

ANGLIA RUSKIN UNIVERSITY
FACULTY OF SCIENCE AND ENGINEERING

**BENEFITS OF SOCIAL BONDS IN DOMESTIC
HORSES (*Equus caballus*).**

**The effect of social context on behaviour and cardiac
activity.**

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A thesis in partial fulfilment of the
requirements of Anglia Ruskin University
for the degree of Doctor of Philosophy

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This work is dedicated to all my equine teachers,
who, with their subtle signs,
inspired me to leave the traditional equestrian arena behind
and to change my perspective.
By stimulating my curiosity about their social lives,
they led me towards this research project,
and also impelled me to develop new handling and training approaches.

Schecki,

Saldino,

Rotschimmel,

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Don,

Laurin,

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Branbo,

and last but definitely not least

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ABSTRACT

Horses (*Equus* spp.) are one of the few species that form close bonds between unrelated and non-reproductive conspecifics. These are characterised by a preference towards specific group members, spatial proximity, and high rates of affiliative interactions and can provide individuals with different benefits. The ultimate benefits are well evidenced, such as increased reproductive success, health, and longevity. However, it is less understood whether these bonds provide benefits on a proximate level, such as social buffering. Thereby, the proximity to, or interactions with, a closely bonded conspecific during or after a stressful situation can provide support and facilitate a decrease of the behavioural and physiological stress response.

The aim of this research was to address this knowledge gap by investigating whether domestic horses (*E. caballus*) benefit from social buffering provided by closely bonded, but unrelated, conspecifics. Through a combination of behavioural observations and heart rate recordings, bond-related buffering effects were investigated in three contexts: (1) during social interactions among group members, (2) during separation from the group, and (3) during novel object exposures. In all contexts, it was assessed whether the interaction with, or the presence of, a closely bonded conspecific would affect the behaviour and cardiac activity of the horses under study.

The analysis showed that group-housed horses primarily engaged in low-intensity agonistic interactions. These did not facilitate a pronounced stress response. Social rank but not social bonds between the horses had a small effect on their heart rate during agonistic interactions. The heart rate in receivers of threats was slightly higher the higher the initiator was in rank. Grooming was the only affiliative interaction that corresponded with a lower heart rate. This effect was independent of the bond strength between the horses. During the separation, the horses' behaviour and heart rate were not affected by the bond relationship to the support provider. Their vigilance during separation was lower when with any conspecific than when alone. Moreover, the rate of affiliative interactions and spatial proximity did not differ between closely bonded and less closely bonded horses and both did not affect their heart rate or heart rate variability. The novel object tests represented a mild stressor. Upon the initial exposure to a novel object, the horses showed proximity seeking to closely bonded conspecifics. Throughout the remainder of the object tests, the horses' behavioural and physiological stress response and their exploratory behaviour were independent of their social context.

These findings make a novel contribution to knowledge as they reveal no strong evidence that close social bonds among unrelated horses provided social buffering in the specified contexts. Rather, the horses' behavioural and physiological responses were generalised towards closely bonded and less closely bonded members of their group or were independent of their social context. Thus, this research has contributed to the understanding of the role of social bonds between domestic horses in different contexts. Moreover, this research has implications for equine science and can inform housing and husbandry routines from an equine welfare perspective. It identifies that in established groups of horses, agonistic interactions are mostly not associated with a pronounced stress response. Furthermore, grooming and potentially other affiliative behaviours can promote positive welfare. These are valuable findings that support the practice of housing horses in groups. Additionally, in contexts of mild stress, horses seem to benefit from the presence of any familiar conspecific, independently of their bond relationship. These findings suggest that horses can benefit from the presence of familiar conspecifics during potentially stressful husbandry routines.

Keywords: Socio-physiology, separation, novel object test, equine welfare, heart rate, heart rate variability.

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LIST OF ABBREVIATIONS

DS	David Score
DS _{Diff}	Difference in David Score
DSI	Dyadic Composite Sociality Index
GLZM	Generalized liner mixed-effects model
HP	High preference/highly-preferred
HPV-axis	Hypothalamic-pituitary axis
HR	Heart rate
HRV	Heart rate variability
ISO	Isolation (Experimental condition)
IQR	Interquartile range
LP	Low preference/less-preferred
LM	General linear model
LMM	Linear mixed-effects model
PI	Preference Index
SAM-axis	Sympathetic-adreno-medullary axis
SE	Standard error
SD	Standard deviation

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I declare that this thesis, and the research that it details, are the result of my own work. I acknowledge the guidance of my supervisors, Dr Claudia A.F. Wascher, Dr Jacob C. Dunn, and Dr Dawn Hawkins. I confirm that where the work of others was used to inform my research, I provided explicit citations. I further state that no part of my work has been submitted for any other qualification, either at Anglia Ruskin University, or at any other institution.

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Signed



.....

Denise Viktoria Hebesberger

CHAPTER 1

Benefits of social bonds in domestic horses (*Equus caballus*) – General introduction and study outline



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1.1 Social bonds and their benefits

In this thesis, it was investigated whether close bonds among unrelated domestic horses (*Equus caballus*), specifically of the same sex, facilitate social buffering of their behavioural and physiological stress response. To introduce this work, social bonds, their benefits, and links to physiological mechanisms will be characterised. Thereafter, gaps in current research will be pointed out, followed by a description of the aim and structure of this thesis.

1.1.1 Social bonds

In species that live in stable social groups, natural selection favours social strategies for which benefits or advantages outweigh their costs or disadvantages (Silk, 2012). One social strategy of group-living animals is the formation of differentiated relationships among the members of a group, thereby determining the group's social structure (Kappeler et al., 2013; Koenig et al., 2013). Relationships are shaped by the patterns of interactions over time, refer to what happens between two individuals (content), and how the interactions are characterised (quality) (Hinde, 1976). For social interactions to become a relationship between two individuals, it is necessary that they know each other and can remember previous interactions (Ostner and Schülke, 2014). Based on this bookkeeping of previous interactions, individuals can adapt their behaviour towards their conspecifics in the present and future (Aureli et al., 2012; Seyfarth and Cheney, 2012).

The two observable relationship pillars of social groups in birds and mammals are rank relationships and social bonds (Sachser, Dürschlag and Hirzel, 1998; Ziegler and Crockford, 2017). Rank relationships refer to the individual's position in the dominance hierarchy, which can be either socially inherited (e.g., in Spotted hyenas, *Crocuta crocuta*: Engh et al., 2000) or the result of winning or losing agonistic interactions (Barrette and Vandal, 1986; Briffa et al., 2013; e.g., in Horses: Van Dierendonck and De Vries, 1995). The relative rank positions in a dominance hierarchy can dictate who has prioritised access to resources such as food (Appleby, 1980; Banks et al., 1979; Giles et al., 2015) or mating partners (Newton-Fisher et al., 2009; Witt, Schmidt and Schmitt, 1981). Social bonds are relationships characterised by selective affiliative interactions with one or more preferred members of the social group (Ziegler and Crockford,

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2017). Bonded individuals show low aggression levels, frequent proximity, and a high rate of affiliative interactions, such as grooming. The interaction patterns between bonded individuals are usually bi-directional (Silk, