as a first step response to unpredictable mild acute stressors. However, in the comparatively more challenging and long-lasting test situations (b and d), IgA concentration may have been a result of downregulated adaptive immune function. In follow-up studies, stronger and more isolated stressors are needed to analyse whether chronic stress results in faecal IgA suppression.

6.   Conclusion

Although further research is needed to fully understand the relationship between the investigated parameters, this study opens up new non-invasive stress parameters for evaluating animal welfare. Sensory laterality is a promising non-invasive parameter that may help to avoid exposing animals to additional stress through invasive sampling procedures. This study indicates that potentially stressful situations change sensory laterality in horses. Further research on a larger sample size is needed to evaluate chronic stress at greater intensities. Other studies have demonstrated that individuals differ in their coping strategies and ability to adapt to stress in novel and/or unnatural situations. The non-invasive parameters used in this study may allow animal welfare to be evaluated on an individual level, and motor and sensory laterality are easy to assess. Laterality may therefore be a promising parameter to help lay persons to identify stress in their horses, eliminate or reduce the cause and improve the horses' welfare.

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STUDY 2

Evidence for Right-Sided Horses Being More Optimistic than Left-Sided Horses

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Simple Summary

Behaviour that indicates stress or poor welfare in horses can be very subtle and, especially for the lay person, difficult to assess. Furthermore, the absence of such behaviour does not necessarily indicate a healthy mental state. Therefore, this study aimed to investigate whether a preference for the left or right forelimb in different tasks (motor laterality) or for left or right sensory organs (sensory laterality) indicates a positive mental state (positive cognitive bias—optimism) or negative mental state (negative cognitive bias—pessimism). This study demonstrates that horses that use the right forelimb more often when starting to move off from a standing position (initial forelimb use) are more likely to expect a neutral stimulus to be positive and to be in an optimistic mental state than horses that use the left forelimb. This knowledge about the horses' mental state can help us to improve their welfare by minimizing negative events. Furthermore, evaluation of the mental state of animals by determining motor laterality is quicker and easier than conventional tests for cognitive bias that include a long period of training.

Abstract

An individual's positive or negative perspective when judging an ambiguous stimulus (cognitive bias) can be helpful when assessing animal welfare. Emotionality, as expressed in approach or withdrawal behaviour, is linked to brain asymmetry. The predisposition to process information in the left or right brain hemisphere is displayed in motor laterality. The quality of the information being processed is indicated by the sensory laterality. Consequently, it would be quicker and more repeatable to use motor or sensory laterality to evaluate cognitive bias than to perform the conventional judgment bias test. Therefore, the relationship between cognitive bias and motor or sensory laterality was tested. The horses (n = 17) were trained in a discrimination task involving a box that was placed in either a "positive" or "negative" location. To test for cognitive bias, the box was then placed in the middle, between the trained positive and negative location, in an ambiguous location, and the latency to approach the box was evaluated. Results indicated that horses that were more likely to use the right forelimb when moving off from a standing position were more likely to approach the ambiguous box with a shorter latency (generalized linear mixed model, $p < 0.01$), and therefore displayed a positive cognitive bias (optimistic).

1. Introduction

Emotions are closely associated with cognition. Cognition can initiate emotions, and emotions influence cognition, resulting in a cognitive bias [1], which leads to an enhanced positive or negative perception or expectation of neutral stimuli. Positive and negative emotions evolved to support the search for valuable resources and to prevent the organism from harm [2,3]. They are the result of attention, perception, and memory [3]. The measurement of cognitive bias can help in the assessment of animal welfare, as the absence of stress parameters does not necessarily indicate a healthy mental state. Unpredictable or unenriched housing has been shown to reduce anticipation of positive events (negative cognitive bias—pessimism) in rats [4,5] and starlings [6], while the change to enriched housing or release from a stressful situation results in enhanced anticipation of positive events (positive cognitive bias—optimism) in non-human primates [7], rats [8], sheep [9], pigs [10], and horses [11,12]. Specific stereotypic behaviours that are indicators of poor animal welfare are associated with

a negative cognitive bias in starlings [13] and mice [14]. The measurement of cognitive bias in animals by conditioning them to discriminate between a positive and negative stimulus is time-consuming and limited by the learning effect relating to the ambiguous stimulus, resulting in low repeatability [15]. Therefore, other parameters are needed.

Motor and sensory laterality may be such indicators. They describe the preferred use of the left or right forelimbs and sensory organs, respectively. In common marmosets, the perception of an ambiguous stimulus as positive has been associated with right-handedness and the perception of an ambiguous stimulus as negative or threatening with left-handedness when picking up food in a relaxed situation [16]. This indicates a link between cognitive bias and motor laterality. The limbs and sensory organs (with the exception of the olfactory organs) are connected to the contralateral brain hemisphere [17]. The right brain hemisphere mainly controls withdrawal behaviour and responses to stress, novelty, social interactions, and predators, and is connected to the left side, whereas the left hemisphere is generally responsible for categorization of stimuli, routine situations, and approach behaviour and is connected to the right side [18–23]. The observation of motor laterality indicates the predisposition to process information in the left or right brain hemisphere [16]. Therefore, motor laterality may be an indicator for cognitive bias when neutral stimuli are presented. Individuals that prefer the left forelimb may have a more pessimistic outlook and treat an ambiguous stimulus as threatening, and individuals that prefer the right forelimb may have a more optimistic outlook and treat the ambiguous stimulus as positive. Sensory laterality is an indicator for brain specialization of perceptual functions [24] and is more flexible, changes faster, and more situation-related than motor laterality [25]. Therefore, it may be a better predictor for cognitive bias, as it better displays the spontaneous reaction to a novel, ambiguous object and indicates the hemisphere that was used first to process the incoming information, according to the individual's positive or negative expectations. Horses (Equus caballus) display motor and sensory laterality depending on their breed [20], age [26], social interaction [23], stress load [25], emotionality [27,28], and the type of incoming or processed information [29]. The strength of motor laterality may also depend on conformation characteristics [30]. Both sensory and motor laterality are easy to assess: motor laterality can be observed while the horse is grazing on pasture or eating hay, and sensory laterality can be tested in a novel object test. Furthermore, horses' laterally positioned eyes and ears facilitate the evaluation of sensory laterality.

The traditional measurement of cognitive bias is restricted due to a learning effect that has been demonstrated in sheep which show significantly fewer approaches to an ambiguous stimulus in repeated tests [15]. In comparison, the assessment of laterality is not restricted and is less time consuming.

Therefore, the aim of the study was to test whether motor and/or sensory laterality is related to cognitive bias. Seventeen horses were trained in a discrimination task to test for cognitive bias using laterality as the parameter. As different methods of measuring motor laterality have been shown not to correlate in their results for preferred forelimb use in horses [22], three different methods were implemented and compared (forelimb position in a relaxed situation, forelimb position in a task-related situation, and the initial forelimb used when starting to move). The following questions were addressed: Which measurement of laterality correlates with cognitive bias, and does right-sidedness indicate a positive cognitive bias?

2. Materials and Methods

2.1. Animals and Location

The study was conducted during winter and spring 2017/2018 with 17 domestic horses at three different facilities (facility 1: N = 5; facility 2: N = 4; facility 3: N = 8) in Nuertingen, Germany. There were ten geldings and seven mares of different types: warmblood (N = 10), pony (N = 6), and thoroughbred (N = 1). As motor laterality starts to increase at two years of age [26], subjects aged three to 26 years (with a median of 13 years) were tested. We assumed the subjects would include a broad range of left-sided, right-sided, and ambilateral horses. The horses were housed either in group housing (N = 8, five horses at facility 1 and three at facility 3), or in individual boxes with access to paddocks at any time, pasture, and contact with conspecifics (N = 9, four horses at facility 2 and five at facility 3). The horses' individual needs for roughage and supplementary feed were covered. All horses were in healthy condition (no lameness, no acute illness) and had been familiar with general handling from the ground for at least one year. They were either unridden, trained only from the ground, or ridden for a maximum of one hour per day.

2.2. Experimental Procedure

The training and testing of cognitive bias was adapted from previous cognitive bias studies on horses [11,12]. The horses were habituated to the experimental box and learned to discriminate two locations: (a) with a reward ("positive location") and (b) with no reward ("negative location"). Afterwards they were tested for their expectations of a positive event [31] when confronted with a third location ("ambiguous location"). In the following three days of training and testing, the motor laterality of each horse was observed and the sensory laterality was calculated from a novel object test.

2.3. Cognitive Bias Test

2.3.1. Apparatus and Test Arena for Cognitive Bias

A wooden box with a lid was used (30 cm 30 cm 22 cm) and magnets held the lid shut. The horses could open the box by pushing the lid with their muzzles, and in the case of the positive location, they would then be able to eat the carrot inside. The lid could be locked by inserting a stick underneath it, which prevented the horse from opening the box and reaching the carrot inside. The box always contained a carrot, but it was set up to be openable at the positive location and to remain locked at the negative and ambiguous locations. The experiment was carried out either in an indoor or outdoor riding arena, in a fenced area of 20 20 m. The box was placed 4 m to the left or right of the midline and 10 m from a starting line, so that the horses had to walk 10 m to each location (right, middle, left). The starting line was marked with two poles laid on the ground to the left and right and a space of 1.5 m (start point) between them for the horses to walk through. All trials were recorded with a camera (Panasonic Lumix DMC-TZ8, Panasonic Marketing Europe GmbH,Wiesbaden, Germany) behind a cover, in direct line with the starting point and the ambiguous location.

2.3.2. Habituation, Habituation Area, and Training

The habituation of the horses to the experimental box was conducted separately for each horse either in the individual's own paddock for the horses living in paddock boxes, or in the grooming area for those in group housing. First, the horse was allowed to eat pieces of carrot from the box with the lid open. Then, in the training phase, a carrot was placed in the box and the lid was closed. The training

criterion was reached when the horse opened the box and ate the carrot three times consecutively. There were 10 training sessions, each consisting of six trials: three with the box in a positive and three in a negative location. The locations were in a pseudorandom order with no more than two consecutive trials with the box in the same location, with the exception of the first training session. In the first session, all horses received the same order (two trials positive location, two trials negative location, one trial positive location, one trial negative location). The horses were randomly assigned to have either the positive location on the left (N = 8) or on the right (N = 9). At the start of a trial, experimenter 1 led the horse on a halter and lead rope to the starting point, where it was stopped at a 90° angle to the start line with its forelimbs on the starting line. Experimenter 1 then turned her back to the experimental setup, removed the lead rope from the horse's halter, and stood still. When the horse was free to move it was allowed 60 s to approach the box. If the horse did not approach the positive location, open the box, and eat the carrot within 60 s on the first training day, experimenter 1 led the horse to the box and allowed it to open the box and eat the carrot. If the horse did not approach the negative location within 60 s on the first training day, experimenter 1 led the horse to the box and allowed it to investigate the box for about 5 s. After each trial, experimenter 1 caught the horse, led it back to the starting line, and held it facing away from the experimental set up. Then, experimenter 2 walked to the box and refilled it with a carrot if necessary, and/or relocated it (according the pseudorandom order for each horse), before the next trial started. Experimenter 2 also recorded all trials with the camera.

2.3.3. Test

Before the horses were confronted with the box in the ambiguous location (i.e., in the middle, between the positive and negative location) they were confronted once with the positive and the negative location of the box for repetition, and randomly assigned to either positive (N = 6) or negative (N = 11) location first. To test the cognitive bias, the box was locked and placed in the ambiguous location. When the horses approached the ambiguous location, we observed the latency of the approach and for how long the horses investigated and tried to open the box within the 60 s. To avoid habituation and learning effects [15,32], the horses were tested only once with the ambiguous location and the horses' spontaneous reactions were recorded.

2.3.4. Analysis of the Horses' Performance

The analyses were based on the video recordings. To establish whether the horses learned to discriminate between the positive and the negative locations in the training trials, latencies were measured from the time of removal of the lead rope at the starting position until the first contact with the box. If the horse did not approach the box within the 60 s, a latency of 60 s was recorded. The horse was considered to have learned to discriminate between the negative and the positive locations when the latency to approach the positive location was significantly shorter than for the negative location in training sessions 8 to 10. When testing the horses' approach to the ambiguous location, the latency was measured in the same way as for the training. For horses that approached the ambiguous box within the 60 s, we observed how long they investigated the box (total time the horse was in contact with the box, i.e., nose, muzzle, hoof). Those that approached the box were categorized as optimistic and those who did not approach the box were categorized as pessimistic.

2.4. Laterality

2.4.1. Motor Laterality

Motor laterality was assessed independently in three different ways. Firstly, we measured motor laterality in a relaxed situation [21] and termed it "relaxed forelimb position". We observed how often the left or right forelimb was placed in front while eating hay in the box and/or grazing on pasture in a relaxed situation. A lateral position was documented when one of the front feet was one hoof length or more in front of the other. Otherwise, an ambilateral forelimb position was documented. Sixty observations per horse were spread over three days and conducted at 30 s intervals. The motor laterality was observed once after the last two training sessions and once after the testing session.

A second motor laterality measurement (termed "initial forelimb use") was taken from the video recordings of the cognitive bias training. We observed which forelimb was used by each horse when it moved off from the starting point [33]. This yielded a maximum of 60 observations and a minimum of 53 for each horse, depending on how many times they moved away from the start point.

A third motor laterality evaluation was measured (termed "task-related forelimb position"), again from video recordings of the cognitive bias training. We observed which forelimb was initially placed in front or whether horses stood ambilaterally while opening or investigating the box. A lateral position was documented when one of the front feet was one hoof length or more in front of the other, otherwise an ambilateral forelimb position was documented [30]. This yielded a maximum of 56 observations and a minimum of 45 observations for each horse, depending on the number of times they ate from or investigated the box.

Laterality indices (LIs) were calculated separately for all three measurements and for each horse [21,34]. The formula $LI = (R - L)/(R + L)$ was used for the initial forelimb use and the formula $LI = (R - L)/(R + L + A)$ was used for relaxed forelimb position and task-related forelimb position. R describes the number of observations of right forelimb use, L the number of observations of left forelimb use, and A the ambilateral use of both forelimbs. A positive LI indicates a preference for the right forelimb and a negative LI indicates a preference for the left forelimb.

2.4.2. Sensory Laterality

Sensory laterality was observed by ad libitum sampling for each horse during a novel object test, conducted either in its paddock or in the grooming area, following the cognitive bias tests. Novel objects were placed individually one to two meters in front of the horse and the side of the head initially used to investigate the object (left, right, or ambilateral) was observed. Lateral sensory organ use was counted when the horse approached the object with a divergence of more than 5° from a straight line (i.e., from a 90° positioning to the object), turned the head more than 5° when inspecting the object, or clearly touched the object with a specific side of the head for inspection with the eye, nose, or ear. The horses were confronted with a total of nine objects in a random order over three days. The objects were a yellow/black (random pattern) cone, a ball, a plastic bottle filled with water, a folded blue plastic tarpaulin, a yellow swimming noodle, a coloured tube, a box with white and red striped barrier tape, a piece of white polystyrene, and a folded silver windscreen cover (all sized 20 cm to 70 cm in height). A laterality index (LI) was calculated as described for the relaxed forelimb position and task-related forelimb position (see Section 2.4.1. Motor laterality) using the following formula: $LI = (R - L)/(R + L + A)$.

2.5. Inter-Observer Reliability

Inter-observer reliability was calculated from two observers' analysis of eight horses' latency to approach the box (training: Spearman's rho = 0.95, and testing: Spearman's rho = 0.98), of the three measurements of motor laterality (when grazing/hay eating (relaxed forelimb position): Spearman's rho = 0.97, when starting to move off from a standing position (initial forelimb use): Spearman's rho = 0.86, and at the experimental box (task-related forelimb position): Spearman's rho = 0.88), and of the sensory laterality observation (Spearman's rho = 0.97).

2.6. Statistical Analysis

RStudio (version 0.99.484, Boston, MA, USA) and the package R commander (version 2.2.1) were used for the statistical analysis. Figures were constructed with Microsoft Excel 2010 and the cs-tool of Microsoft Excel (Microsoft Corporation, Washington, DC, USA). As the data were not normally distributed (Shapiro-Wilk test: most $p < 0.05$), non-parametric tests were used. The Spearman rank correlation was used to test whether the three different measurements of motor laterality were correlated. The Wilcoxon signed rank test for repeated measurement was used to analyse whether the horses learned to discriminate between the positive and the negative locations by comparing the latency to approach the two boxes. A Wilcoxon signed rank test for repeated measurement was used to compare the time spent investigating the ambiguous box with the mean time spent investigating the box at the negative location in the last three training sessions. A generalized linear mixed model (GLMM) with random effects (age, facility, breed, housing, sex) and fixed effects (positive side, last location before testing with ambiguous location) was used to test whether the latency to approach the ambiguous box was affected by the fixed or random factors, i.e., the laterality measurement: formula = latency to approach ambiguous box ~ positive side + last location before testing with ambiguous location + laterality/(age + facility + breed + housing + sex), family = Gaussian (identity). Non-significant random factors were removed step-wise to simplify the models and to improve the models' goodness of fit only when their deletion did not cause a significant reduction of the models' goodness of fit. The results are presented after model simplifications with the best goodness of fit. The complete full models and the reduced model are presented in the supplementary materials (STUDY 2: Results S1). All tests were two-sided. The significance level was set at 0.05.

3. Results

3.1. Correlation between the Three Measurements of Motor Laterality

No significant correlations between the three measurements of motor laterality (relaxed forelimb position, initial forelimb use, and task-related forelimb position) were found (Spearman N = 17, all p > 0.05). However, there was a weak correlation trend between the relaxed forelimb position and the task-related forelimb position (Spearman N = 17, rho = 0.42, p = 0.09). We therefore proceeded to analyse the test parameters separately for each motor laterality measurement.

3.2. Training Criterion

All horses reached the training criterion of learning to distinguish between the positive and the negative locations of the feed box; the latency to approach the positive location (median = 8 s) was significantly shorter than the latency to approach the negative location (median = 60 s) in all horses (Wilcoxon test N = 9, V < 3.5, p < 0.05 for all horses).

3.3. Latency to Approach Ambiguous Box and Laterality

Horses that were faster to approach the ambiguous box showed a significantly higher laterality index in the initial forelimb use, indicating that they were more right-sided in their forelimb choice when starting to walk (GLMM: ambiguous box ~ initial forelimb use, N = 17, t = -3.71, p = 0.002, Study 2 - Figure 1). Furthermore, horses in facility 1 (group housing, N = 5) that were quicker to approach the ambiguous box also had a significantly higher sensory laterality index, i.e., they were more lateralized to right sensory organ use (GLMM: ambiguous box ~ sensory laterality/(facility), N facility b = 5, t facility = -2.73, p facility b = 0.02). However, there was no significant relationship between the latency to approach the ambiguous box and the relaxed forelimb position (GLMM: ambiguous box ~ relaxed forelimb position, N = 17, t = 0.38, p = 0.71) or the task-related forelimb position (GLMM: ambiguous box ~ task-related forelimb position, N = 17, t = -0.82, p = 0.42), nor was there any relationship across all facilities between the latency to approach the ambiguous box and the horses' sensory laterality (GLMM: ambiguous box ~ sensory laterality/(facility), N = 17, t = 1.00, p = 0.34).
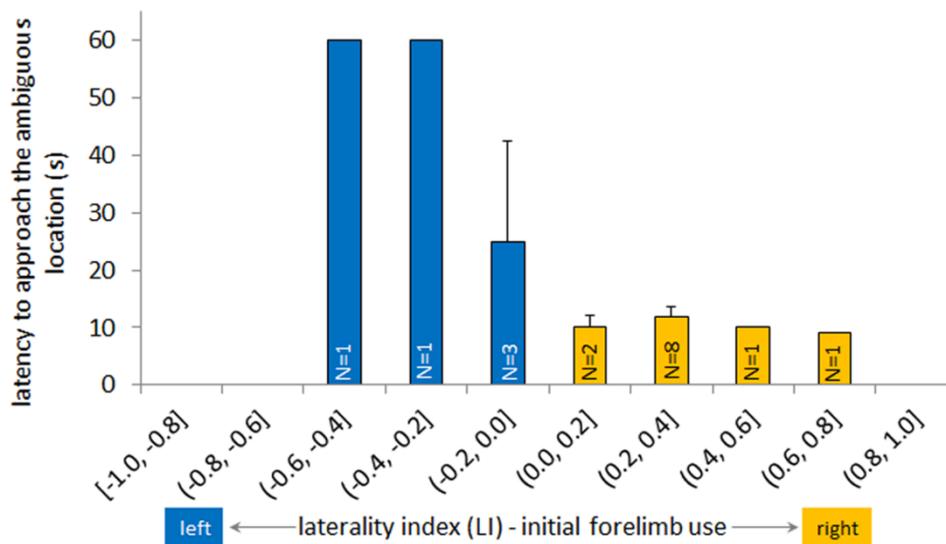
Study 2 - Figure 1: Mean latency to approach the test box in the ambiguous location compared to the initial forelimb use. Horses with a laterality index of the initial forelimb use lower than 0 (blue) started walking from the starting position more often with the left forelimb, and horses with an index higher than 0 (yellow) started more often with the right forelimb (GLMM: ambiguous box ~ initial forelimb use, N = 17, t = −3.71, p = 0.002). Horses which needed more than 60 s were considered not to have horses: one horse at (−0.6, −0.4], one horse at (−0.4, −0.2], one horse at (−0.2, 0.0]). The mean latency and the standard error are shown. GLMM: generalized linear mixed model.

3.4. Time Spent Investigating the Ambiguous Box

Fourteen horses approached the ambiguous box, and the time spent investigating it was significantly longer than the time spent investigating the negative box during the last three training sessions (Wilcoxon test N = 14, V = 92, p = 0.01, Study 2 - Figure 2).
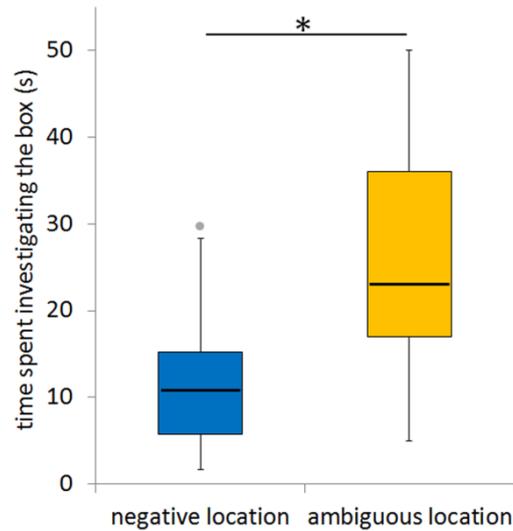
Study 2 - Figure 2: Time spent investigating the box at the ambiguous location (i.e., the novel location where the horses did not know whether it could be opened) compared with mean time spent investigating the box at the negative location (i.e., the location where the horses knew it was locked) during the last three training sessions (N = 14). * p < 0.05.

4. Discussion

Horses that used the right forelimb more often when starting to move from the starting position (initial forelimb use) were more likely to treat the ambiguous box as positive and to approach it. The horses that approached the box at the ambiguous location seemed to expect an unlocked box that would allow them to eat the carrot inside. They investigated and tried to open the box for significantly longer than when the box was at the negative location, demonstrating an optimistic manner. Therefore, these horses can be considered more optimistic than horses that used the left forelimb more often. The latter hesitated or did not approach the box within the given time of 60 s. In common marmosets, the handedness in a relaxed situation (picking up food) is correlated with a cognitive bias [16]. In horses, cognitive bias was not related to the relaxed forelimb position or to the task-related forelimb position; it therefore remains debatable whether motor laterality measurement can be compared between species [35]. In future research on horses, a careful choice should be made on the method used to assess motor laterality, as the three methods of the present study did not correlate, as already demonstrated in other studies [22]. The forelimb preference seems to be dependent on the task, situation, and the strength of laterality on conformation characteristics [30].

Sensory laterality may not be a reliable measure of an overall cognitive bias in horses, as it changes too quickly and is too flexible [25]. The effect of the facility on the cognitive bias and the sensory laterality may have been a result of management, environment, and/or environmental changes, and these factors require further investigation. Nonetheless, further research is needed to answer the question of whether right-sided sensory laterality predicts a more optimistic manner in horses in general, or whether the strength of sensory laterality predicts the emotionality of a horse, as a preference for left sensory organs has been observed in both positive and negative situations [23,28,29].

Cognitive bias testing using a judgment bias test is limited because of the learning effect [15] and the time-consuming training procedure; as such, the measurement of initial forelimb use may be a promising method for assessing cognitive bias in horses, and especially in the repeated assessment of

cognitive bias. Furthermore, the use of a judgment bias test to test for cognitive bias has limited application in the assessment of animal welfare because the cognitive bias training/testing procedure is a release from the investigated stressful situation. That stress release may result in a positive cognitive bias, even though the stress hormones may still be elevated from the investigated stressful situation (sheep: [9], horses: [11]). It remains to be seen whether a stress-induced left-shift in motor laterality [25] is indicative of the development of a pessimistic cognitive bias, and which type of motor laterality measurement may be a more reliable parameter. In rats, there is a link between vulnerability to stress-induced pessimism and cognitive bias [36]. Therefore, motor laterality may be a promising indicator not only of the horses' cognitive bias but also of the vulnerability to stress-induced pessimism, and may be helpful in the selective breeding of less stress-prone horses and in improving animal welfare. This needs to be investigated in future research. Another interesting topic for future research would be whether the initial forelimb choice can be manipulated, e.g., by positioning the forelimbs in special relation to each other (standing square, left or right forelimb advanced), as in the present study we did not manipulate or train the horses to stop and stand in a special manner.

5. Conclusions

A preference for the use of the right forelimb when moving off from a standing position (initial forelimb use) indicates an optimistic manner/positive cognitive bias in horses in specific context and object investigation. There was no relation between the cognitive bias and the other laterality measurements (task-related forelimb position, relaxed forelimb position, sensory laterality). The knowledge of the animals' cognitive bias, and therefore its emotions, can help to improve welfare by enabling negative events to be minimized [2], but further research is needed on the accurate measurement of motor laterality.

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STUDY 3

Sensory laterality in affiliative interactions in domestic horses and ponies (Equus caballus)

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Abstract

Many studies have been carried out into both motor and sensory laterality of horses in agonistic and stressful situations. Here we examine sensory laterality in affiliative interactions within four groups of domestic horses and ponies (N = 31), living in stable social groups, housed at a single complex close to Vienna, Austria, and demonstrate for the first time a significant population preference for the left side in affiliative approaches and interactions. No effects were observed for gender, rank, sociability, phenotype, group, or age. Our results suggest that right hemisphere specialization in horses is not limited to the processing of stressful or agonistic situations, but rather appears to be the norm for processing in all social interactions, as has been demonstrated in other species including chicks and a range of vertebrates. In domestic horses, hemispheric specialization for sensory input appears not to be based on a designation of positive versus negative, but more on the perceived need to respond quickly and appropriately in any given situation.

Keywords: Laterality; Equine; Affiliative; Behaviour

Introduction

Specialization in the functions of the two hemispheres of the brain has been well catalogued and is believed to have its origins in brain asymmetry in early vertebrates (Mac- Neilage et al. 2009). There is therefore increasing interest in the details of asymmetry of brain function and the different ways in which information is processed and interpreted by each hemisphere. Asymmetry has been observed in many taxa, including mammals, birds, fish, and even insects (for overview see Rogers 2017), and may be expressed as motor laterality (usually limb preference), or sensory laterality (preferential use of a sensory organ on one side of the body). It has been shown that in most situations motor laterality and sensory laterality are not correlated in horses (Austin and Rogers 2012; McGreevy and Rogers 2005), fish (Biazza and Brown 2011; Takeuchi and Hori 2008), new born humans (Cioni and Pellegrinetti 1982), and rhesus monkeys (White et al. 1994).

The close connection between humans and horses as sport and leisure partners makes the understanding of laterality in horses important, as it potentially has wide ranging implications for the welfare and safety of both the horses and the humans. For example, if horses have a preferred side for social interaction, this could be an indication of how training and handling can be carried out most effectively and safely. Rogers (2004) found that chicks that were not lateralized were slower to respond to a potential predator than lateralized chicks and proposed that lateralisation of the brain may have an evolutionary benefit for animals with sideplaced eyes, as it allows for dual attention. This enables, for example, simultaneous attention to be given to foraging and predator vigilance. Additionally, lateralisation may facilitate appropriate reaction to unexpected stimuli as proposed by Austin and Rogers (2007).

To date, research in this field has focussed mainly on aggressive behaviour, stressful situations and negative emotions, in which a preference for left side, and therefore dominance of the right brain hemisphere, has been found consistently. Larose et al. (2006) found emotionality in horses to be linked to using the left eye to observe a novel object, and Austin and Rogers (2007) found stronger reactions to an unexpected stimulus (an opening umbrella) when it was presented on the horse's left side. Additionally, Smith et al. (2016) observed a left eye bias and an increased heart rate when horses were presented with photographs of an angry-faced human, while Austin and Rogers found a left bias in agonistic and vigilance behaviour in free roaming feral "Brumby" horses (2012) and Przewalski horses

(2014). Similar left biases have also been shown in male tree lizards (Hews and Worthington 2001), dogs (Siniscalchi et al. 2010), Australian magpies (Koboroff 2008), and cattle (Robins and Phillips 2010).

In humans, it has been proposed that there is a hemispheric divide in the processing of emotion with the left hemisphere processing positive emotion, and the right hemisphere processing negative emotion (Davidson and Tomarken 1989; Canli et al. 1998; Godfrey and Grimshaw 2016). However, other studies such as Borod et al. (1998) have suggested that the right hemisphere may be used for all emotional processing, and according to Davidson (1992) frontal and anterior areas of the brain differ in the processing of positive and negative emotions, with the right frontal region more strongly active for negative emotions, and posterior regions of the right hemisphere more strongly involved in the perception of positive emotions. Kilgore and Yurgelun-Todd (2007), on the other hand, propose that these various hypotheses may not actually be in opposition, but may instead reflect different facets of a complex distributed emotion processing system.

In non-human primates, numerous studies have shown emotion of all types to be processed in the right hemisphere (overview Lindell 2013), but in horses, while there is a large body of research on laterality in stressful and agonistic situations, there has so far been no dedicated research into sensory laterality in positive interactions between conspecifics. Farmer et al. (2010) observed that domestic horses had a preference to have humans on their left side, and that this preference was stronger in conventionally trained horses, which are handled mostly from the left, than in bilaterally trained horses. Although it cannot be discounted that the presence of the human may have represented a form of stress which could have influenced the lateral preference shown, Karenina et al. (2017) found a left bias in mother–infant interactions across several mammalian species, including horses, which suggests that the right hemisphere is indeed used for positive as well as negative emotions.

Here we examine laterality in affiliative interactions in individuals, comparing groups of different social compositions and breeds. Domestic horses have diverse genealogies and phenotypes and are broadly categorised into (1) race and riding horses, (2) ponies and (3) draught horses (Pirault et al. 2013; Petersen et al. 2013). We therefore considered it possible that the hemispheric specialization might differ between the riding horses and ponies in this study. The groups included a mixed-sex group of riding horses, an all-female group of Mini-Shetland pony mares and foals, a Mini-Shetland pony harem group of one stallion and several mares, and an all-male group of Mini-Shetland pony stallions and colts. The specific questions we addressed were: (1) is sensory laterality in affiliative interactions normally distributed, or is one side preferred over the other; (2) if there is a side preference, is this affected by age, rank, sociability, gender, or phenotype of the individuals; (3) if there is a side preference, is it affected by the social composition of the group?

Methods, materials, and subjects

Subjects

Thirty-one privately owned horses and ponies took part in the study, all of them housed at the Aktivstall Mauerbach complex in the Vienna Woods, Austria. There were four groups, each of different social composition.

Group 1, riding horses (N = 10), comprised 4 geldings and 6 mares, aged from 2 to 22 years. They included 4 Warmbloods, 1 Sorraia Mustang, 1 Pryor Mountain Mustang, 2 Quarter Horses, 1 Icelandic horse, and 1 Haflinger. The group was housed in a "Hit Aktivstall", designed to cover the needs of horses as well as possible. The stabling covered approximately 2.5 hectares, (2500 m2 per horse) and included a rest and sleeping shelter (300 m2, enclosed on three sides, with three open doorways on the eastern side). The horses had 24-h access to grass pasture, straw fodder, and water from an

automatic dispenser. An automatic group hay feeder opened for 15 min, 16 times per day. There was also an individual automatic hay feeder and an individual automatic pellet feeder, which were programmed according to each horse's needs, with individual rations varying between 500 g and 2 kg per day. The feeders automatically portioned and dispensed the hay or pellets when activated by a transponder, worn either on a collar around the horse's neck, or woven into the horse's mane. To reach the pellet dispenser, the horses had to walk around a track of approximately 700 m. The stabling area also included three grass pastures, covering a total of 2 hectares, which were open to the horses 24 h a day. The horses shared their living quarters with two female donkeys, but as only one interaction was observed between a horse and the donkeys, the donkeys were not considered in the analysis.

Group 2, mares and foals (N = 8), comprised 5 Mini-Shetland pony mares, 3 with foals at foot. The foals, 2 fillies and 1 colt, were all between 3 and 6 months old at the time of observation, while the mares ranged from 1 to 20 years old. The group was housed on approximately 6400 m2 of grass pasture and woodland (800 m2 per pony) with two shelters, each of 20 m2, which were enclosed on three sides. There was a covered hay station providing ad libitum hay, and water was supplied in large buckets. The area was divided into two grass pastures, a sand enclosure where the hay station was positioned, and an area of woodland which offered shade. The ponies also received approximately 150 g of grain once a day.

Group 3, harem (N = 8), comprised 1 stallion and 7 mares, all Mini-Shetland ponies, aged 3–14 years old. The group was housed on 0.7 hectares of mixed grass pasture and woodland, (970 m2 per pony). Hay was provided in hanging dispensers and nets, as well as in fixed stands. Fresh water was available from a stream, as well as in large buckets. The ponies also received approximately 150 g of grain once a day. There were two shelters, each enclosed on three sides: one of 72 m2, one of 48 m2. The stallion was removed from the group for management purposes the evening before the final observation period; however, as the absence of a stallion has been shown to slightly increase social interaction in mares (Sigurjónsdóttir et al. 2003), we continued to collect data on the mares.

Group 4, stallions (N = 5), comprised 3 mature stallions and 2 yearling colts, all Mini-Shetlands, aged from 1 to 20 years old. The group was housed on a 2-hectare grass pasture (4000 m2 per pony), with shade provided by trees along one side and a small grove in the centre. There were two shelters each measuring 48 m2 and enclosed on three sides. The ponies shared this pasture with nine sheep, but there was very little contact between the sheep and the ponies. The grass was so plentiful that additional hay was not considered necessary, but the ponies did receive approximately 150 g of grain once a day. Water was supplied in buckets and automatic drinkers.

Observation

Groups 1, 2 and 3 were observed for 12 h each, and group 4 (which had fewer individuals) for 10 h, between July 4th and July 21st, 2017. Observation was carried out between 10 a.m. and 6 p.m., in periods of between 1.5 and 2.5 h. The observation periods for each group were randomized across the times of day, and no group was observed more than once on any 1 day. Observer 1 (KF) recorded each observation verbally on the voice recorder of a Samsung A3 mobile phone, and Volunteer 1 made video recordings of the observations on an iPhone6 as a backup and cross reference. The data from the recordings were transferred to an Excel 2013 sheet on a Packard Bell "Easy Note" laptop immediately after the observation period. The recordings and data sheets were then backed up on USB sticks. Volunteer 2 transcribed the voice recordings into text.

All the horses and ponies were already acclimatised to the presence of people, and the observation points were based between 10 and 30 m away from each group, although the precise distance depended on the movement of the horses/ ponies, and whether the observers had to move in closer

to see the details of an interaction. There was no point at which the horses and ponies appeared to be disturbed by the presence of the observers. When horses or ponies spontaneously approached the observers, they were gently encouraged to move away and return to other members of their group.

Volunteer 1 simply recorded the video and did not make any rating or comment and so could not be used for an interobserver rating. Therefore, a sample of 10% of the videos was shown to volunteer 3, who made an independent assessment of the behaviour. There was a high level of agreement between observer 1 and volunteer 3, with a Cohen's Kappa coefficient of k = 0.932.

Data collection

Affiliative interactions: approaches and interactions

We defined affiliative approaches by considering the behaviour of the approached horse. If the approached horse retreated more than two metres from the approaching horse, the approach was considered non-affiliative. If the approached horse did not move, moved towards the approaching horse, or moved less than 2 m to make room for the approaching horse, the approach was considered affiliative, as described by Schneider and Krüger (2012). Affiliative interactions typically included allo-grooming, swishing flies for each other, and standing in a proximity of less than 2 m for at least 15 s while grazing or resting. The side placement of equine eyes makes it easy to see whether one eye or the other is being preferred in any interaction. One horse approaching another with its left eye to the approached horse scored one point under "affiliative left" for the approach, or "affiliative right" if the approach was with the right eye. A further point was allocated both to the approaching and to the approached horse if the approach led to allo-grooming, nose to tail fly swishing, or just relaxing and standing within 2 m of each other for at least 15 s, according to the side of the interaction.

If a pair of horses switched sides, further points were allocated to each horse accordingly, and if a pair positioned themselves side by side, affiliative points were awarded to each horse according to the eye used for viewing the conspecific.

Interactions where a lateral choice could not be established (for example, a head-on approach) were not scored for the sensory laterality data but were included in the rank dominance calculations if appropriate. As head-on approaches only occurred in agonistic encounters, these were excluded from the affiliative laterality analysis.

Rank dominance observations

Rank dominance points were awarded based on retreats by either the approaching or the approached horse. The retreating horse was allocated one point under "lose" for a retreat, and the horse that was retreated from was awarded a point under "win". Non-affiliative interactions were defined as approaches with the ears pinned back and the nose extended, retreats, threats to bite or kick, bites, kicks and chases as described by McDonnell and Haviland 1995, and McDonnell 2003. Approaches and interactions were scored under the categories (1) affiliative left, (2) affiliative right, (3) win, and (4) lose.

Data and statistical analysis

Affiliative laterality index (ALI): an ALI was calculated for each horse, using the standard formula of (right eye score − left eye score)/total lateral interactions, as used by Austin and Rogers (2012). This gives scores between − 1 and + 1 with negative scores showing a left bias, and positive scores a right bias.

Social index (SI): an index was calculated for each horse using all interactions, where the SI = (affiliative interactions − non-affiliative interactions)/total interactions. This gives a number from − 1 to + 1, with positive numbers indicating relatively more affiliative behaviour.

Dominance Index: An average dominance index (ADI) was calculated as recommended by Hemelrijk et al. (2005). ADI = $1/N \Sigma j[x_{ij}/(x_{ij} + x_{ji})]$; N the number of interaction partners, x_{ij} the number of times the individual i won against conspecific j, x_{ji} the number of times individual i lost against conspecific j. ADI values range from 0 to 1, with a high value indicating a high rank in the group. Individuals were counted as a winner when their interaction partner retreated one step or more. Pairs that were not involved in an encounter with each other were excluded from the analysis.

The R Studio and R commander (version 3.4.1, 2017) were used to analyse the data and compare the laterality indices across groups, gender, rank, and social index. Figures and tables were compiled in Microsoft Excel 2016. The ALI was not normally distributed (Shapiro Wilk test). We therefore continued using non-parametric tests. We considered the numbers of literately indices to the left and the right for each individual and used a binomial test to analyse the level of bias on population and individual levels. Multivariate factor analysis [GLM, formula = ALI ~ age + phenotype + gender + group + rank + social index, family = Gaussian (identity)] was used to compare the four groups with respect to the variables of phenotype and group composition, and to compare the variables of age, gender, social index, and rank within the groups. All the tests used were two sided and the significance level was set at 0.05.

Results

A total of 2475 interactions (2043 affiliative and 432 nonaffiliative) were recorded among the 31 horses and ponies. Details of the interactions and categorizations are shown in Study 3 - Figure 1, and the raw data table is included in the supplementary material.

The ALI values within each group were independent of age, sex, rank, social index, phenotype, and group composition (GLM: N = 31, all $p > 0.05$). However, there was a weak trend for the riding horses to be more strongly lateralized (Wilcoxon rank sum test: N = 31, W = 65, $p = 0.09$) and this is illustrated in Fig. 2 in the supplementary material.

A binomial test indicated that the proportion of animals showing a left bias of 0.9 was higher than the expected 0.5 (binomial test, two sided: N = 31, $p < 0.001$). Additionally, 4 horses and 5 ponies, showed significant individual left preferences in their affiliative interactions (binomial test, two sided: all $p < 0.05$). See Study 3 - Table 1.
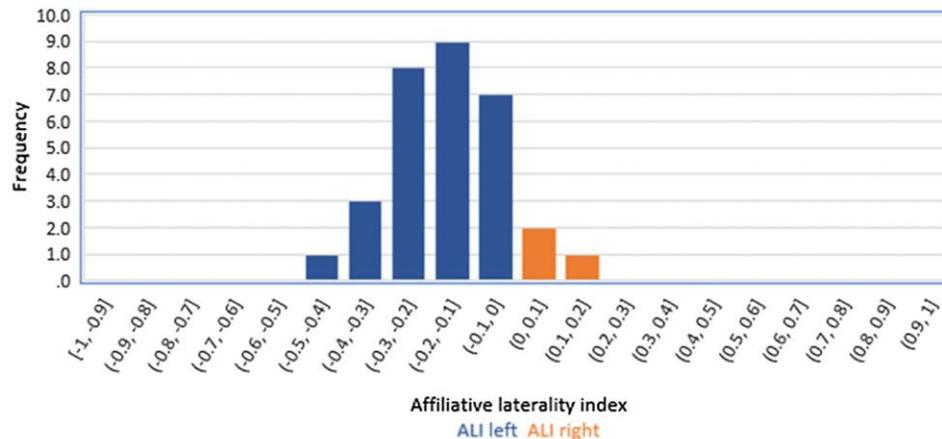
Study 3 - Figure 1: Distribution of affiliative laterality indices showing a clear bias to the left

Discussion

Our results do not support the hypothesis that lateral choices in affiliative interactions are normally distributed, but instead indicate a consistent and significant bias to the left. We found no evidence that this left bias is affected in direction or strength by age, rank, sociability, phenotype, or sex. The weak trend for the riding horses to be more strongly lateralized than the ponies was not significant, but further research with larger sample sizes is required to investigate this more thoroughly. McGreevy and Thompson (2006) found that motor laterality varied according to breed in performance horses, and Larose et al. (2006) found that a more emotional breed of horse (French Saddlebred) showed stronger sensory laterality in a novel object test than a more phlegmatic breed (Trotter), so it is certainly possible that sensory laterality in affiliative interactions may vary also according to breed and type.

Interestingly, the strength and distribution of the left bias we observed in the horses' affiliative interactions corresponds very closely to the left biases in agonistic and vigilance behaviour observed by Austin and Rogers (2012, 2014) in feral and Przewalski horses. This supports the theory that the right hemisphere is preferred for the processing of both positive and negative emotions as proposed by Davidson (1992).

Numerous studies have found that allo-grooming, and even grooming by humans, can significantly reduce a horse's heart rate (e.g. Feh and Mazières 1993; Normano et al. 2003), and activities such as allo-grooming and swishing flies do not appear to be stressful (Feh and Mazières 1993). These interactions are shown in this study to be lateralised to the left, as has been shown in comparable interactions in fish (Sovrano et al. 1999), chicks (Vallortigara and Andrew 1994) and numerous vertebrates (Karenina et al. 2017). This again suggests that the right hemisphere may specialise in processing social interactions and emotions, both positive and negative. In fact, de Boyer des Roches et al. (2008) reported that horses preferred to use their left eye to observe an object with a negative emotional association (a vet's jacket) and for an object with positive association (a feed bucket), while the right eye was preferred for a neutral object (a traffic cone).

Rogers (2017) proposes that the strength of laterality is of greater significance than the direction, and it has been shown that laterality increases with the level of concentration and task complexity in vervet monkeys (Harrison and Byrne 2000). It is therefore possible that the observed laterality in affiliative interactions is simply an indicator of how much attention the horse or pony is applying, and

how much emotional involvement it is experiencing. It is not necessarily an indicator of the nature of the attention or emotion, or whether the horse or pony is experiencing stress or eustress. Further research is needed into the factors that may influence the strength of sensory laterality in affiliative behaviour, and into the influence of specific breeding and training. This may then prove to be useful, together with physiological parameters, in the assessment of animal welfare.

Study 3 - Table 1: Raw data collected in July 2017 from horses and ponies at Aktivstall Mauerbach, Austria

Horse ID and gender	Age	Gender	Rank index	Social index	Total affiliative approaches and interactions	Left side affiliative approaches interactions	Affiliative and laterality index	* p<0.05, ** p<0.01
GROUP 1: Riding horses								
Alia	13	Mare	0,86	-0,17	22	12	-0,09	
Amaluna	2	Mare	0,17	0,89	102	55	-0,80	
Annie	19	Mare	0,32	0,76	87	58	**-0,33**	**
Bayladora	6	Mare	0,45	0,26	29	21	**-0,45**	*
Baika	22	Mare	0,55	-0,30	31	21	**-0,35**	*
Billy	14	Gelding	0,83	-0,45	16	10	-0,25	
Eco	11	Gelding	0,95	0,45	103	58	-0,13	
Kyakur	15	Gelding	0,25	0,87	119	64	-0,08	
Moon	16	Gelding	0,37	0,75	48	29	-0,21	
Sharon	2	Mare	0,02	0,94	96	61	**-0,27**	**
GROUP 2: Mini-pony mares and foals								
Zenith	11	Mare	0,93	0,75	47	27	-0,15	
Choco Chino	6 mths	Colt	0,25	0,96	102	59	-0,16	
Magreeth	20	Mare	1,00	0,66	29	14	0,03	
Cinne Bun	3 mths	Filly	0,22	0,97	134	83	**-0,24**	**
Sita	15	Mare	0,59	0,68	48	26	-0,08	
Buttercup	4 mths	Filly	0,47	0,92	136	82	**-0,21**	*
Tiramisu	1	Mare	0,14	0,78	41	25	-0,23	
Sara Jane	7	Mare	0,65	0,56	21	11	-0,05	
GROUP 3: Mini-pony harem								
Versace	9	Stallion	0,85	0,35	21	12	-0,14	
Sun Suena	3	Mare	0,26	0,67	61	34	-0,11	
Funny Honey	3	Mare	0,19	0,80	80	50	**-0,25**	*
Andromeda	3	Mare	0,72	0,24	50	28	-0,12	
Dusky	3	Mare	0,29	0,64	54	32	-0,19	
Blissful	3	Mare	0,08	0,88	47	27	-0,15	
Goldie	7	Mare	0,81	0,28	66	41	**-0,24**	*
Mascara	14	Mare	0,88	0,27	54	32	-0,19	
GROUP 4: Mini-pony stallions and colts								
Horatio	20	Stallion	0,40	0,90	59	32	-0,08	
Versace	9	Stallion	0,90	0,82	71	46	**-0,30**	**
**Amazonic	10	Stallion	0,83	0,85	63	28	0,11	
Frappuccino	1	Colt	0,11	0,98	100	49	0,02	
Toffee Popcorn	1	Colt	0,00	1,00	106	57	-0,08	

Conclusion

Based on the sample of 31 riding horses and Mini-Shetland ponies, this study shows for the first time that affiliative behaviour in horses and ponies is significantly left lateralised. This adds a new dimension to research into sensory laterality in equids which has, to date, focused on agonistic encounters, which also show a left bias. The fact that there is now evidence that processing of all social interactions is left lateralised means that, in practical and welfare terms, a low level of left laterality is to be expected and does not have implications as to whether a particular experience is positive or negative. The bias for social processing on the left is consistent with the traditional belief that new tasks should usually be taught from the left before transferring to the right. Recognising the horse's preference in this could potentially reduce stress and make training safer and more successful. A further study on a larger number of animals, including equines of different breeds and types, and under different types of human management and training, is needed to investigate this in detail.

Compliance with ethical standards

Ethical statement: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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STUDY 4

Preservation of fecal glucocorticoid metabolites and immunoglobulin A through silica gel drying for field studies in horses

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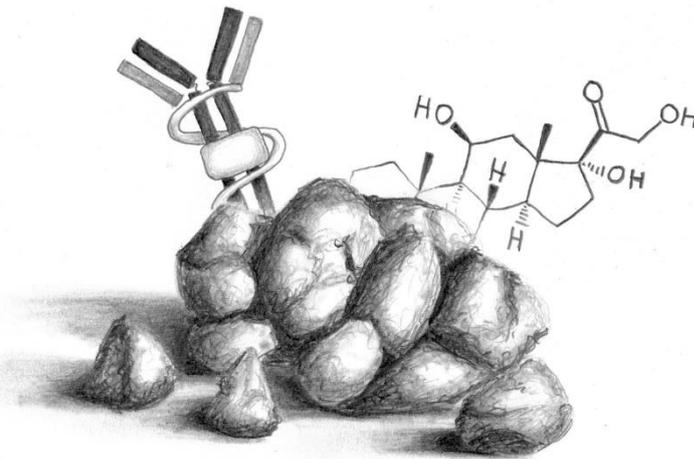
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Stability of stress hormone metabolites, fecal glucocorticoid metabolites (FGMs), and immunoglobulin A (IgA) was evaluated in horse feces dried in an air tight tube on silica gel, for improving stress analysis with fecal samples collected under field conditions with uncontrollable humidity. IgA showed a significant loss, but FGMs were stable and appeared to be reliably preserved.

Abstract

Non-invasive methods enable stress evaluation through measuring fecal glucocorticoid metabolites (FGMs), and immunoglobulin A (IgA) in the feces avoiding stressful blood drawing or stressful restraining of animals in the field. However, FGMs and IgA are mostly analysed in freshly frozen samples, which is difficult when fresh samples cannot be frozen immediately or frozen samples cannot be stored or transported. Good results were also derived from air-dried fecal samples, which are hampered by unstable air humidity in the field. These difficulties may be overcome, when drying of samples could be induced with colorless silica gel (SiO2) granules in a secure set-up, such as an air tight tube. We determined the speed of drying 1.5 g of a fresh fecal sample from six horses on air and on silica gel. Furthermore, FGMs and IgA were analysed in differently stored subsamples from 12 horses: in frozen fecal samples, in air- or silica gel-dried samples stored for 1 day and for 7 days, and in wet fecal samples kept in a tube at room temperature for 7 days. FGM levels remained stable in feces dried on air or on silica gel for 7 days, whereas IgA quantities showed a significant loss. Under field conditions, when freezing or transporting the frozen samples is not possible and humidity hampers air drying, drying samples on silica gel in air tight tubes appears to be very helpful and reliable for analysing FGMs.

Key words: Drying on silica gel, Equus caballus, fecal glucocorticoid metabolites, field studies, horse, immunoglobulin A, noninvasive sampling

Introduction

The present study investigates whether two stress parameters, fecal glucocorticoid metabolites (FGMs) and immunoglobulin A (IgA), are well preserved in dried samples taken from fecal heaps in a herbivore, such as the horse (McGhee and Mestecky, 1990; Möstl and Palme, 2002; Palme, 2019).

Secretion of glucocorticoids (adrenal stress hormones) is enhanced when stressful events activate the hypothalamic–pituitary–adrenal axis. Glucocorticoids stimulate the carbohydrate, protein, and lipid metabolism, as well as the immune response (Möstl and Palme, 2002). As prolonged stress may cause glucocorticoid levels to decrease to baseline values (Thun and Schwarz-Porsche, 1994; Pawluski et al., 2017), a combination with other parameters (such as immunological ones) is helpful. The immune system responds to stress with an increased production of immune cells in acute stress situations. Under chronic stress, the immune system will be depressed and the production of immune cells will be significantly inhibited and decline below baseline values (Siegel, 1987; Herbert and Cohen, 1993). IgA appears to be a suitable parameter for measuring the immune response in the gut, as it constitutes the main antibody in local immune defence in many mammals. IgA inhibits the binding of bacteria and viruses at the outer epithelial layers and reduces infections (McGhee and Mestecky, 1990). FGM and IgA quantities may increase with age as reported for dogs (IgA: Zaine et al., 2011) and may differ between sexes (FGMs: Gorgasser et al., 2007; IgA:Weber-Mzell et al., 2004) and between individuals (FGMs: Möstl et al., 1999; IgA: Paramastri et al., 2007).

Glucocorticoid metabolites (GMs) and IgA can be analysed in the blood or via non-invasive sampling in horse feces (FGMs: Flauger et al., 2010; review: Palme, 2012, 2019; IgA: May, 2007). For several species, non-invasive sampling provides reliable measurement of FGMs and IgA avoiding stressful blood drawing and enables field researches to collect samples without restraining the animals (Sheriff et al., 2011). In the past, FGM and IgA quantities were usually analysed in frozen fecal samples (IgA and FGM: rats: Eriksson et al., 2004; horses: May, 2007; mice: Moon et al., 2015), in fresh fecal samples (only IgA: dogs: Zaine et al., 2011), or in fecal samples stored on alcohol (only FGM: Palme et al., 2013). Freezing prevents further bacterial metabolism of glucocorticoid metabolites (Möstl and Palme, 2002) and the destabilization of IgA (Hau et al., 2001) best. However, immediate freezing may not be possible in the field or the transportation of frozen samples may be too difficult and costly.

Some studies acquired good results for conserving FGMs and IgA by drying samples in the lab (FGMs: review: Palme et al., 2013; cats: Ramos et al., 2013; macaques: Gholib et al., 2018; IgA: human: Vetvik et al., 1998). In humans, IgA quantities are reduced through air drying but can reliably be quantified by extrapolating IgA amounts from dried to frozen or fresh samples (Vetvik et al., 1998). We tested whether the preservation of FGMs and IgA through drying could be achieved in the field. We wondered whether drying of fecal samples can be performed with colorless silica gel (SiO_2) granules in a water tight, secure set-up, such as an air tight tube. Adding silica gel for preservation through water absorption is widely applied in scientific fields, such as botany (Chase and Hills, 1991) and for genetic analysis (Taberlet et al., 1999; Murphy et al., 2002; Engelhardt et al., 2017). Whether fecal FGM and IgA quantities are stable in samples dried on silica gel over time has not been evaluated.

The horse is a good model organism for testing the stability of FGMs and IgA in feces dried on silica gel as methods for their measurement have been well established (FGMs: Möstl et al., 1999; Merl et al., 2000; Flauger et al., 2010; Palme, 2012; Wolter et al., 2014, Yarnell et al., 2015) or at least reported (IgA: Vaerman et al., 1971, May, 2007). In the present study, we collected fresh fecal samples from 18 warmblood horses. We measured the speed at which given fecal quantities can be dried under lab conditions on air and in an air tight tube on silica gel.We compared concentrations of FGMs and IgA in frozen samples with those of air or silica gel-dried samples after 1 and 7 days and in samples kept in tubes at room temperature for 7 days without drying. We aimed to evaluate whether IgA and FGMs can be preserved reliably when dried on silica gel.We assumed (i) that IgA will destabilize significantly, but reproducibly when dried with silica gel, and (ii) that FGM quantities dried on silica gel should be well preserved and stable when the drying is as effective as air drying under controlled conditions in the lab.

Materials and Methods

Animals and Location

Eighteen horses (9 mares, 8 geldings, and 1 stallion) were used for the study. All 18 were warmblood horses and were aged between 1 and 27 years (median = 8 years). The feces for the study were sampled at the stable of the University of Applied Sciences Nürtingen-Geislingen and an adjacent private stable. Horses were given hay ad libitum and amounts of oat feed and mineral supplements were adjusted to individual needs.

Sample collection and preservation

Single samples were collected from six horses in April, June, and July 2016 and from further 12 horses in November 2017 between 7:00 a.m. and 8:00 a.m. from dung heaps defecated within 1 hour

before collection. Pool samples from five different locations of the dung heap were collected with one-way gloves, stored in unused freeze bags, and homogenized by kneading for 2–3 minutes.

Speed of air drying and drying on silica gel

From the samples of the six horses collected in 2016, we evaluated how fast 1.5 g of feces lost humidity under the following conditions: (i) when spread out in a petri dish and air dried at room temperature (20∘C) in a lab without air conditioner or controlled air flux (air drying = AD) and (ii) when given in a paper tea bag and dried in an air tight tube on 20 mL of colorless silica gel granules (silica gel drying = SD; Study 4 - Figure 1). For each horse, we measured the weight loss (i.e. humidity loss) of the samples after 12, 24, 48, and 72 hours of drying.
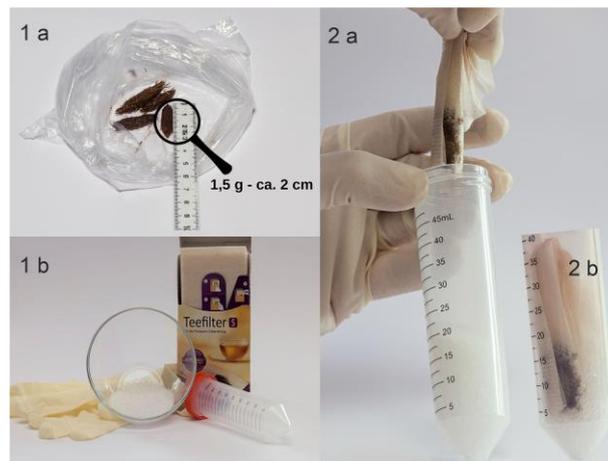
Study 4 - Figure 1: Drying fecal samples on silica gel. 1a, From the fecal pool samples, an aliquot of 1.5 g was taken and formed to an approximate 2-cm-long roll to increase surface for faster drying. 1b, One way gloves, 20 mL of colorless silica gel granules, a small paper tea bag, and a 50-mL tube were used. 2a, The samples were placed in a tea bag, and the tea bag coiled, its ends folded, and placed in a tube with 20 mL of silica gel. 2b, The lid was screwed on the tube with the sample and tilted a couple times until silica gel was all around the tea bag with the sample.

Preservation method

The samples taken from 12 horses in 2017 were aliquoted for all preservation procedures and the following preservation methods were applied: (i) 1.5 g of fresh feces were frozen at −20∘C (frozen sample = FR), (ii) 1.5 g of fresh feces were placed in an airtight tube and kept for 7 days at room temperature without drying (wet sample room temperature = WR), (iii) 1.5 g of fresh feces were spread out in a glass petri dish and air dried for 7 days at room temperature (air dry = AD), or (iv) 1.5 g of fresh feces were placed in a paper tea bag and dried for 7 days in an air tight tube on 20 mL of colorless silica gel granules (silica gel drying = SD; Study 4 - Figure 1).

Analyses of fecal samples

Fecal glucocorticoid metabolites

Glucocorticoid metabolites were extracted from horse feces with the simplified method described by Flauger et al., (2010). We used 0.5 g of wet feces. In the dried samples, we balanced the weight loss from drying by weighting the sample after drying and using the corresponding dry mass to 1.5 g of

fresh feces for each sample (for complete data, see Supplementary STUDY 4: Table S2; for calculation for balancing weight loss from drying, see Supplementary STUDY 4: File S1).

Thereafter, 0.5 g of wet feces (or the balanced dried fecal samples) plus 1 mL of water and 4 mL of methanol were vortexed for 2 minutes, kept at room temperature for 15 minutes, and vortexed again for 1 minute. The methanolic suspension was centrifuged. An aliquot of the supernatant was diluted in assay buffer and frozen until analysis. FGMs were quantified using an 11-oxoetiocholanolone enzyme immunoassay (EIA; for details, see Möstl and Palme, 2002), which has been validated for horses (Flauger et al., 2010). All differently stored subsamples of a horse were analysed (in duplicate; coefficient of variance (CV): <10%) consecutively on two microtitre plates in total (6 horses per plate).

Immunoglobulin A

We used 1 g of wet feces and dried samples, balanced for the weight loss from drying similar as for the FGM extraction only that the weight of each samples corresponded to 1 g of fresh feces (for complete data, see Supplementary STUDY 4: Table S2; for calculation for balancing weight loss from drying, see Supplementary STUDY 4: File S1).

The particular masses for each dried sample were dissolved in 10 mL of PBS at pH 7.4. After shaking strongly, the samples were vortexed for 3 minutes and allowed to stand for 15 minutes. The shaking and vortexing procedure was repeated once. Afterwards, the samples were centrifuged for 20 minutes at 1600 g at room temperature. A supernatant of 800 μL was transferred into 1.5-mL tubes.

Now, the 1.5-mL tube, containing the supernatant, was centrifuged for 15 minutes at 3260 g at room temperature. From this, a supernatant of 500 μL were transferred into 1.5-mL tubes and frozen at −20∘C until analysis. IgA was determined using a Horse IgA ELISA Quantitation Kit (Cat. No. E70-116, Lot No. E70-116-14; Bethyl Laboratories, Inc.; https://www.bethyl.com/product/E70-116). The Elisa Kit was validated for the detection of IgA in horse serum and plasma. The company suggests that feces that contain horse IgA are suitable samples for the application of the Horse IgA ELISA Quantitation Kit. The order of the samples (CV of duplicates: <2%) on the two plates was the same as for FGMs.

Statistical Analysis

FGM quantities are given in ng/g and IgA quantities in μg/g. The statistical analysis and the figures were done with the R-Project statistical environment, package R commander (R Development Core Team 2018). Some of the data (for complete data, see Supplementary STUDY 4: Table S3) were not normally distributed (Shapiro–Wilk Test). Therefore, non-parametric generalized linear models (GLMs) were used for analysing the effect of the fixed factor 'treatment' on the FGM and IgA distributions (for complete GLM models, see Supplementary STUDY 4: File S4). Thereafter, Spearman rank correlation tests were used to compare the preservation methods pairwise. Sequential Bonferroni corrections after Holm for multiple testing were applied to adjust the P values (for complete correlation data, see Supplementary STUDY 4: File S4). All tests were two-tailed and the significance level was set at 0.05.

Ethical considerations

The sampling person was not in contact with the animals from which she collected fecal samples. The non-invasive sampling of horses fecal samples did not cause the animals any harm, pain, or suffering (as defined in § 1, 3, and 7 of the German Animal Welfare Law) and did not require permission by the regional Animal Welfare Board, Tübingen.

Results

Speed of drying the fecal samples

The fecal samples (1.5 g) lost most of their humidity during the first 12 hours of drying, both when dried on air at room temperature (n = 6, weight loss median = 75%, min. = 73%, max. = 78%) and when dried on 20 mL of silica gel (n=6, weight loss median = 75%, min. = 73%, max. = 76%). Only very little further reduction of the weight (i.e. humidity) was observed after drying the samples for 24 hours (n = 6; weight loss air drying: median = 77%, min. = 74%, max. = 79%; weight loss silica gel: median = 77.5%, min. = 77%, max. = 79%) and no further weight loss occurred afterwards (48 and 72 hours).

FGM stability at room temperature and in dried samples

Individual animals varied in their fecal FGM concentrations (GLM: n = 72, SE = 0.43, t = 10.64, P<0.001; Supplementary STUDY 4: File S4).FGM concentrations did not differ significantly from concentrations in frozen samples when feces were air dried for 7 days or dried on silica gel for 1 or 7 days (GLM: n = 72, all P>0.05, Study 4 - Figure 2; Supplementary STUDY 4: File S4). However, FGM quantities in samples dried on air for 1 day (GLM: n = 72, SE = 5.11, t = 1.74, P = 0.09) and those kept in wet samples at room temperature for 7 days (GLM: n = 72, SE = 5.11, t =−1.77, P = 0.08) tended to vary from those in frozen samples.

Furthermore, FGM concentrations in frozen samples correlated (Study 4 - Figure 3) with those of the samples of both drying procedures after 1 and 7 days of drying (Spearman rank correlation test: n = 12, all P<0.05; for full statistical data, see Supplementary STUDY 4: File S4). In addition, FGM quantities correlated between both time points of air and silica gel drying (Spearman rank correlation test, n = 12, AD: rs = 0.874, P = 0.003; SD: rs = 0.853, P = 0.006). However, FGM concentrations in air-dried samples were not correlated with those of silica gel-dried ones (Spearman rank correlation test: n = 12, all P>0.05).
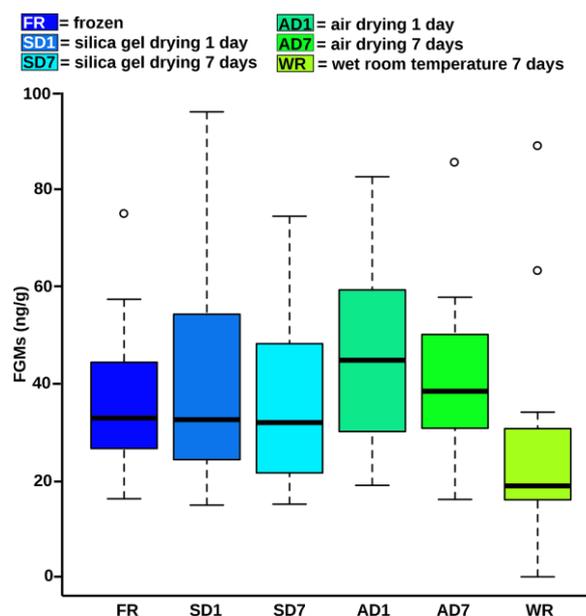
Study 4 - Figure 2: Concentrations of FGMs. The whisker boxplots depict the data of the particular preservation method. The line in the box represents the median and the circles above the box represent outliers. For full statistical data, see Supplementary STUDY 4: File S4
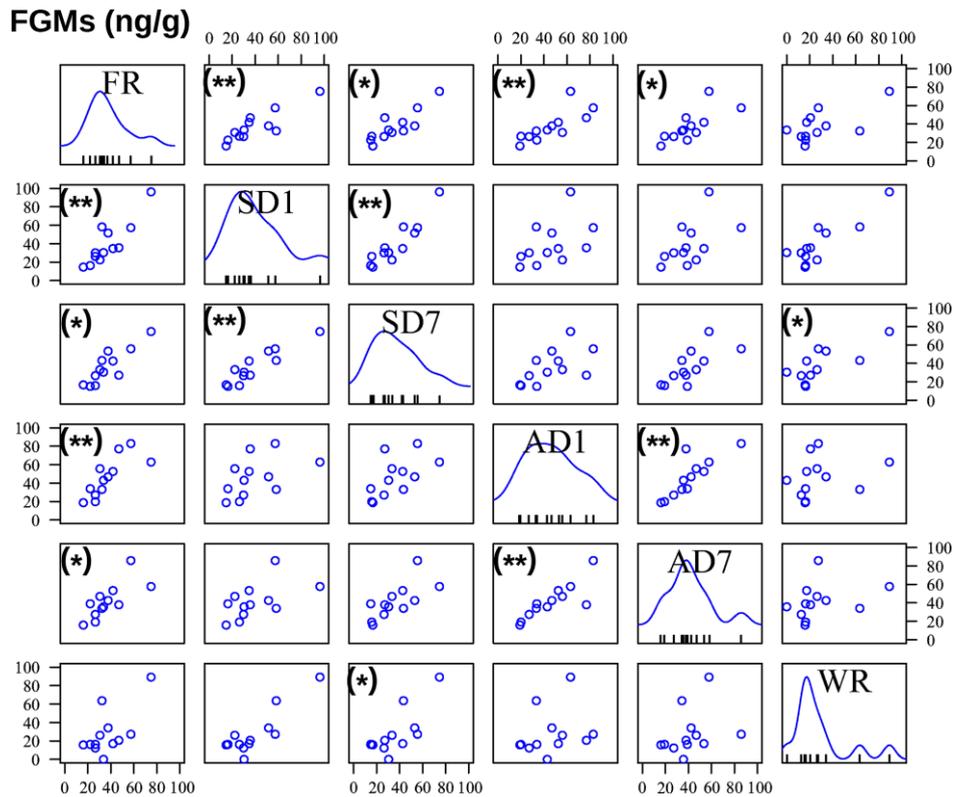
Study 4 - Figure 3: Correlations of FGM concentrations between preservation procedures. On the diagonal from the upper left to the lower right, the frequency distributions of FGM concentrations are shown for the 12 samples of each preservation procedure: FR = frozen samples, SD1 = silica gel-dried sample 1 day, SD7 = silica gel-dried samples 7 days, AD1 = air-dried sample 1 day, AD7 = air-dried samples 7 days, WR=wet sample at room temperature 7 days. FGM concentrations are given in ng/g for the x axis at each column. The 12 lines on the x axis within each preservation graph indicate the FGM concentration of each of the 12 samples. The scatterplots depict the correlations between FGM concentrations of pairs of preservation procedures (for full statistical data, see Supplementary STUDY 4: File S4). For the correlations, y and x axes quantify the concentrations of FGMs in the respective column (x axis) or row (y axis). Significant correlations are given with (*) = P<0.05, (**) = P<0.01.

IgA stability at room temperature and in dried samples

Individuals also varied in their fecal IgA concentrations (GLM: n = 72, SE = 0.05, t = 9.65, P<0.001). IgA concentrations (Study 4 - Figure 4) did not differ significantly between frozen samples and samples dried for 1 day on air or on silica gel (GLM: n = 72, both P>0.05). However, IgA concentrations were lower in samples dried 7 days on air (GLM: n = 72, SE = 0.59, t =−3.5, P<0.001), in samples dried for 7 days in silica gel (GLM: n = 72, SE = 0.59, t =−2.63, P = 0.01), and in wet samples kept at room temperature for 7 days (GLM: n = 72, SE = 0.59, t =−5.14, P<0.001) than in frozen samples.

Even though IgA quantities declined from frozen samples when they were dried on air for 1 and 7 days (Study 4 - Figure 4), frozen sample quantities correlated well with those dried on air for 1 day (Spearman rank correlation test: n = 12, rs = 0.769, P = 0.04) and for 7 days (Spearman rank correlation test: n = 12, rs = 0.853, P = 0.005; Study 4 - Figure 5; for full statistical data, see Supplementary STUDY 4: File S4). IgA quantities in frozen samples did not correlate with those in silica gel-dried samples (Spearman rank correlation test: n = 12, P both >0.05). However, IgA quantities tended to be correlated between both time points of air drying and correlated between both time points of silica gel drying (Spearman rank correlation test, n = 12, AD: rs = 0.748, P = 0.06; SD: rs = 0.860, P = 0.005). Finally, IgA concentrations in wet samples kept at room temperature for 7 days did not correlate with IgA concentrations in frozen samples or in samples from any of the drying procedures (Spearman rank correlation test: n = 12, all P>0.05, Study 4 - Figure 5).
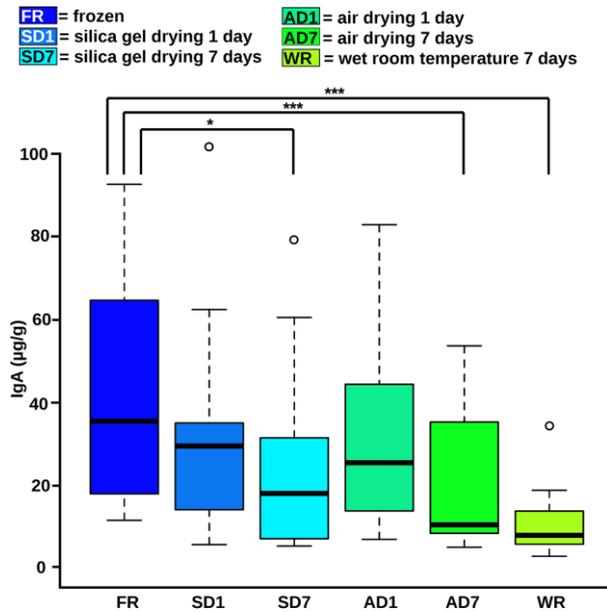
Study 4 - Figure 4: IgA concentrations. The boxes comprise 50% and each whisker 25% of the data of the particular preservation method. The line in the box represents the median and the circles above the box represent outliers. For full statistical data, see Supplementary STUDY 4: File S4. *P<0.5, ***P<0.001.
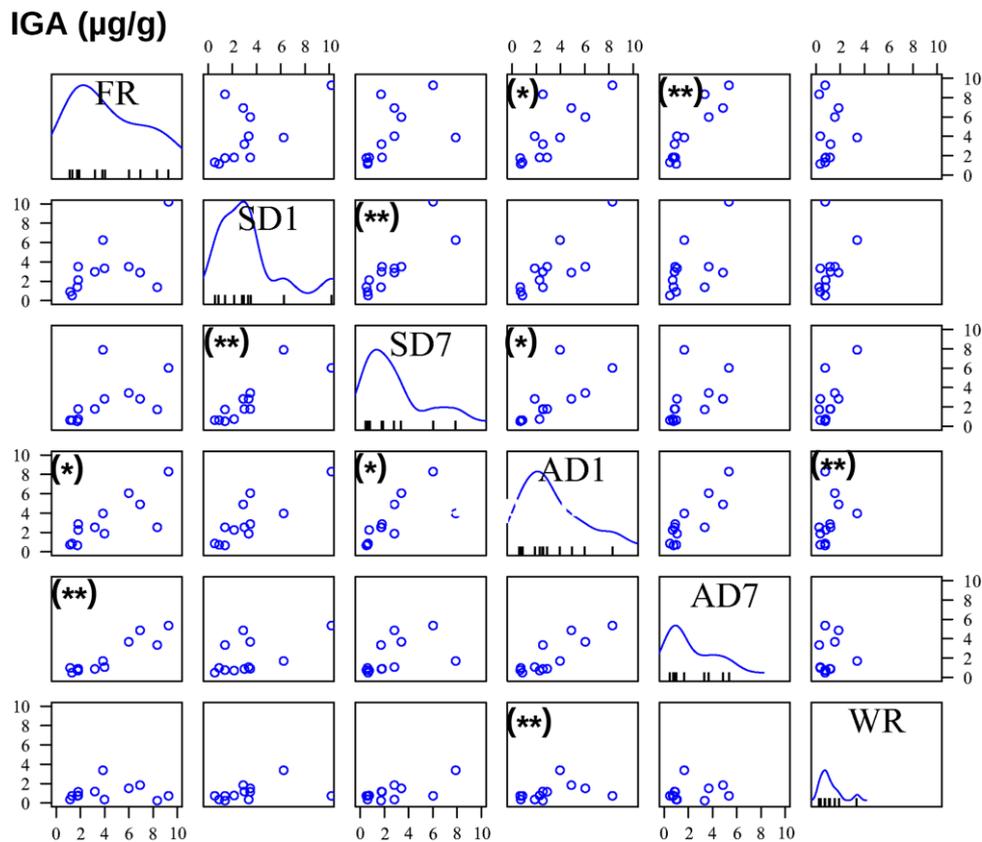
Study 4 - Figure 5: Correlations of IgA concentrations between preservation procedures. On the diagonal from the upper left to the lower right, the frequency distributions of IgA concentrations are shown for the 12 samples of each preservation procedure: FR = frozen samples, SD1 = silica gel-dried sample 1 day, SD7 = silica gel-dried samples 7 days, AD1 = air-dried sample 1 day, AD7 = air-dried samples 7 days, WR=wet sample at room temperature 7 days. IgA concentrations are given in µg/g for the x axis at each column. The 12 lines on the x axis within each preservation graph indicate the IgA concentration of each of the 12 samples. The scatterplots depict the correlations between IgA concentrations of pairs of preservation procedures (for full statistical data, see Supplementary STUDY 4: File S4). For the correlations, y and x axes quantify the concentrations of IgA in the respective column (x axis) or row (y axis). Significant correlations are given with (*) = P<0.05, (**) = P<0.01.

Discussion

Fecal glucocorticoid metabolites

In the present study, bacterial decay of FGMs was prevented for up to 7 days when samples were air dried under controlled laboratory conditions or dried on silica gel under controlled conditions in an air tight tube. Only when wet fecal samples were kept for 7 days without drying, FGM concentrations declined. Thus, for FGM analysis, drying feces on air under controlled laboratory condition is convenient. However, when fresh samples cannot be frozen immediately or when drying samples on air is not applicable (e.g. in field studies), our results suggest that drying fecal samples on silica gel in air tight tubes is a reliable and convenient alternative to preserve FGMs. In a similar way, silica gel has also been used for the preservation of botanical specimens (Chase and Hills, 1991) and for samples for genetic analysis (Taberlet et al., 1999; Murphy et al. 2002; Engelhardt et al., 2017).

Immunoglobulin A

When samples were air dried or dried on silica gel, an insignificant loss of IgA quantities occurred after 1 day, but a significant loss after 7 days. Keeping feces at room temperature without drying them is the least suitable method for measuring IgA, as this procedure produced the strongest decay in IgA concentrations and the sample quantities did not correlate well with the quantities in fresh samples or to the quantities in any of the drying procedures. We therefore suggest to use fresh samples (Zaine et al., 2011) or to conserve fecal samples for IgA analysis through freezing (Hau et al., 2001) whenever possible. When field conditions do not allow for generating fresh or frozen samples, extrapolating the median loss of IgA through drying over a defined duration to the quantities expected for fresh samples has been suggested for air-dried human fecal samples (Vetvik et al., 1998). Drying horse fecal samples on air produced a reliable, quantifiable loss of IgA, as reduced quantities in the samples dried on air for one and for 7 days correlated well with quantities in the frozen samples. Drying the feces on silica gel reduced IgA decay, but only in some individuals as IgA quantities in silica gel drying could not be correlated with IgA quantities in frozen or air-dried samples.

Influences on FGM and IgA preservation

FGM and IgA quantities differed between individuals as previously reported and because many factors influence their levels (FGM: Möstl et al., 1999; Gorgasser et al., 2007; Palme, 2019; IgA: May, 2007; Palm et al., 2016). However, a diverse set of samples is advantageous for stability testing to be able to draw broad conclusions. The speed of drying was sufficient to prevent bacterial decay of FGMs (Möstl and Palme, 2002). Drying samples on air or on silica gel within 12 hours kept FGM concentrations in the samples stable.

The analysis of FGMs and IgA showed inconsistencies between preservation procedures, as some samples had even higher concentrations after running through preservation procedures than measured in the fresh samples. Since bacterial enzymes are mainly involved in the further metabolism of FGMs, the diversity of the individual gut microbiome may exert a strong influence here (Palme, 2019). Some metabolites may be better detected by the EIA than their precursors and this phenomenon can account for higher levels (e.g. Möstl et al., 1999; Lexen et al., 2008). Furthermore, those inconsistencies could be explained by difficulties to establish equal fecal subsample compositions. Although the samples were well homogenized and visually homogenous, minor differences in amounts of undigested materials could not be excluded.

FGM and IgA degradation processes may differ between silica gel drying and air drying as FGM and IgA concentrations correlated well between time points within silica gel or air drying but did not correlate between the two preservation methods. While studies on mechanisms of FGM degradation during air drying were not conducted, possibly because of the satisfying stability of FGMs, Griebenow and Klibanov (1995) demonstrated an alteration of the secondary protein structure during dehydration of proteins in immunoglobulin G. Adding sorbitol and trehalose improved the storage stability of IgG after spray drying of protein solutions (Maury et al., 2005). Future studies may evaluate whether adding sorbitol or trehalose enhances IgA preservation in fecal samples dried on silica gel. By applying ELISA kits validated specifically for the application to horse fecal samples, IgA detection may also be improved, while variability in IgA decay over time and preservation methods may remain.

Conclusion

FGMs of horse feces dried on silica gel remained stable. Thus, drying samples on silica gel in air tight tubes adds to preserving fecal FGMs through freezing, air drying, or storage on alcohol (Palme et al., 2013) and appears to be very helpful to preserve FGMs under field conditions, where freezing is not possible and changing humidity and temperature prevent air drying. However, for analysing immune responses to long lasting stress (Siegel, 1987; Herbert and Cohen, 1993), IgA preservation by freezing samples remains to be the most reliable method. IgA quantities in horse fecal samples dried on air can be extrapolated to the quantities in frozen samples, as reported for human fecal samples (Vetvik et al., 1998).

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STUDY 5

The Importance of the Strength of the Laterality

Unpublished data

Introduction

Different studies on sensory and/or motor laterality have concentrated on the direction of laterality, as the preference for the left or right side depends on the type of information being processed (A. Hook-Costigan and J. Rogers, 1998) and/or the individual predisposition to process information in a particular hemisphere (Gordon and Rogers, 2015). To cite some of the research on horses: They prefer their left side/right brain hemisphere in situations of aggression and novelty (Austin and Rogers, 2014), and of reactivity and vigilance (Austin and Rogers, 2012). Horses preferred their left eye/right hemisphere when they were confronted with a novel object (plastic cone) or an object associated with negative emotions (white shirt of a veterinary surgeon), whereas no preference was shown when the object was associated with positive emotions (feed bucket) (De Boyer Des Roches et al., 2008). Left-sided sensory laterality is associated with a higher emotionality index (Larose et al., 2006). Untrained horses show more extreme negative reactions when humans approached from the left side/right hemisphere (e.g. escape), and more positive reactions when they were approached from the right side/left hemisphere (Sankey et al., 2011). Nonetheless, the left side/right hemisphere is also preferred in positive emotions such as affiliative interactions (Farmer et al., 2018). In motor laterality, it was demonstrated under stressful conditions such as trailer loading, that horses with higher negative emotions (anxiety) were more likely to use the left forelimb (right hemisphere) to step onto the ramp (Siniscalchi et al., 2014). However, studies on the strength of laterality reveal that both the direction and the strength, of laterality may play an important role regarding stress reactivity, fearfulness, behavioural activity and arousal (for literature see chapter "Strength of laterality"). In summary, animals with a weakly lateralized brain seem to be more fearful, more excitable, show a weaker performance in multitasking and a weaker response to novel objects compared to animals with a strongly lateralized brain. Laterality influences social life and the inter-hemispheric communication is less effective (summarized by Rogers, 2017).

Therefore, the aim of Study 5 was to analyse whether:
1. the strength of laterality in relaxed situations is correlated with faecal glucocorticoid metabolite (FGM) concentration (basal stress level), faecal immunoglobulin A (IgA) concentration (basal activity of immune system), or to sensory or motor laterality indices (equal distribution of animals with left and right-sided preference) in situations without stressors
2. the strength of laterality in relaxed situations is correlated with FGM concentration or faecal IgA concentration in stress situations
3. the strength of laterality in relaxed situations is correlated with changes in FGM concentration (stress reactivity) and IgA concentration.
4. changes in the strength of laterality indicate stress responses. As outlined in STUDY 1, to evaluate stress in animals it is important to observe changes in the parameter of interest, because of a high inter-individual difference in animals with normal genetic diversity
5. the strength of laterality in relaxed situations is correlated with cognitive bias
6. the strength of laterality in relaxed situations correlated with age

Material and Methods

Animals, sampling, and data collection

Data were used from STUDY 1 and STUDY 2, so for detailed information on animals, sampling procedure, and data collection please see these chapters.

To answer the questions above the following data were used:

1. Study 1: basal values of the experiment before the change of housing condition
2. Study 1: basal values and related values of the stress situations: the change from group housing to individual stabling (24 h and 48 h), one week of individual stabling, initial training (24 h and 48 h) and two months of individual stabling with initial training
3. Study 1: basal values related to the changes/differences between basal situation and the stress situations
4. Study 1: absolute values of motor and sensory laterality from all investigated situations
5. Study 2: absolute value of motor and sensory laterality indices related to the time the horses took to approach the ambiguous box
6. Study 2: absolute value of motor and sensory laterality indices related to the age of the horses

Statistical Analysis

RStudio (version 0.99.484, Boston, MA, USA) and the package R commander (version 2.2.1) were used for the statistical analysis. Figures were constructed with Microsoft Excel 2010 (Microsoft Corporation, Washington, DC, USA). A generalized linear model (GLM) was used to test whether strength of laterality was correlated with the different parameters, and whether the strength of laterality changed with the different test situations of study 1. Non-significant parameters were removed stepwise to simplify the models and to improve the models' goodness of fit only when their deletion did not cause a significant reduction in the models' goodness of fit. The results are presented after model simplifications with the best goodness of fit. All tests were two-sided, and the significance level was set at 0.05.

Results

1. The higher the absolute value of basal motor laterality, the more often the right limb had been placed in front during grazing (GLM: $|ML\_basal| \sim ML\_basal$, family = gaussian(identity), t = 8.3, $p < 0.001$), and the higher the absolute value of basal sensory laterality the more often the right sensory organs in the situation without stressors and before the change of housing conditions (GLM: $|SL\_basal| \sim SL\_basal$, family = gaussian(identity), t = 2.3, p = 0.047), indicating that the absolute values of the motor laterality and sensory laterality indices were not evenly distributed between animals with a preference for the left and animals with a preference for right side. There was no significant correlation between the absolute sensory or motor laterality and FGM or IgA in basal situation before the change of housing conditions (all $p > 0.05$).

2. The higher the absolute value of sensory laterality the lower were the FGM concentrations after one week of individual stabling (GLM: |SL_basal| ~ FGM _1weekBox, family = gaussian(identity), t = -2.3, p = 0.045, Study 5 - Figure 1). There were no other correlations between the absolute sensory or motor laterality indices and FGM or IgA in the investigated stress situations (all p > 0.05)
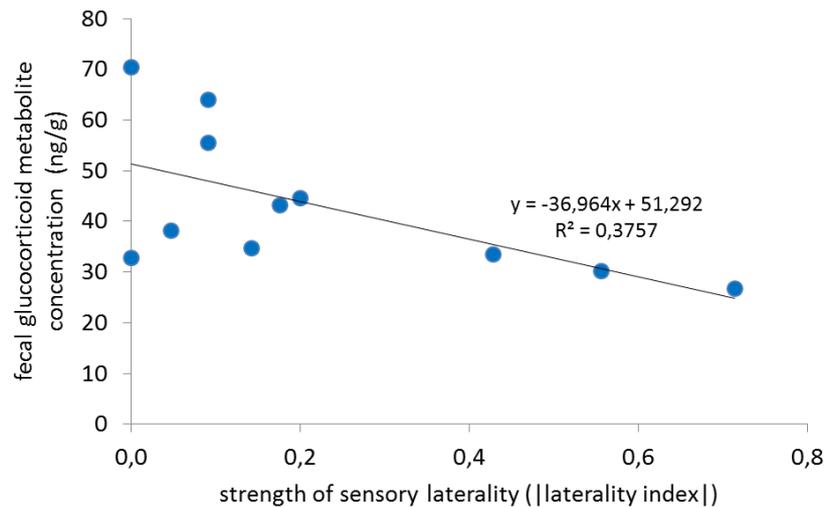
Study 5 - Figure 1: Relation between strength of sensory laterality of basal situation before the change of housing conditions and FGM concentration after one week individual stabling (GLM: |SL_basal| ~ FGM _1weekBox, family = gaussian(identity), t = -2.3, p = 0.045, STUDY 1).

3. The greater the difference between faecal IgA basal and faecal IgA measured 48h after the initial training and during individual housing, the higher the absolute value of basal sensory laterality was (GLM: |SL_basal| ~ IgA difference basal_ 48h training, family = gaussian(identity), t = 2.3, p = 0.044, Study 5 - Figure 2). There were no other significant correlations between the absolute value of motor or sensory laterality indices and the change/difference in FGMs or IgA between base values and stress situations (all p > 0.05).
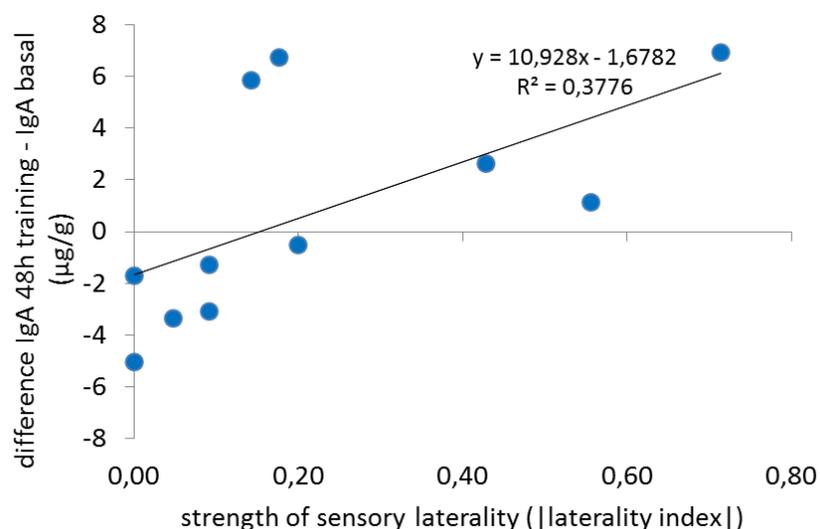
Study 5 - Figure 2: Correlation between basal strength of sensory laterality and the difference between faecal IgA concentration of basal situation and at 48 h after the initial training and individual stabling (GLM: |SL_basal| ~ IgA difference basal_ 48h training, family = gaussian(identity), t = 2.3, p = 0.044, STUDY 1).

4. The absolute value of sensory laterality tended to increase after the initial training (GLM: absolute sensory laterality index ~ test situation, N = 11, t = 1.29, p = 0.06, Study 5 - Figure 3). The absolute value of motor laterality was significantly increased after two months of individual stabling (GLM: absolute motor laterality index ~ test situation, N = 11, t = 2.23, p = 0.03, Study 5 - Figure 3) and tended to increase after the change from group housing to individual stabling (GLM: absolute motor laterality index ~ test situation, N = 11, t = 1.69, p = 0.098, Study 5 - Figure 3).

a. change from group to individual housing - b. 1 week individual stabling
c. initial training - d. two month regular training & individual stabling
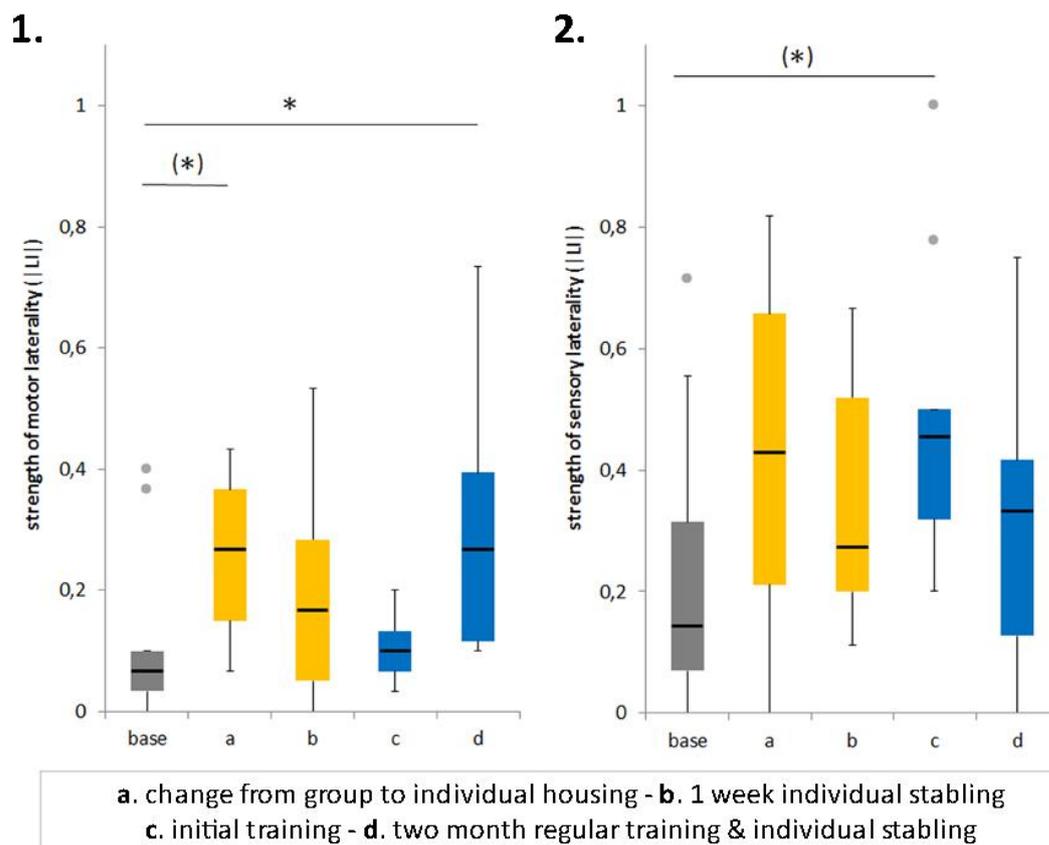
Study 5 - Figure 3: Absolute values of motor and sensory laterality indices from data of STUDY 1: Strength of motor laterality (1) and strength of sensory laterality (2) in test situation without stressors (base, during group housing) and after the change of housing condition (a), one week of changed housing condition (b), after initial training (c), and after 2 months of regular training and individual stabling (d). Yellow: changed housing conditions, blue: combination of individual housing and initial/regular training. Box plots display the medians, interquartile ranges from 25% to 75%, whiskers (minimum and maximum values), and outliers (dots) for values higher or lower than 1.5 interquartile range. * p < 0.05, (*) p < 0.10

5. There was no significant correlation between the absolute values of the motor or sensory laterality indices and the time the horses took to approach the ambiguous location (all p > 0.05).

6. The absolute value of motor laterality (grazing stance) indices increased with the age of the horses (GLM: Age ~ |ML_grazing stance|, family = gaussian(identity), t = 2.5, p = 0.023, Study 5 - Figure 4).
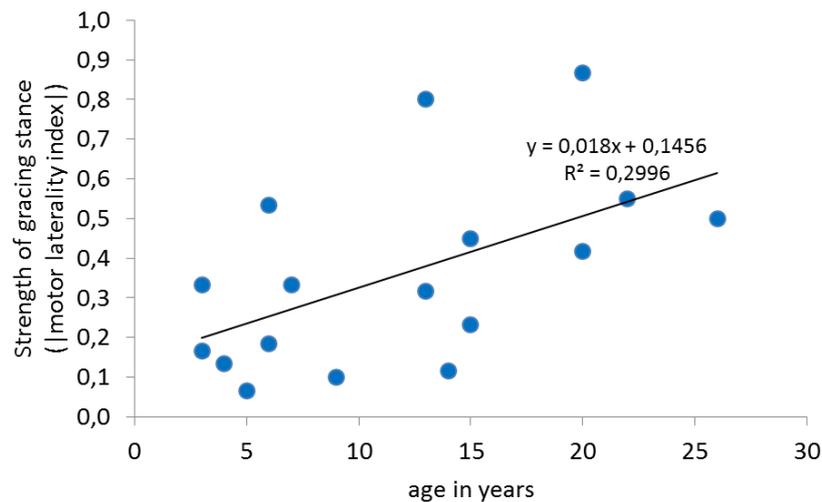
Study 5 - Figure 4: Correlation between strength of motor laterality (grazing stance) and age of horses (GLM: Age ~ |ML_grasing stance|, family = gaussian(identity), t = 2.5, p = 0.023, STUDY 2).

Discussion

The relationship between age and an increasing strength of grazing stance motor laterality that was demonstrated by McGreevy and Rogers, (2005) was confirmed by the present data. The older the horses, the stronger the grazing stance preference was. But only 30% of the grazing stance preference variation was explained by age, indicating a more complex relationship between age and laterality (Leliveld, 2019). Age might result in a stronger grazing stance preference, but there may be other factors as well. Generally, all factors that influence the direction of laterality, simultaneously influence the strength of laterality. For example, stress, that influences the direction and thereby also the strength of laterality as demonstrated in STUDY 1. The results show that the strength of motor and sensory laterality changes during the different test situations, but there is no clearly recognizable correlation with the other investigated stress parameters (FGM, faecal IgA, motor laterality, sensory laterality) in STUDY 1. It seems that the strength of laterality only increases in a particular test situation when the inter-individual difference of the total laterality indices from the same test situation increased. Therefore, the inter-individual differences may have caused the statistically significant changes in the strength of laterality, irrespective of a stress response.

Furthermore, there was no correlation between the strength of laterality and the strongest stressor as indicated by the other measures (the change of housing condition, according to STUDY 1). However, one week after the change of housing condition, the horses with stronger basal sensory laterality had lower FGM concentrations. Similarly, in domestic dogs a weaker paw preference was associated with an increased reactivity towards sounds (Branson and Rogers, 2006). In unfamiliar or fear inducing situations, dogs displaying weaker motor lateralization were more restless and reactive than those with stronger lateralization (Batt et al., 2009; Branson and Rogers, 2006). Therefore, more strongly lateralized animals may be less reactive towards novel stress-inducing objects/situations and may adapt more quickly to novel situations. In this case, the more strongly lateralized horses may have adapted faster to the novel situation of individual stabling, resulting in lower FGMs one week after the changed housing conditions (results of STUDY 1). However, this did not apply for motor laterality, as observed in the cited studies, only for sensory laterality. Furthermore, a correlation between the strength of laterality and FGMs was not observed in the other investigated stress situations. Additionally, 48h after the commencement of initial training, horses with only weak basal sensory

laterality showed a decrease in faecal IgA concentration, whereas more strongly lateralized horses showed increased faecal IgA concentrations. One explanation could be that more strongly lateralized horses may have coped more actively, resulting in an activation of the SAM axis typical for active coping strategies (Koolhaas et al., 1999). Overall, the horses were already slightly stressed by the changed housing conditions, as indicated by significantly increased stress hormones (see results of STUDY 1), so for some weakly lateralized horses the additional stress of initial training could have led to slight reactive coping strategies and hence a slight suppression of faecal IgA. These results are comparable with the results for dogs in an unfamiliar environment (Batt et al., 2009; Branson and Rogers, 2006), but in the present study there was no correlation between motor laterality and IgA, or laterality and FGMs, indicating that the strength of sensory and motor laterality is not a reliable parameter for measuring stress reactivity. Additionally, weakly lateralized ewes and lambs, showed weaker arousal during separation tests (Barnard et al., 2016) in contrast with the findings above, and in Port Jackson sharks there was a link between weakly lateralized individuals and decreased stress reactivity during rotational swimming (Barnard et al., 2016). Furthermore, the data of the present study were not evenly distributed with respect to the strength of laterality, because animals showing a right bias in motor and sensory laterality were more strongly lateralised than animals with a left-sided preference in the basal condition. This could also be why more strongly lateralized animals adapted faster to the novel situation of individual stabling as shown in the results after one week, and coped actively in novel situations, as animals with e right-sided preference are less stress-sensitive and cope better (Sullivan, 2004).

Nevertheless, if the strength of laterality is of greater importance than the direction of laterality, the direction of laterality may have played an indirect role and the relationships between strength of laterality and the investigated parameters were present anyway. Furthermore, no correlation was found between the strength of motor or sensory laterality and cognitive bias. Therefore, it is assumed that the strength of laterality may have only a small impact on stress sensitivity, reactivity, and coping strategies and the direction of laterality may be of greater importance regarding animal welfare evaluation.

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GENERAL DISCUSSION AND PERSPECTIVES

All five studies of this thesis demonstrated that, with a few limitations, laterality may serve as a reliable, repeatable, and objective animal welfare indicator that is easy to determine in horses and further investigations are needed. In summary, the direction of laterality changes in accordance with other well-established stress parameters such as glucocorticoid metabolites (STUDY 1), the direction of laterality can provide insight into the cognitive bias of horses (STUDY 2), the direction of laterality is relevant to positive emotions such as affiliative interactions (STUDY 3), but no reliable correlation has been found between the strength of laterality and stress sensitivity or cognitive bias (STUDY 5). Nonetheless, like other stress parameters, laterality shows some limitations in indicating stress and state of welfare, and a combined analysis of different parameters is recommended (König v. Borstel et al., 2017). Non-invasive methods are especially recommended, as they do not incur additional stress due to the sampling procedure (Balcombe et al., 2004; Morrow et al., 2002; Palme et al., 1996; Säkkinen et al., 2004; Sheriff et al., 2011). By using novel conservation methods such as drying faecal samples in silica gel, the implementation of physiological measurements, especially in field studies, can be simplified as demonstrated in STUDY 4. One limitation of laterality is the high inter-individual variation in animals with a normal genetic diversity such as the horses that were used in the present studies. In situations without stressors, they displayed left and right biases in both sensory and motor laterality, depending on the individual. This inter-individual variation is a function of age, breed, stress susceptibility, dominance, emotionality etc. (Batt et al., 2009; Larose et al., 2006; McGreevy and Rogers, 2005; McGreevy and Thomson, 2006; Phillips et al., 2015), as demonstrated by STUDY 2. Horses from similar, good housing conditions displayed positive as well as negative cognitive bias that was related to initial forelimb use (motor laterality). This suggests that motor laterality also seems to be a trait of personality. Therefore, when evaluating welfare, observation of changes in laterality that can indicate stress, such as an increasing use of the left side (organs, limbs) as demonstrated in STUDY 1, is recommended. In situations in which stress hormones were increased, the investigated horses displayed a clear shift to the left side in both sensory and motor laterality, irrespective of the direction of their initial laterality. The context in which laterality is displayed is also important. An increased use of the left side does not necessarily indicate stress or poor welfare, as STUDY 3 demonstrated a left side preference in affiliative interactions between conspecifics. Whereas stress is an emotional arousal that mostly involves negative emotions, affiliative interactions are assumed to elicit positive emotions and are natural needs of horses in the form of contact to conspecifics. Therefore, observation of additional stress parameters and their changes, alongside changes in laterality, can give a better insight when evaluating welfare. Models of cerebral lateralization may improve understanding of the influence of different emotions on cerebral lateralization as displayed in sensory and motor laterality and changes of emotional states.

Lateralization and emotional processing - Models of cerebral lateralization

In STUDY 1 emotional arousal was caused by stress situations such as the change of housing conditions and training. This challenged the horses as they experienced restriction in free movement and in social interactions. Emotional arousal and, therefore, a change in emotionality, was indicated by significantly increased stress hormones. In STUDY 2 emotions of different valence (positive or negative cognitive bias) that were associated with an ambiguous situation were related to different directions of laterality, and in STUDY 3, possible positive emotions induced by affiliative interactions were also associated with cerebral lateralization. In all three studies, emotional arousal and the valence of emotions were linked to cerebral lateralization, and these supports one or more of the five models of cerebral lateralization of emotions (summary: Table 2)

1. The right hemisphere model

The right hemisphere model (Borod et al., 1998) is supported by STUDY 1. Emotional arousal was indicated by increased stress hormones and occurred with a shift to the left in sensory and motor laterality. Information processing increased in the right hemisphere, which is responsible for processing, perception and expression of emotions according to the hypothesis of the model (Borod et al., 1998). Wittling and Pflüger (1990) also assumed that with arousal under stress situations, the cortisol release is controlled by the right hemisphere. This assumption is supported by a study in domestic cats that displayed a higher right tympanic temperature during emotional stress, indicated by high cortisol levels, than less stressed conspecifics (Mazzotti and Boere, 2009). Both studies support the right hemisphere model. STUDY 2, however, does not support the hypothesis, because according to the hypothesis the valence of emotions is irrelevant. Depending on positive or negative cognitive bias/emotions the horses were right or left-sided in their initial forelimb use (motor laterality) indicating a processing, perception and expression of emotions by one hemisphere or the other.

In contrast to these findings, other studies support the model. For example, a stronger left bias in sensory laterality was associated with increased emotionality in domestic horses (Larose et al., 2006), and horses with a left-sided sensory laterality displayed increased fearfulness and reactivity (Austin and Rogers, 2007). Furthermore, a potentially threatening object that caused emotional arousal in marmosets, resulted in a shift to a left-sided sensory organ use (A. Hook-Costigan and J. Rogers, 1998). In addition, it seems that some types of threat and arousing interaction between individuals of the same species, such as agonistic interactions, are processed by the right hemisphere, and are independent of the animals' social organisation. A left-eye preference has been observed in various species (Austin and Rogers, 2014, 2012; Deckel, 1995; Hews et al., 2004), with some exceptions, such as domestic pigs and cows, where no lateralization on population level could be found in agonistic interactions (Camerlink et al., 2018; Phillips et al., 2015). However, in most of these examples, emotional arousal involved negative emotions which were associated with the right hemisphere. In horses, it is not only agonistic interactions that may induce negative emotions (Austin and Rogers, 2014), but also affiliative interactions, that are assumed to elicit positive emotions, that are linked to the right hemisphere, as demonstrated in STUDY 3.

STUDY 1 and STUDY 3 demonstrate that emotions may be linked to the right hemisphere irrespective of their valence, and this may be indicated in sensory laterality. But STUDY 2 suggests that the left hemisphere is important as well, and motor laterality appears to be linked to the expectation of positive or negative events.

2. The valence model

The valence model (Demaree et al., 2005; Silberman and Weingartner, 1986) is supported by STUDY 1, where the negative valence of the new housing conditions, which restricted some natural needs, is experienced by the right hemisphere and expressed in the increased left biased sensory and motor laterality. STUDY 2 also supports this hypothesis, because a positive cognitive bias (positive valence) is associated with the left brain hemisphere, whereas negative cognitive bias (negative valence) is associated with the right brain hemisphere, as described by the model. Many other studies support the model, too. A left-eye preference, and therefore processing by the right hemisphere, is associated with some types of negative emotion, such as agonistic interactions in non-social and social living animals (Austin and Rogers, 2014, 2012; Deckel, 1995; Hews et al., 2004), reactivity and fearfulness (Austin and Rogers, 2012, 2007; Phillips et al., 2015), and negative emotional arousal caused by potentially threatening object (A. Hook-Costigan and J. Rogers, 1998; Robins and Phillips, 2010; Siniscalchi et al., 2010). When situations or objects became familiar, and consequently the negative emotions changed to more positive or neutral emotions, a right eye preference (left hemisphere) was observed in different species (A. Hook-Costigan and J. Rogers, 1998; Phillips et al., 2015; Robins and Phillips, 2010; Siniscalchi et al., 2010). Nonetheless, this valence model seems not to be supported by STUDY 3, where positive feelings were also processed by the right brain hemisphere and affiliative interactions were associated with a left eye preference. Wittling and Pflüger (1990) assumed that in arousal situations (that could be eustress or distress) the cortisol release is controlled by the right hemisphere. Demaree et al. (2005), Silberman and Weingartner (1986) wrote that the balance between both hemispheres is controlled by the right hemisphere, as well. Possibly, sensory laterality is not only influenced by the valence of emotions, but also by the level of arousal, and that may be an additional parameter that controls the balance between both hemispheres and is controlled by the right hemisphere (Wittling and Pflüger, 1990). Affiliative interactions may be arousing in a positive context, too, which results in a left eye preference (right hemisphere).

To conclude, STUDY 1 and STUDY 2 demonstrated that negative emotions may be linked to the right hemisphere and positive emotions may be linked to the left hemisphere, but STUDY 3 demonstrated that there is no clear hemispheric responsibility for either positive or negative emotions. Especially in social contexts, both hemispheres seems to be involved (summarized by Leliveld, 2019, examples are domestic pig (Camerlink et al., 2018) and saiga antelopes (Giljov et al., 2019)). Further parameters such as arousal may influence whether information/emotions are processed in the left or right hemisphere.

3. The approach-withdrawal model

The approach-withdrawal model (Davidson et al., 1990) is only partly supported by STUDY 1, because, according to the model, negative emotions are controlled by the right hemisphere and stress increases left side preference in sensory and motor laterality (information processing by the right hemisphere). Whether these horses also start to show more withdrawal behaviour must be investigated in future research. STUDY 2 completely supports the model. Right-sided initial forelimb use, controlled by the left hemisphere, was associated with positive cognitive bias as these horses approached the ambiguous location. Whereas a left-sided initial forelimb use, that is of control by the right hemisphere, was associated with a negative cognitive bias, resulting in a kind of withdrawal behaviour (no approach). Comparably, in Australian magpies approach behaviour towards a predator was associated with the left hemisphere (right eye use) and withdrawal behaviour was linked to the right hemisphere (left eye use) (Koboroff et al., 2008). Therefore, in situations that elicit an emotional response, sensory laterality may predict either approach (low arousal) or withdrawal behaviour (high arousal). In neutral situations, motor laterality may give insight into the predisposition to approach or

withdraw, described as cognitive bias, which also depends on the predisposition for high or low emotional arousal.

Similar to the Valence model, the approach-withdrawal model cannot be supported by STUDY 3 where positive feelings were also processed by the right brain hemisphere and were not associated with withdrawal behaviour as would be predicted by this model. Instead, the investigated horses showed a left lateral bias in approach behaviour during social interaction. Other studies also only partly support the approach-withdrawal hypothesis. As outlined above, in agonistic interactions a left eye preference was observed (Austin and Rogers, 2014, 2012; Deckel, 1995; Hews et al., 2004). Agonistic interactions start with approach behaviour and end with withdrawal behaviour by the loser. In this case withdrawal seems to be controlled by the right hemisphere and is not in accordance with the model's hypothesis. Maybe agonistic interactions need to be investigated in more detail, separating the start and the finish of the interaction, which may show different lateralization. The mentioned studies all investigated the start of an agonistic interaction, which is an approach with an agonistic intent. This was linked to a left side preference (right hemisphere) and, therefore, does not provide evidence in support of the model hypothesis that the right hemisphere is linked to withdrawal behaviour. But the link between a left-side preference in sensory laterality and increased fearfulness and reactivity that leads to withdrawal behaviour in other studies (Austin and Rogers, 2007), supports the hypothesis. Also, right-sided pigs were bolder and more explorative and this supports the hypothesis of the model (Goursot et al., 2018).

To conclude, STUDY 1 and STUDY 2 demonstrated, that negative emotions and withdrawal behaviour may be linked to the right hemisphere and positive emotions and approach behaviour may be linked to the left hemisphere, but STUDY 3 suggests that the valence of emotions and the approach/withdrawal behaviour is not completely separated between left and right hemisphere in social situations (Giljov et al., 2019, for summary see Leliveld, 2019). It is also possible that when the model is applied to different studies there may be additional parameters that may influence the cerebral lateralization of emotions.

4. The behavioural activation (BAS) and behavioural inhibition system (BIS)

Negative feelings are associated with the BIS, which is sensitive to novelty (Carver and White, 1994; Demaree et al., 2005). This can be compared with the investigated stress situations of STUDY 1, where the horses were confronted with a novel situation that induced stress (increased stress hormones) and may have also elicited negative feelings through the restriction of some natural needs. BIS is controlled by the right hemisphere. The investigated horses also showed a shift to the left side in sensory and motor laterality that is controlled by the right hemisphere. But in the present study it was not investigated whether the activation of the BIS is also associated with enhanced anxiety and this should be the subject of future research. Studies on dairy cows demonstrated a link between the right hemisphere and anxious behaviour that might be the BIS (Amira et al., 2018). Apart from the significant increase of stress hormones and shift to the left side in laterality on population level, in STUDY 1 high inter-individual variation was observed, which may be a result of different coping strategies. A distinction must be made between two types of coping strategies that influence the neuroendocrine functions differently. Proactive coping animals show more active behavioural responses. Their HPA axis activity, HPA and parasympathetic reactivity are low, and their sympathetic reactivity is high (Koolhaas et al., 1999), and this seems to be a BAS, that is linked to the left hemisphere. However, reactive coping animals tend to show immobility behaviour, are more adaptive and flexible, and their HPA axis activity, HPA and parasympathetic reactivity are higher, while their sympathetic reactivity is lower (Koolhaas et al., 1999). This seems to be the BIS, that is linked to the right hemisphere. Both

coping strategies may be linked to the BAS and BIS, to the left and right hemispheres, and lead to different emotions and motivations (Tops et al., 2017). They could be the reason for the inter-individual variation in stress responses and the shifts in laterality, and this would support the BIS/BAS model.

STUDY 2 also supports the model. The BAS is sensitive to reward, enhances behaviour, and is associated with positive feelings and the left brain hemisphere. This could be observed in the horses that approached the ambiguous stimulus displaying a positive cognitive bias, which is a positive feeling. Those horses showed a right side preference for the initial forelimb use, indicating information-processing by the left hemisphere. Whereas the horses that did not approach the ambiguous stimulus displayed a negative cognitive bias, which is a negative feeling. They preferred to use the left forelimb in the initial forelimb use (right hemisphere). This correlation is predicted by the BIS, which is linked to the right hemisphere, inhibits behaviour and is associated with negative feelings. But STUDY 2 did not investigate whether positive cognitive bias/BAS and negative cognitive bias/BIS are related to enthusiasm and anxiety respectively, and this should be investigated in future research. However, other studies demonstrated a link between fearfulness, reactivity and the right brain hemisphere (Austin and Rogers, 2012, 2007; Phillips et al., 2015) and would support the hypothesis of the model. Furthermore, a link between viewing an unfamiliar object and the right hemisphere had been described (Robins and Phillips, 2010; Siniscalchi et al., 2010). This could be explained by the BIS that is linked to the right brain hemisphere and sensitivity to novelty. When the objects became familiar, a shift to the left hemisphere was recorded (Robins and Phillips, 2010; Siniscalchi et al., 2010), and this could be described by the BAS. It shows that after the first reaction to novelty, that may initially be immobile behaviour (BIS), this can change to enhanced behavioural activity (BAS) linked to more positive emotions when the novelty becomes familiar. This kind of learning behaviour, that shifts from the left to the right side, frees up the left eye to scan the environment for new potential threats/predators (Robins and Phillips, 2010; Siniscalchi et al., 2010) and supports the hypothesis. It also reveals how important the time point of observation could be. When observing sensory laterality, which depends on information processing and changes quickly, if the animals' decision is made within a short timeframe there may be different results if the sensory laterality is recorded too late. This could be a reason for contradictory results of some researches. Nonetheless, motor laterality shows the predisposition to rely on the left or right brain hemisphere, as demonstrated in dogs. Right-pawed dogs were calmer and less aroused in unfamiliar situations (Batt et al., 2009), and that corresponds with the left hemisphere and the definition of BAS. However, STUDY 3 does not support the hypothesis of this model. In STUDY 3 affiliative interactions, which are supposed to elicit positive feelings and a kind of enthusiasm (BAS), were linked to the right brain hemisphere, and not the left hemisphere as would be predicted by the hypothesis of the model. Other studies also demonstrate that the model does not completely explain the observations. Agonistic interactions are linked to the right brain hemisphere (Austin and Rogers, 2014, 2012; Deckel, 1995; Hews et al., 2004), and that would assume that the BIS is active. Of course, agonistic interactions may involve negative emotions, but do not necessarily inhibit behaviour. In bison a left hemisphere dominance in more aggressive and dominant behaviour, and a right hemisphere dominance for more positive and/or inhibition of aggression was found (Giljov and Karenina, 2019). This study only partly supports the model, because right hemisphere is associated with behaviour that aims to decrease aggression (inhibit behaviour/aggression – BIS) and the left hemisphere is associated with dominant behaviour (active behaviour – BAS). But the emotional valence of the behaviour studied by Giljov and Karenina (2019) is not in accordance with the model hypothesis.

To conclude, STUDY 1 and STUDY 2 demonstrated that BIS may be linked to the right hemisphere and BAS may be linked to the left hemisphere. But STUDY 3 suggests that BIS and BAS are not

completely separated by the left and right hemispheres and additional parameter may influence the cerebral lateralization of emotions.

5. The circumplex-dominance model

The above discussed models included one or two factors that may influence the cerebral lateralization. The results of the aforementioned studies suggest the existence of further factors that influence the cerebral lateralization of emotions. The circumplex-dominance model proposes the dominance of a feeling as a third factor (Demaree et al., 2005) and is supported by STUDY 1. Stress responses depend on the perception of the stress situation, for example whether the organism feels the situation to be controllable (dominance) or uncontrollable (submissiveness). Both will initiate a physiological stress response, but the feeling of controllability will influence the coping strategy and the further stress response that is a result of the animal's personality, its different emotions and motivations (Tops et al., 2017). In STUDY 1 the restrictions of movement and social contact resulted in enhanced stress hormones in most horses, leading to an increase of information processing by the right hemisphere that was shown in left-sided sensory and motor laterality. According to the model, this indicates that most horses may have perceived the situation as uncontrollable, because the right hemisphere is related to submissiveness and the feeling of uncontrollability.

Taking the circumplex-dominance model into consideration when discussing STUDY 2, a negative cognitive bias, which is linked to the right hemisphere, would correspond with submissive feelings/uncontrollability, whereas a positive cognitive bias is linked to the left hemisphere and would correspond with dominant feelings/controllability. Further research is needed to investigate whether cognitive bias is linked to specific feelings that can be differentiated in their dominance.

The horses of STUDY 3 demonstrated a link between affiliative interactions, that are supposed to elicit positive emotions, and the right brain hemisphere, and at first, this seems to be contradictory to the model. But when considering the model, the results of the study could also be explained as follows: affiliative interactions may cause both less dominant (right hemisphere) and positive feelings (left hemisphere), that may be arousing (right hemisphere) in a positive context leading to overall enhanced information processing by the right brain hemisphere. This demonstrates that the information processing by the left or right hemisphere may be a result of different factors that may enhance or reduce the processing in a particular hemisphere. Some feelings and emotions may not be restricted to only one hemisphere, but involve both hemispheres in different degrees leading to more strongly or weakly lateralized responses (Rogers, 2010).

STUDY 1, STUDY 2, and STUDY 3 may support the hypothesis of the circumplex-dominance model, but further research is needed to investigate the dominance of feelings. When considering studies on agonistic interaction between conspecifics, this model also seems to have some limitations in explaining the relationship between emotions and cerebral lateralization. Agonistic interactions are linked to the right brain hemisphere in several species (Austin and Rogers, 2014, 2012; Deckel, 1995; Hews et al., 2004). When considering the model, the involved emotions would be submissive from the beginning, that is contradictory because the animals act actively with approach behaviour. However, in domestic pigs no population bias in lateralization of agonistic interactions could be found (Camerlink et al., 2018), so this does not support the model's hypothesis, although a left hemisphere dominance in more aggressive and dominant behaviour, and a right hemisphere dominance for more positive and/or inhibition of aggression, were found in bison (Giljov and Karenina, 2019). The authors concluded that less fearful and dominant behaviour is controlled by the left hemisphere, and positive behaviour and behaviour that aims to decrease aggression is controlled by the right hemisphere. This

study would support the circumplex-dominance model, but because of the contrasting results of different studies involving different species and circumstances, more detailed and specific contexts should be considered in future research, for example such as the intention of the social interactions (reduce the aggression or dominating conspecifics), the relative dominance of the involved animals, and the motivation of a social response (Giljov et al., 2019). Different species and/or different circumstances may result in different motivational and emotional backgrounds (Giljov et al., 2019; Giljov and Karenina, 2019).

To summarize, as outlined in the other models, besides the valence, activity, approach/ withdrawal behaviour etc. there may be one or more additional parameters which influence the cerebral lateralization of emotions that could be explained by the dominance of emotions. Furthermore, some emotions may involve both hemispheres in various degrees, leading to more weakly or strongly lateralized responses on the left or right side.

The cerebral lateralization of emotional processing seems to be a result of the emotional valence, the level of arousal, and the dominance of the feeling. Further factors that may also influence lateralization, such as motivation (Giljov et al., 2019), cannot be excluded, as all the discussed models showed some limitations. Possibly, lateralization is a sum of all influencing factors and together they lead to weaker or stronger lateralization.

The hypothesis that the strength of laterality is an important indicator for stress sensitivity and emotional arousal could not be reliably confirmed by the results of the STUDY 5, and it also could not be supported by any of the five discussed models of cerebral lateralization. All models include a division of different types of emotion and/or the dominance of that feeling in the left and right, or only in the right, hemispheres. The hypothesis that weakly lateralized animals are more reactive (emotional arousal, summarized by Rogers, 2017) would suggest that direction of lateralization is less important. According to the results of the present studies and the described models, the direction of lateralization seems be important, although additional parameters, such as the dominance and the circumstances, may also have an influence. Possibly, the strength of laterality does not result in a specific reaction or behaviour. The strength of laterality may be a result of the involvement of different factors that are linked to the left or right hemisphere, and lead to strongly or weakly lateralized behaviour. To better understand possible influences on the strength of laterality, data should be analysed for animals with a preference for the left side and for animals with a preference for the right side separately, and should include the strength of laterality on the particular side.

To summarize, only the circumplex-dominance model seems to be supported by the three studies of the thesis on directional laterality. Therefore, the hypothesis that considers more than two factors seems to be most appropriate for the explanation of the relationship between emotions and cerebral lateralization. But it has its limitations, too. Therefore, further detailed research is needed. Cerebral lateralization is displayed in sensory and motor laterality, which are easy to observe. Sensory laterality may give insight into the emotional processing, whereas motor laterality shows the predisposition to rely on the left or right hemisphere and its emotional processing (A. Hook-Costigan and J. Rogers, 1998; Gordon and Rogers, 2015). The two parameters do not correlate with each other, but they may influence each other.

Table 2: Summary of the present publications, that support (black) the five models of cerebral lateralization of emotional processing, are contradictory (red) and/or leave questions which were not investigated by the particular publication (green).

right brain hemisphere

Pub. 1: stress - emotional arousal
Pub. 2: negative cognitive bias - emotions
Pub. 3: affiliative interactions - emotions (positive)

1. Right brain hemisphere model
(Borod et al., 1998)

left brain hemisphere

Pub. 2: positive cognitive bias - emotions

Pub. 1: stress -negative emotional arousal
Pub. 2: negative cognitive bias
Pub. 3: affiliative interactions - positive emotions

2. Valence model
(Demaree et al., 2005; Silberman and Weingartner, 1986)

Pub. 2: positive cognitive bias

Pub. 1: stress - negative emotions - withdrawal behaviour?
Pub. 2: negative cognitive bias - no approach
Pub. 3: affiliative interactions - approach behaviour

3. Approach - withdrawal model
(Davidson et al., 1990)

Pub. 2: positive cognitive bias - approach behaviour

Pub. 1: stress - novel environment - negative feelings - reactive coping? - anxiety?
Pub. 2: negative cognitive bias - inhibit behaviour - anxiety?
Pub. 3: affiliative interactions - positive emotions - active behaviour

4. behavioural activation (BAS) & behavioural inhibition system (BIS) model
(Carver and White, 1994)

Pub. 2: positive cognitive bias - reward - active behaviour - dedication?

Pub. 1: stress - negative emotional arousal - uncontrollability of changed environment?
Pub. 2: negative cognitive bias - feeling of submission? - uncontrollability?
Pub. 3: affiliative interactions - positive emotions - feeling of submission? - positive arousing?

5. Circumplex - dominance model
(Demaree et al., 2005)

Pub. 2: positive cognitive bias - feeling of dominance? - controllability?

Sensory laterality

Sensory laterality may provide insight into the processing of information, the emotions involved, the emotional valence of the processed information, the feeling of dominance and other, not yet identified, parameters (for example see A. Hook-Costigan and J. Rogers, 1998; Larose et al., 2006). Sensory laterality is an objective parameter that is repeatable and easy to observe in horses. It can help to identify stressors and assess welfare, with some limitations, by identifying the emotional background.

As demonstrated in STUDY 1 sensory laterality was more flexible and shifted to the left side in temporal proximity to increasing stress hormones. In domestic horses the right eye was used to view a novel neutral object, the left eye was used for known negative objects, and a binocular view was observed when a known positive object was presented (De Boyer Des Roches et al., 2008). According to these findings, it was expected that the investigated horses would prefer the right side in the novel object test of STUDY 1, but in the neutral situation without a stressor the use of the left and right sensory organs was equally distributed. This demonstrates that the novel objects may have elicited negative or positive feelings depending on the individual's perception, therefore leading to equal use of both sides on a population level. Consequently, the shift to the left in stressful situations may indicate that the horses started to perceive the neutral and novel objects more negatively, as demonstrated by Austin and Rogers (2007), in which domestic horses with a left-side preference in sensory laterality showed higher levels of fear and reactivity. A correlation between a left-side preference in interactions and increased fearfulness was also observed in cows (Phillips et al., 2015). The shift to the left side in stressful situations is in accordance with studies on marmosets (A. Hook-Costigan and J. Rogers, 1998). In contrast to horses (De Boyer Des Roches et al., 2008), marmosets showed a right eye preference on population level when viewing familiar food objects (positive). This population right-sidedness in sensory laterality changed when viewing a fear-inducing object. Some individuals clearly shifted to a left-eye preference, others changed to no significant preference compared with the control situation, and others changes to an increased use of the left side, although there was no left eye preference. Therefore, left shifts in sensory laterality can indicate, and help to evaluate, short-term stressors in animals. Short-term stress may enhance the activity of the right brain hemisphere for information processing due to emotional arousal, and that can be measured as sensory laterality, but the context of the observed sensory laterality is important, as demonstrated by STUDY 3. Left sidedness in sensory laterality was observed in affiliative interactions that did not induce stress. According to these results, the right hemisphere seems to control the emotions irrespective of the valence (Borod et al., 1998) and/or it displays the activity of the right hemisphere when controlling the balance between both hemispheres (Demaree et al., 2005; Silberman and Weingartner, 1986). Taking this into consideration, sensory laterality on its own cannot reliably indicate the valence of emotions. Nonetheless, changes in sensory laterality can indicate stress responses, and this should be supported by other parameters such as non-invasive stress hormone analysis (STUDY 4). Furthermore, it seems that sensory laterality cannot reliably predict the cognitive bias of a horse, as demonstrated by STUDY 2. In this case motor laterality may be more indicative. A change to a negative cognitive bias could also be an indication of compromised welfare (Harding et al., 2004; Rogers, 2010). Therefore, motor laterality may be a more reliable parameter to evaluate long-term stress and welfare.

Motor laterality

Motor laterality is more robust, and changes more slowly than sensory laterality, as demonstrated in STUDY 1. Motor laterality changed after one week of individual stabling, and was still changed after two months of individual stabling and regular training. It therefore seems to be a useful parameter to determine stress responses in horses. As with sensory laterality, a change to use the left side more often in comparison with the control situation was observed for motor laterality. The shift to the left in motor laterality is in accordance with other studies involving potentially stressful changes in environment, for example a reduction in space availability (Zucca et al., 2011). A left bias in motor laterality indicates a predisposition to process information in the right hemisphere, and rhesus monkeys displaying high right-sided, and no lateral frontal, electroencephalographic activity had significant higher basal blood cortisol concentration than animals displaying a high left frontal activity (Kalin et al., 1998). Furthermore, in common marmosets, a left forelimb preference was also associated with negative cognitive bias (Gordon and Rogers, 2015). Therefore, for evaluating animal welfare, motor laterality may be more reliable than sensory laterality, although it changes more slowly and it may not indicate acute stress. Additionally, acute stress that is controllable for the organism may not compromise welfare, but long-term and uncontrollable stress may do so (Mendl et al., 2009). Although motor laterality does not indicate acute stress, it may be a promising parameter because shifts to the left indicate long-term stress. However, it seems to change before the horses clearly display compromised welfare (STUDY 1), and therefore, further parameters should be investigated that might support these findings.

Furthermore, motor laterality measured as initial forelimb use indicates the cognitive bias of a horse (STUDY 2), and that may help to identify compromised welfare. Nonetheless, further research is needed to investigate which method of measuring motor laterality is the most reliable. The grazing stance that was observed in STUDY 1, when evaluating stress did not indicate a cognitive bias in STUDY 2, but the initial forelimb use did. Grazing stance preference and the initial forelimb use were not correlated, so future research should evaluate whether the initial forelimb use changes to the left side in stressful situations as stress hormones increase, and whether the shift to the left in grazing stance may correlate with changes in cognitive bias in stressful situations. Changes to a more negative cognitive bias would indicate compromised welfare. Therefore changes to the left side in the initial forelimb use would be an issue. To conclude, motor laterality is a promising welfare indicator that is easy to assess, objective and repeatable. But, as for sensory laterality, changes in motor laterality should be supported by the measurement of other indicators such as non-invasive stress hormone analysis (STUDY 4). Nonetheless, further research is needed to understand the relationship between emotionality, cerebral lateralization, preferred grazing stance, and initial forelimb use, although the direct measurement of cognitive bias by judgment bias testing in the meantime also has some limitations.

Cognitive bias

In addition to using sensory and motor laterality to evaluate animals' stress and welfare, it is important to observe changes in cognitive bias. Animals differ in their personalities, experiences and perceptions, and this results in inter-individual variation in cognitive bias. Therefore, animals with normal genetic diversity display positive and negative cognitive bias depending on the individual's personality. Nevertheless, a change to a more negative cognitive bias can indicate compromised welfare and can occur as a result of long-term stressors (Harding et al., 2004; Mendl et al., 2009). However, after acute stress, a higher sensitivity to reward was displayed, but cognitive bias did not

change (Hernandez et al., 2015). Similar to motor laterality, cognitive bias does not change after controllable acute stress, but changes after uncontrollable long-term stress that leads to compromised welfare. Cognitive bias also seems to be a useful parameter in evaluating welfare, but the determination of cognitive bias by using judgment bias tests has its limitations as outlined in STUDY 2 (long training period and not repeatable). However, initial forelimb use (motor laterality) seems to be a reliable indicator of cognitive bias. Left-sided motor laterality indicated negative cognitive bias and that may be a welfare issue if an animal's negative cognitive bias increases. Until the relationship between emotionality, cerebral lateralization, cognitive bias and welfare is completely understood, the use of two or more parameters to reliably determine stress in animals is recommended, and in particular a combination of behavioural and physiological parameters such as laterality and stress hormones, as demonstrated in STUDY 1.

Simplifying non-invasive evaluation of physiological welfare parameters

When studying animal welfare, the use of two or more parameters is recommended for reliable evaluation (König v. Borstel et al., 2017). In particular, behavioural parameters should be supported by physiological parameters. Sampling for non-invasive faecal stress hormone analysis was simplified and improved with a novel drying method in silica gel (STUDY 4), which broadens the possibilities for research on wild horses.

A population bias in motor laterality was observed, especially in domestic horses. Breeds such as Thoroughbred and Standardbred horses showed a population level left-sided bias in grazing stance (McGreevy and Rogers, 2005; McGreevy and Thomson, 2006), but this has not been found in wild horses (Austin and Rogers, 2014, 2012), in which motor laterality was observed only on individual level. Therefore, it would be interesting to investigate whether the differences in motor laterality are related to stress hormones. Furthermore, the investigation of wild horses' coping strategies in stress situations could be instructive for the understanding of horses' natural ways of coping with stress. This knowledge could be applied to improve housing systems for domestic horses and reducing suffering from stress. For such studies, stress hormone analysis is recommended, and this is now easier with the novel conservation method for faecal samples. This is important because, especially in wild animals, it is difficult to get blood samples. The procedure of anaesthetising the animal would lead to stress both in the animal involved and in the whole group, and the resulting increase in stress hormones would falsify the results (Balcombe et al., 2004; Morrow et al., 2002; Palme et al., 1996; Säkkinen et al., 2004; Sheriff et al., 2011). When sampling faeces in the field, it has been necessary to freeze the samples as soon as possible. This limits the time available to observe the wild horses' behaviour of interest (for example laterality). Therefore, drying faecal samples by using silica gel would simplify the study of stress in wild horses, for example stress-related behaviour and/or coping strategies.

Strength of laterality

The strength of laterality did not reliably indicate stress or cognitive bias in horses, as demonstrated in STUDY 5. The strength of neither motor nor sensory laterality reliably correlated with stress hormones or IgA, changes in stress hormones or IgA, or cognitive bias. In STUDY 1, only under the condition at one week after the change of housing did the horses with stronger basal sensory laterality have a lower faecal stress hormone concentration, and in the condition 48 h after the first training session the faecal IgA concentration correlated positively with the strength of sensory laterality from basal situation. But on population level, there was no change in faecal IgA concentration compared

with basal situation. Both these results could be explained by the uneven distribution of left-sided and right-sided animals, with right-sided animals being more strongly lateralized. From this, it is possible to conclude that it is not the strength, but the direction that is important, and that would explain why animals with a preference for the right side are less stress-sensitive and cope better/more actively (Sullivan, 2004).

Furthermore, as outlined for all other parameters, to evaluate animal welfare it is important to observe changes in the stress parameters. In practice, when working with animals that have normal genetic diversity, personality traits influence the basal situations of the stress parameters, and this leads to a greater range of basal stress hormone concentrations, basal sensory and motor laterality indices, and basal cognitive biases as demonstrated by the first three studies in this thesis. The strength of laterality also seems to be linked to different characteristics of the individual such as age, stress sensitivity etc. (McGreevy and Rogers, 2005; Rogers, 2017), and this leads to inter-individual variation. As with the other investigated aspects, changes in the strength of motor or sensory laterality did not reliably indicate stress responses, and only seem to correlate with increasing inter-individual differences in laterality indices in the particular test situation. Nonetheless, as demonstrated in STUDY 1, stress leads to changes in stress parameters, especially to a shift in sensory and motor laterality to the left. Donkeys also showed increased use of the left forelimb in the potentially stressful situation of a reduction in space availability (Zucca et al., 2011). Before, the donkeys displayed a population right sidedness and afterwards no bias at the population level. When considering the strength of laterality, the donkeys shifted from a stronger to a weaker laterality. In contrast, the present results of STUDY 1 showed a shift from weaker to a stronger lateralization in some test situations. Left shifts in laterality will cause a weaker or stronger lateralization depending on the basal lateralization. Because these results demonstrate that stress may cause a shift to the left in laterality regardless of the basal bias, the strength of motor or sensory laterality does not seem to be a reliable parameter for the evaluation of stress and animal welfare. Nonetheless, it cannot be excluded that the strength of laterality may provide insight into other parameters or personality traits, and may be useful in the evaluation of predispositions for some characteristics or reactions in specific situations. For example, a more strongly lateralized brain could help protect the individual from injuries during fights with conspecifics (Leliveld, 2019), but further research is needed to understand the full meaning of the strength of laterality.

Perspectives

Although there is a growing interest in laterality and many studies have already been done, research into laterality is still in its infancy. Results of different studies seem to be contradictory, and there are many different methods of measuring laterality. Therefore, the next step should be to try to standardize the methods of measuring motor and sensory laterality to make the results more repeatable and comparable. However, standardization will have limitations, as different species have developed different motor skills, and this may result in different manifestations of motor laterality (Leliveld, 2019). As motor laterality is generally task-related in animals, one possibility could be creating a standard task that will be done in similar ways by different species. Therefore, the initial forelimb use when moving off from a standing position, that was observed in STUDY 2, could be a task that has widespread potential as a standard measurement of motor laterality as there are many quadruped species. Further research is needed on this topic as, until now, results of different studies were not always comparable or applicable to other studies and species.

Furthermore, factors that possibly influence laterality, such as emotionality, valence of emotions, dominance, motivation, personality, stress, circumstances etc., should be included in laterality research. These factors should be investigated more detailed, to understand how they influence each other and how they influence the direction and/or the strength of laterality.

Nonetheless, there are some observations that occurred repeatedly in different species and suggest that in most species stress involves the right brain hemisphere and enhances its dominance. This demonstrates the potential of laterality as an animal welfare indicator both on the population and the individual level. It may help improve selective breeding for less stress-sensitive animals that cope better in human management regimes, and it may help improve housing conditions and management to optimize animal welfare. For example, cows that preferred to lie down on their left side, or to pass a person to their left side, produced more milk per day (Goma et al., 2018; Večeřa et al., 2016). Goma et al. (2018) assumed that high milk production may have caused higher stress in cows resulting in more anxious and left lateralized behaviour. In this case, the determination of laterality could help to optimize milk production without compromising the cows' welfare by indicating which cows are stressed because their daily milk production is too high. Therefore, laterality can improve the selective breeding by excluding animals that become stressed by a high meat, milk, or egg production rate and this improve welfare for all livestock. But there is still a lack of knowledge regarding exactly how high production rates affect laterality. It is also still unclear whether laterality, or the tendency to lateralization on a particular side, is inherited. Another topic is the investigation of the perception and emotions of animals when interacting with humans, during human treatment, and in a human management regime. A greater understanding of these factors would help to reduce stress in animals by improving handling and housing conditions.

Conclusion

It has been demonstrated that laterality is a promising welfare indicator, especially in horses, as it changes in line with increasing stress hormone concentrations and correlates with the cognitive bias. As with the already established welfare indicators, it is necessary to record changes in the parameters to reliably evaluate the welfare of an animal, because inter-individual differences in genetics, personality, age etc. lead to a high variation of laterality, even in basal situations without stressors and with good welfare. Furthermore, the use of additional stress parameters, such as non-invasive stress hormone analysis from faecal samples, is recommended to clarify the meaning of a shift in laterality. Compared to established welfare indicators, the evaluation of laterality is less time consuming (compared to cognitive bias testing), inexpensive (compared to stress hormone analysis), objective (compared to other behavioural parameters), and repeatable (compared to cognitive bias testing). Therefore, laterality seems to be a promising welfare indicator not only for scientific research, but also for lay persons. Nonetheless, further research is necessary to improve and develop the measurement, especially for motor laterality, to create more reliable and comparable results in future research. Further research is also needed to better understand the relationship between emotional processing and cerebral lateralization that might explain apparently contradictory research results. Several factors may influence the cerebral lateralization of emotional processing, such as arousal level (high / low), valence (positive / negative), activity level (active / inhibited), approach / withdrawal behaviour, feeling of dominance (dominant / submissive), feeling of controllability (controllable / uncontrollable), and possibly not yet identified parameters, that result in either left, right or no preference of limbs or sensory organs.

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CURRICULUM VITAE

PERSONAL DATA

Name	**Isabell Marr**
Date and city of birth	1st of July 1987, Leipzig

UNIVERSITY EDUCATION

since 05/2015   Dissertation **Agricultural Sciences** (**Dr. sc. agr.**) at the University of Hohenheim and at the Nürtingen - Geislingen University
Topic: *„Non-invasive welfare evaluations in horses: The usefulness of laterality"*

10/2012 – 01/2015   **Master of Science Animal Biology and Biomedical Sciences** at the University of Veterinary Medicine Hannover, Foundation (TiHo)
Topic: *„Expression von Tight Junctions im Dünndarm von wachsenden Ziegen mit unterschiedlicher N- und/oder Ca-Versorgung"* [Expression of tight junction proteins in the small intestine of young goats offered a reduced N and/or Ca diet]

10/2009 – 10/2012   **Bachelor of Science Biology** at the Leibniz University Hannover
Topic: *„Begrüßungsverhalten unter in Gruppen gehaltenen männlichen Pferden (Hengste/Wallache)"* [Greeting behaviour of group housed male horses (stallions/geldings)]

SCHOOL EDUCATION AND TRAINING

08/2006 – 07/2008   training as professional horsewoman/equestrian (breeding and equine management) at Circle-L-Ranch Wenden, Justus-von-Liebig-School Hannover

08/1998 – 06/2006   Abitur Robert-Schumann-Gymnasium, Leipzig

WORK EXPERIENCE

Research assistant and lectureship:

Nürtingen - Geislingen University

09/2015 – 07/2019   lectureship for presentations in the following subjects: „Ausbildung von Reitern", „Ausbildung von Pferden", „Pferdehaltung und spezielle Ethologie"; tutor for „Wissenschaftliches Arbeiten"

University of Veterinary Medicine Hannover, Foundation, Department of Physiology

11/2014 – 12/2014   tutor for the course
„Physiologische und ernährungsphysiologische Übungen"
(main topic intestinal motility)

University of Veterinary Medicine Hannover, Foundation, Institute of Animal Welfare and Ethology

09/2013 – 09/2014   tutor for the course
„Klassische Lerntheorien"

Equine training, equine care und show service:

since 10/2011	freelance work: IM-Westerntraining – horse training and riding lessons Show-Secretary and Manager for AQHA- and DQHA-Shows
12/2008 – 09/2011	horse training and care at different employers
08/2006 – 03/2011	Show Secretary for AQHA and EWU shows of the Reitverein Wenden

STAY ABROAD

09/2008 – 11/2008	Jim-Dudley-Quarter-Horses, Columbia, USA, horse training and care

INTERNSHIP

08/2009	Gestüt Eckershausen, horse training and care
20/02 – 24/02/2006	Medizinische Tierklinik, University of Leipzig, animal care

PRESENTATIONS

21/09 – 24/09/2018	14th Conference of International Equitation Science, Rome, Italy "Evidence that right sided horses are more optimistic than left-sided horses"
15/01 – 16/01/2018	8. Leipziger Tierärztekongress, Leipzig, Germany "Non-invasive Stress Analyse bei Pferden"
07/03 – 08/03/2017	Netzwerktagung Pferd 2017, Nürtingen Germany "Optimierung der Haltung und des Trainings von Pferden anhand des Stressindikators 'Lateralität'"
14/02 – 15/02/2017	8. Pferdeworkshop DGfZ, Bad Bevensen, Germany "Veränderungen der sensorischen Lateralität als Indikator für haltungsbedingten Stress sowie stress bei beginnendem Training von Pferden (Equus caballus)"
17/11 – 19/11/2016	49. Internationale Tagung Angewandte Ethologie, Freiburg, Germany "Sensorische Lateralität als Indikator für haltungsbedingten Stress sowie Stress bei beginnendem Training von Pferden (Equus caballus) [Sensory laterality as an indicator for stress in horse (Equus caballus) management and initial training]"
06/05 – 08/05/2016	33. annual meeting FFP, Freisingen, Germany "Gibt die sensorische Lateralität im Objekttest Aufschluss über das Interieur, den aktuellen Gemütszustand, oder den Trainingszustand der Pferde"

POSTER PRESENTATIONS

27/02 – 28/02/2018	Göttinger Pferdetage, Göttingen, Germany "Non-invasive Stress Analyse bei Pferden"
12/07 – 15/07/2016	8th European Conference of Behavioural Biology, Vienna, Austria "Sensory laterality, stress hormones and immunoglobulin A in horses (*Equus caballus*) during changes in semi-natural environments and human management regimes"

Nürtingen, 27.07.2020

SUPPLEMENTARY DATA

Evidence for Right-Sided Horses Being More Optimistic than Left-Sided Horses
Isabell Marr, Kate Farmer and Konstanze Krüger

STUDY 2: Results S1

Generalized linear mixed model with random effects

LatAmb: latency to approach ambiguous location in s
Facility: facility a, b, and c
BoxbeforeAmb: last location before testing with ambiguous location; positive (p) or negative (n)
Breed: warmblood, pony, and thouroughbred
Housing: group housing or box with paddock
Pos.Side: trained positive location of the box; left (l) or right (r)
Sex: mare (m) or gelding (g)
ML: motor laterality (laterality index)
SL: sensory laterality (laterality index)

Initial forelimb use (MLstart):

```
glm(formula = LatAmb ~ PosSide + BoxBeforeAmb + MLstart/(Age + Facility + Breed +
housing + Sex), family = gaussian(identity), data = Datenmatrix)

Deviance Residuals:
    Min       1Q    Median       3Q       Max
-19.7084   -6.1635   0.9422   4.4886   25.9218

Coefficients:
                                Estimate Std. Error t value Pr(>|t|)
(Intercept)                       19.519     12.199   1.600    0.161
PosSide[T.r]                      10.539     18.094   0.582    0.581
BoxBeforeAmb[T.p]                 14.282     13.821   1.033    0.341
MLstart                         -156.998    121.395  -1.293    0.243
MLstart:Age                        5.541      4.778   1.160    0.290
MLstart:Facility[T.f1]           -87.303     96.268  -0.907    0.399
MLstart:Facility[T.f2]           -24.721     88.628  -0.279    0.790
MLstart:Breed[T.Thouroughbred]    60.591    107.757   0.562    0.594
MLstart:Breed[T.Warmblood]         6.856     68.645   0.100    0.924
MLstart:housing[T.group housing] 100.889    111.959   0.901    0.402
MLstart:Sex[T.m]                  13.059     40.735   0.321    0.759

(Dispersion parameter for gaussian family taken to be 339.5188)
    Null deviance: 6291.5  on 16  degrees of freedom
Residual deviance: 2037.1  on  6  degrees of freedom
  (61 observations deleted due to missingness)
AIC: 153.61
Number of Fisher Scoring iterations: 2
```

Simplified model with the best goodness of fit
```
Call:
glm(formula = LatAmb ~ MLstart, family = gaussian(identity), data = Datenmatrix)

Deviance Residuals:
```

```
     Min        1Q     Median        3Q        Max
-24.4113   -8.2052   -0.5927    8.9316    27.2017
Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   27.089      4.155   6.519 9.7e-06 ***
MLstart      -44.858     12.083  -3.713  0.00208 **
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1


(Dispersion parameter for gaussian family taken to be 218.5852)
    Null deviance: 6291.5  on 16  degrees of freedom
Residual deviance: 3278.8  on 15  degrees of freedom
  (61 observations deleted due to missingness)
AIC: 143.7
Number of Fisher Scoring iterations: 2
```

Relaxed forelimb position (MLrelaxed)

```
Call:
glm(formula = LatAmb ~ PosSide + BoxBeforeAmb + MLrelaxed/(Age + Breed + Facility +
housing + Sex), family = gaussian(identity), data = Datenmatrix)

Deviance Residuals:
      Min        1Q     Median        3Q        Max
-23.8939   -11.8452    0.1796    5.7358    30.3379

Coefficients:
                                   Estimate Std. Error t value Pr(>|t|)
(Intercept)                          18.737     13.411   1.397    0.212
PosSide[T.r]                         11.832     17.116   0.691    0.515
BoxBeforeAmb[T.p]                   -17.632     17.974  -0.981    0.364
MLrelaxed                            21.675     88.385   0.245    0.814
MLrelaxed:Age                        -4.991      4.500  -1.109    0.310
MLrelaxed:Breed[T.Thouroughbred]    -72.389    106.153  -0.682    0.521
MLrelaxed:Breed[T.Warmblood]         28.317     55.439   0.511    0.628
MLrelaxed:Facility[T.f1]             80.599    143.591   0.561    0.595
MLrelaxed:Facility[T.f2]            121.926     78.234   1.558    0.170
MLrelaxed:housing[T.group housing]  -28.689    148.233  -0.194    0.853
MLrelaxed:Sex[T.m]                  -28.335     58.245  -0.486    0.644

(Dispersion parameter for gaussian family taken to be 604.7097)
    Null deviance: 6291.5  on 16  degrees of freedom
Residual deviance: 3628.3  on  6  degrees of freedom
  (61 observations deleted due to missingness)
AIC: 163.42
Number of Fisher Scoring iterations: 2
```

Simplified model with the best goodness of fit
```
Call:
glm(formula = LatAmb ~ MLrelaxed, family = gaussian(identity), data = Datenmatrix)

Deviance Residuals:
    Min        1Q     Median        3Q        Max
-15.280   -10.191    -9.438    -1.028    42.896

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)    18.27       5.63   3.246  0.00543 **
MLrelaxed       5.01      13.21   0.379  0.70978
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

(Dispersion parameter for gaussian family taken to be 415.4507)
    Null deviance: 6291.5 on 16 degrees of freedom
Residual deviance: 6231.8 on 15 degrees of freedom
  (61 observations deleted due to missingness)
AIC: 154.62
Number of Fisher Scoring iterations: 2

Task related forelimb position (MLbox):

Call:
glm(formula = LatAmb ~ PosSide + BoxBeforeAmb + MLbox/(Sex + housing + Facility + Breed + Age), family = gaussian(identity), data = Datenmatrix)

Deviance Residuals:
     Min   1Q   Median   3Q   Max
-16.9130   -5.7045   0.2229   4.9464   27.4710

Coefficients:
                              Estimate Std. Error t value Pr(>|t|)
(Intercept)   25.913   9.571   2.707   0.0352 *
PosSide[T.r]   3.113   10.250   0.304   0.7716
BoxBeforeAmb[T.p]   -22.437   13.700 -1.638   0.1526
MLbox   -229.231   140.779 -1.628   0.1546
MLbox:Sex[T.m]   408.932   171.137   2.389   0.0541
MLbox:housing[T.group housing] -313.324 207.044 -1.513   0.1810
MLbox:Facility[T.f1]   257.044   196.064   1.311   0.2378
MLbox:Facility[T.f2]   288.342   188.371   1.531   0.1767
MLbox:Breed[T.Thouroughbred] -468.070   229.614 -2.039   0.0876
MLbox:Breed[T.Warmblood]   180.220   139.136   1.295   0.2428
MLbox:Age   1.513   7.744   0.195   0.8516

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

(Dispersion parameter for gaussian family taken to be 322.9118)
    Null deviance: 6291.5 on 16 degrees of freedom
Residual deviance: 1937.5 on 6 degrees of freedom
  (61 observations deleted due to missingness)
AIC: 152.75
Number of Fisher Scoring iterations: 2

Simplified model with the best goodness of fit
Call:
glm(formula = LatAmb ~ MLbox, family = gaussian(identity), data = Datenmatrix)

Deviance Residuals:
    Min   1Q   Median   3Q   Max
-17.561 -11.128   -7.712   -0.362   40.232

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   21.128   5.349   3.95 0.00128 **
MLbox   -15.295   18.644   -0.82 0.42486

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

(Dispersion parameter for gaussian family taken to be 401.4253)
    Null deviance: 6291.5 on 16 degrees of freedom
Residual deviance: 6021.4 on 15 degrees of freedom
  (61 observations deleted due to missingness)

```
AIC: 154.03
Number of Fisher Scoring iterations: 2
```

Sensory laterality

```
Call:
glm(formula = LatAmb ~ BoxBeforeAmb + PosSide + SL/(Sex + housing + Facility + Breed
+ Age), family = gaussian(identity), data = Datenmatrix)

Deviance Residuals:
    Min       1Q    Median       3Q       Max
-20.891   -2.226    0.958     5.185    18.954

Coefficients:
                                Estimate Std. Error t value Pr(>|t|)
(Intercept)                       18.512      8.051   2.299   0.0611
BoxBeforeAmb[T.p]                -16.591     12.878  -1.288   0.2451
PosSide[T.r]                       3.316     11.099   0.299   0.7752
SL                                49.811     98.999   0.503   0.6328
SL:Sex[T.m]                     -112.856     66.097  -1.707   0.1386
SL:housing[T.group housing]       72.000    115.301   0.624   0.5553
SL:Facility[T.f1]               -155.273    100.962  -1.538   0.1750
SL:Facility[T.f2]                -78.376     44.225  -1.772   0.1267
SL:Breed[T.Thouroughbred]        137.191    141.769   0.968   0.3706
SL:Breed[T.Warmblood]            -64.578     74.220  -0.870   0.4177
SL:Age                             2.529      3.187   0.794   0.4577
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1


(Dispersion parameter for gaussian family taken to be 224.4657)
    Null deviance: 6291.5  on 16  degrees of freedom
Residual deviance: 1346.8  on  6  degrees of freedom
  (61 observations deleted due to missingness)
AIC: 146.57
Number of Fisher Scoring iterations: 2
```

Simplified model with the best goodness of fit
```
Call:
glm(formula = LatAmb ~ SL/(Facility), family = gaussian(identity),
    data = Datenmatrix)

Deviance Residuals:
    Min       1Q    Median       3Q       Max
-23.639   -7.606    -4.575    6.328    33.378

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)          17.61       4.33   4.066  0.00134 **
SL                   15.09      15.08   1.001  0.33508
SL:Facility[T.f1]   -96.24      35.29  -2.727  0.01728 *
SL:Facility[T.f2]   -53.92      27.60  -1.954  0.07256
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1


(Dispersion parameter for gaussian family taken to be 267.899)

    Null deviance: 6291.5  on 16  degrees of freedom
Residual deviance: 3482.7  on 13  degrees of freedom
  (61 observations deleted due to missingness)
AIC: 148.72
Number of Fisher Scoring iterations: 2
```

Preservation of fecal cortisol metabolites (FGM) and Immunoglobulin A (IgA) through silica gel drying for field studies in horses

Konstanze Krueger, Isabell Marr, Andrea Dobler, Rupert Palme

STUDY 4: File S1

Calculation of sample weight –balancing weight loss through drying

FGMs

Glucocorticoid metabolites were extracted from horse feces with the simplified method described by Flauger et al., (2010). We used 0.5 g of wet feces, which is equivalent to 33.33 % of 1.5 g wet feces from the frozen sample. In the dried samples we balanced the weight loss from drying, by weighting each sample within its filter and using the exact dry mass without the filter which, for FGM extraction, corresponded to 1.5 g of fresh feces in each sample (complete data see Table S1).For the calculation of the dry mass necessary for extraction, we applied the following formula:

weight dry sample for FGM extraction = weight dry feces * 33.33 / 100

Example calculation dry samples FGM (Supplementary Table S1):
original sample = weight original feces (1.51 g) + weight tee filter (0.25 g) = 1.76 g
weight dry sample = weight dry feces(0.26 g) + weight tee filter (0.25 g) = 0.51 g
weight dry feces = weight dry sample (0.51 g) -weight tee filter (0.25 g) = 0.26 g
weight loss = weight original feces (1.51 g) + weight tee filter (0.25 g) -weight dry feces (0.26) = 1.25g
% weight loss particular sample =weight loss 1.25 g * 100 / weight original feces (1.51 g) = 82,78%
weight dry sample for FGM extraction= weight dry feces (0.26) * 33.33 / 100 = 0.0867g

IgA

We used 1 g wet feces, which is equivalent to 66.67%of 1.5gwet feces from the frozen sample. In dried samples we balanced the weight loss from drying similar as for the FGM extraction only that the weight of each samples corresponded to 1 g of fresh feces (complete data see Table S1). We applied the following formula to calculate the dry mass that is necessary for the extraction:

weight dry sample for IgA extraction = weight dry feces * 66.67 / 100

STUDY 4: Table S2

FGM	horse number	horse 1	horse 2	horse 3	horse 4	horse 5	horse 6	horse 7	horse 8	horse 9	horse 10	horse 11	horse 12
1SD = 1 day silica gel drying	weight original feces	1,51	1,5	1,52	1,5	1,51	1,5	1,5	1,52	1,5	1,52	1,51	1,52
	weight tee filter	0,25	0,25	0,24	0,25	0,24	0,25	0,25	0,25	0,25	0,24	0,24	0,25
	weight dry feces + filter	0,51	0,64	0,54	0,61	0,53	0,61	0,56	0,5	0,57	0,52	0,58	0,5
	weight dry feces	0,26	0,39	0,3	0,36	0,29	0,36	0,31	0,25	0,32	0,28	0,34	0,25
	diffrence	1,25	1,11	1,22	1,14	1,22	1,14	1,19	1,27	1,18	1,24	1,17	1,27
	% loss	82,781	74,000	80,263	76,000	80,795	76,000	79,333	83,553	78,667	81,579	77,483	83,553
	weight dry sample for extraction	0,087	0,130	0,100	0,120	0,097	0,120	0,103	0,083	0,107	0,093	0,113	0,083
7SD = 7 days silica gel drying	weight original feces	1,52	1,5	1,5	1,5	1,52	1,5	1,5	1,52	1,5	1,51	1,51	1,52
	weight tee filter	0,24	0,25	0,25	0,25	0,25	0,24	0,24	0,24	0,24	0,24	0,25	0,24
	weight dry feces + filter	0,51	0,62	0,52	0,6	0,52	0,6	0,54	0,49	0,54	0,48	0,58	0,48
	weight dry feces	0,27	0,37	0,27	0,35	0,27	0,36	0,3	0,25	0,3	0,24	0,33	0,24
	diffrence	1,25	1,13	1,23	1,15	1,25	1,14	1,2	1,27	1,2	1,27	1,18	1,28
	% loss	82,237	75,333	82,000	76,667	82,237	76,000	80,000	83,553	80,000	84,106	78,146	84,211
	weight dry sample for extraction	0,090	0,123	0,090	0,117	0,090	0,120	0,100	0,083	0,100	0,080	0,110	0,080
1AD = 1 day air drying	weight original feces	1,5	1,5	1,52	1,51	1,51	1,5	1,5	1,51	1,5	1,51	1,52	1,51
	weight petri dish	84,03	74,96	74,35	83,94	76,54	82,52	79,32	84,81	38,17	81,36	9,34	37,82
	weight dry feces + petri dish	84,35	75,36	74,68	84,29	76,86	82,9	79,64	85,1	38,49	81,67	9,68	38,13
	weight dry feces	0,32	0,4	0,33	0,35	0,32	0,38	0,32	0,29	0,32	0,31	0,34	0,31
	diffrence	1,18	1,1	1,19	1,16	1,19	1,12	1,18	1,22	1,18	1,2	1,18	1,2
	% loss	78,667	73,333	78,289	76,821	78,808	74,667	78,667	80,795	78,667	79,470	77,632	79,470
	weight dry sample for extraction	0,107	0,133	0,110	0,117	0,107	0,127	0,107	0,097	0,107	0,103	0,113	0,103
7AD = 7 days air drying	weight original feces	1,51	1,5	1,52	1,51	1,51	1,5	1,5	1,52	1,5	1,5	1,51	1,5
	weight petri dish	74,96	79,56	82	73,96	81,14	84,94	77,83	82,15	37,01	76,7	8,3	76,02
	weight dry feces + petri dish	75,31	79,97	82,34	74,34	81,46	85,32	78,15	82,5	37,31	77	8,62	76,31
	weight dry feces	0,35	0,41	0,34	0,38	0,32	0,38	0,32	0,35	0,3	0,3	0,32	0,29
	diffrence	1,16	1,09	1,18	1,13	1,19	1,12	1,18	1,17	1,2	1,2	1,19	1,21
	% loss	76,821	72,667	77,632	74,834	78,808	74,667	78,667	76,974	80,000	80,000	78,808	80,667
	weight dry sample for extraction	0,117	0,137	0,113	0,127	0,107	0,127	0,107	0,117	0,100	0,100	0,107	0,097
WR = wet sample room temperature	weight original feces	1,51	1,5	1,52	1,53	1,52	1,5	1,5	1,52	1,5	1,5	1,51	1,51
	weight airtight tube	14,84	14,78	15,26	14,9	14,87	14,89	14,81	14,75	14,93	14,75	14,81	15,21
	weight dry feces + airtight tube	no difference to original feces weight + weight airtight tube											
	weight dry feces												
	diffrence												
	% loss												
	weight sample for extraction	equal to original feces weight											

IgA	horse number	horse 1	horse 2	horse 3	horse 4	horse 5	horse 6	horse 7	horse 8	horse 9	horse 10	horse 11	horse 12
1SD = 1 day silica gel drying	weight original feces	1,52	1,5	1,5	1,52	1,51	1,5	1,5	1,51	1,5	1,52	1,5	1,5
	weight tee filter	0,25	0,25	0,24	0,24	0,24	0,25	0,24	0,25	0,24	0,24	0,24	0,25
	weight dry feces + filter	0,56	0,63	0,52	0,61	0,53	0,62	0,57	0,54	0,61	0,51	0,55	0,47
	weight dry feces	0,31	0,38	0,28	0,37	0,29	0,37	0,33	0,29	0,37	0,27	0,31	0,22
	diffrence	1,21	1,12	1,22	1,15	1,22	1,13	1,17	1,22	1,13	1,25	1,19	1,28
	% loss	79,605	74,667	81,333	75,658	80,795	75,333	78,000	80,795	75,333	82,237	79,333	85,333
	weight dry sample for extraction	0,207	0,253	0,187	0,247	0,193	0,247	0,220	0,193	0,247	0,180	0,207	0,147
7SD = 7 days silica gel drying	weight original feces	1,52	1,5	1,51	1,5	1,51	1,5	1,5	1,5	1,5	1,51	1,5	1,52
	weight tee filter	0,24	0,25	0,25	0,25	0,25	0,24	0,24	0,24	0,24	0,25	0,25	0,24
	weight dry feces + filter	0,53	0,62	0,53	0,62	0,51	0,6	0,55	0,54	0,56	0,51	0,52	0,47
	weight dry feces	0,29	0,37	0,28	0,37	0,26	0,36	0,31	0,3	0,32	0,26	0,27	0,23
	diffrence	1,23	1,13	1,23	1,13	1,25	1,14	1,19	1,2	1,18	1,25	1,23	1,29
	% loss	80,921	75,333	81,457	75,333	82,781	76,000	79,333	80,000	78,667	82,781	82,000	84,868
	weight dry sample for extraction	0,193	0,247	0,187	0,247	0,173	0,240	0,207	0,200	0,213	0,173	0,180	0,153
1AD = 1 day air drying	weight original feces	1,51	1,5	1,52	1,5	1,51	1,5	1,5	1,52	1,5	1,52	1,52	1,51
	weight petri dish	82,97	75,3	82,74	75,77	83,96	70,43	78,28	85,75	78,06	77,45	9,36	8,3
	weight dry feces + petri dish	83,3	75,68	83,09	76,12	84,27	70,8	78,6	86,06	78,37	77,71	9,69	8,59
	weight dry feces	0,33	0,38	0,35	0,35	0,31	0,37	0,32	0,31	0,31	0,26	0,33	0,29
	diffrence	1,18	1,12	1,17	1,15	1,2	1,13	1,18	1,21	1,19	1,26	1,19	1,22
	% loss	78,146	74,667	76,974	76,667	79,470	75,333	78,667	79,605	79,333	82,895	78,289	80,795
	weight dry sample for extraction	0,220	0,253	0,233	0,233	0,207	0,247	0,213	0,207	0,207	0,173	0,220	0,193
7AD = 7 days air drying	weight original feces	1,52	1,5	1,52	1,52	1,51	1,5	1,5	1,52	1,5	1,51	1,53	1,5
	weight petri dish	80,11	73,7	79,83	87,22	82,55	77,51	76,07	97,4	47,81	79,97	8,3	47,16
	weight dry feces + petri dish	80,44	74,1	80,17	87,61	82,92	77,86	76,41	97,71	48,15	80,26	8,64	47,47
	weight dry feces	0,33	0,4	0,34	0,39	0,37	0,35	0,34	0,31	0,34	0,29	0,34	0,31
	diffrence	1,19	1,1	1,18	1,13	1,14	1,15	1,16	1,21	1,16	1,22	1,19	1,19
	% loss	78,289	73,333	77,632	74,342	75,497	76,667	77,333	79,605	77,333	80,795	77,778	79,333
	weight dry sample for extraction	0,220	0,267	0,227	0,260	0,247	0,233	0,227	0,207	0,227	0,193	0,227	0,207
WR = wet sample room temperature	weight original feces	1,5	1,5	1,51	1,5	1,5	1,5	1,5	1,51	1,5	1,52	1,52	1,52
	weight airtight tube	14,81	14,82	14,76	14,85	14,82	14,9	14,83	14,82	14,86	14,82	14,85	14,91
	weight dry feces + airtight tube	no difference to original feces weight + weight airtight tube											
	weight dry feces												
	diffrence												
	% loss												
	weight sample for extraction	equal to original feces weight											

STUDY 4: Table S3

horse_num	treatment	GCMs ng/g	IgA µg/g	horse_num	treatment	GCMs ng/g	IgA µg/g
1	FR	26,62	3,87	7	FR	37,80	1,81
1	SD1	26,20	6,23	7	SD1	51,50	3,49
1	SD7	15,88	7,89	7	SD7	53,28	1,79
1	AD1	19,80	3,95	7	AD1	46,88	2,87
1	AD7	19,32	1,68	7	AD7	42,50	0,90
1	WR	16,15	3,39	7	WR	34,12	1,16
2	FR	33,57	8,33	8	FR	22,29	6,93
2	SD1	30,36	1,39	8	SD1	16,30	2,88
2	SD7	30,56	1,71	8	SD7	15,05	2,84
2	AD1	42,86	2,52	8	AD1	33,86	4,90
2	AD7	35,51	3,34	8	AD7	39,05	4,86
2	WR	0,00	0,26	8	WR	16,32	1,86
3	FR	16,17	1,74	9	FR	32,23	3,18
3	SD1	14,85	1,39	9	SD1	58,23	2,98
3	SD7	16,54	0,51	9	SD7	43,30	1,77
3	AD1	18,96	0,67	9	AD1	33,24	2,53
3	AD7	16,04	0,78	9	AD7	34,19	0,86
3	WR	15,80	0,75	9	WR	63,45	1,18
4	FR	41,93	4,00	10	FR	46,95	1,30
4	SD1	34,76	3,33	10	SD1	35,57	0,54
4	SD7	42,68	2,84	10	SD7	27,13	0,64
4	AD1	52,58	1,86	10	AD1	76,93	0,86
4	AD7	53,44	1,06	10	AD7	37,84	0,48
4	WR	17,09	0,36	10	WR	20,57	0,76
5	FR	26,55	9,27	11	FR	75,15	5,98
5	SD1	29,96	10,20	11	SD1	96,32	3,49
5	SD7	26,51	6,04	11	SD7	74,69	3,42
5	AD1	26,95	8,29	11	AD1	63,01	6,06
5	AD7	27,39	5,35	11	AD7	57,90	3,68
5	WR	12,52	0,75	11	WR	89,19	1,53
6	FR	57,49	1,80	12	FR	30,73	1,13
6	SD1	57,31	2,14	12	SD1	22,35	0,91
6	SD7	55,62	0,73	12	SD7	33,35	0,60
6	AD1	82,87	2,25	12	AD1	55,86	0,72
6	AD7	85,95	0,71	12	AD7	46,97	0,99
6	WR	27,26	0,78	12	WR	26,33	0,34

STUDY 4: File S4

Full Statistical Data

GLM and Correlation for FGMs Analysis

```
Shapiro-Wilk normality test
data:  FGMs
W = 0.93117, p-value = 0.0007006


Call:
glm(formula = FGM ~ horse + treatment, family = gaussian(identity),data = Dataset)

Deviance Residuals:
     Min       1Q   Median       3Q      Max
 -24.271   -7.410    0.246    4.475   35.957

Coefficients:
                   Estimate Std. Error t value Pr(>|t|)
(Intercept)           7.747      4.554   1.701   0.0937 .
Horse                 4.545      0.427  10.644 7.02e-16 ***
treatment[T.AD1]      8.861      5.106   1.735   0.0874 .
treatment[T.AD7]      4.052      5.106   0.793   0.4304
treatment[T.WR]      -9.057      5.106  -1.774   0.0808 .
treatment[T.SD1]      2.187      5.106   0.428   0.6698
treatment[T.SD7]     -1.074      5.106  -0.210   0.8340
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1


(Dispersion parameter for gaussian family taken to be 156.4348)
    Null deviance: 30037  on 71  degrees of freedom
Residual deviance: 10168  on 65  degrees of freedom
AIC: 576.75


Spearman rank correlations FGMs:
        FR      AD1      AD7      WR      SD1      SD7
FR   1.0000   0.8531   0.7483   0.6154   0.8462   0.8112
AD1  0.8531   1.0000   0.8741   0.5594   0.5664   0.6503
AD7  0.7483   0.8741   1.0000   0.6154   0.5175   0.7133
WR   0.6154   0.5594   0.6154   1.0000   0.7343   0.7692
SD1  0.8462   0.5664   0.5175   0.7343   1.0000   0.8531
SD7  0.8112   0.6503   0.7133   0.7692   0.8531   1.0000
 Number of observations: 12
 Pairwise two-sided p-values:
        FR      AD1      AD7      WR      SD1      SD7
FR            0.0004   0.0051   0.0332   0.0005   0.0014
AD1  0.0004            0.0002   0.0586   0.0548   0.0220
AD7  0.0051   0.0002            0.0332   0.0849   0.0092
WR   0.0332   0.0586   0.0332            0.0065   0.0034
SD1  0.0005   0.0548   0.0849   0.0065            0.0004
SD7  0.0014   0.0220   0.0092   0.0034   0.0004
 Adjusted p-values (Holm's method)
        FR      AD1      AD7      WR      SD1      SD7
FR            0.0059   0.0461   0.1658   0.0063   0.0150
AD1  0.0059            0.0030   0.1658   0.1658   0.1322
AD7  0.0461   0.0030            0.1658   0.1658   0.0644
WR   0.1658   0.1658   0.1658            0.0523   0.0345
SD1  0.0063   0.1658   0.1658   0.0523            0.0059
SD7  0.0150   0.1322   0.0644   0.0345   0.0059
```

GLM and Correlation for IgA Analysis

```
Shapiro-Wilk normality test
data:  IgA
W = 0.84018, p-value = 0.0000002461


Call:
glm(formula = IgA ~ horse_data_ranking + treatment, family = gaussian(identity),
data = Dataset)


Deviance Residuals:
    Min       1Q    Median       3Q       Max
-2.9575  -0.8581  -0.1246   0.7106    4.3451


Coefficients:
                    Estimate Std. Error t value Pr(>|t|)
(Intercept)          1.02740    0.52463   1.958 0.054486 .
horse_data_ranking   0.47478    0.04919   9.652 3.58e-14 ***
treatment[T.AD1]    -0.99085    0.58823  -1.684 0.096888 .
treatment[T.AD7]    -2.05656    0.58823  -3.496 0.000856 ***
treatment[T.WR]     -3.02078    0.58823  -5.135 2.77e-06 ***
treatment[T.SD1]    -0.86603    0.58823  -1.472 0.145775
treatment[T.SD7]    -1.54685    0.58823  -2.630 0.010657 *
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1


(Dispersion parameter for gaussian family taken to be 2.07608)
    Null deviance: 394.24  on 71   degrees of freedom
Residual deviance: 134.95  on 65   degrees of freedom
AIC: 265.56


Number of Fisher Scoring iterations: 2


Spearman rank correlations IgA:
        FR       AD1      AD7      WR       SD1      SD7
FR    1.0000   0.0769   0.7692   0.5594   0.8531   0.7413
WR    0.0769   1.0000   0.5315   0.4476   0.0769   0.5175
AD1   0.7692   0.5315   1.0000   0.7692   0.7483   0.8881
SD1   0.5594   0.4476   0.7692   1.0000   0.5385   0.8601
AD7   0.8531   0.0769   0.7483   0.5385   1.0000   0.7413
SD7   0.7413   0.5175   0.8881   0.8601   0.7413   1.0000
 Number of observations: 12
 Pairwise two-sided p-values:
        FR       AD1      AD7      WR       SD1      SD7
FR             0.8122   0.0034   0.0586   0.0004   0.0058
WR    0.8122            0.0754   0.1446   0.8122   0.0849
AD1   0.0034   0.0754            0.0034   0.0051   0.0001
SD1   0.0586   0.1446   0.0034            0.0709   0.0003
AD7   0.0004   0.8122   0.0051   0.0709            0.0058
SD7   0.0058   0.0849   0.0001   0.0003   0.0058
 Adjusted p-values (Holm's method)
        FR       AD1      AD7      WR       SD1      SD7
FR             1.0000   0.0414   0.4101   0.0054   0.0522
WR    1.0000            0.4254   0.4338   1.0000   0.4254
AD1   0.0414   0.4254            0.0414   0.0512   0.0017
SD1   0.4101   0.4338   0.0414            0.4254   0.0046
AD7   0.0054   1.0000   0.0512   0.4254            0.0522
SD7   0.0522   0.4254   0.0017   0.0046   0.0522
```

AFFIDAVIT

Affidavit
pursuant to Sec. 8(2) of the University of Hohenheim's doctoral degree regulations for Dr.sc.agr.

1. I hereby declare that I independently completed the doctoral thesis submitted on the topic
Non-invasive welfare evaluations in horses: The usefulness of laterality

2. I only used the sources and aids documented and only made use of permissible assistance by third parties. In particular, I properly documented any contents which I used - either by directly quoting or paraphrasing - from other works.

3. I did not accept any assistance from a commercial doctoral agency or consulting firm.

4. I am aware of the meaning of this affidavit and the criminal penalties of an incorrect or incomplete affidavit.

I hereby confirm the correctness of the above declaration. I hereby affirm in lieu of oath that I have, to the best of my knowledge, declared nothing but the truth and have not omitted any information.

………………………………………………………   ……………………………………………………........
(Place, date)   (Signature)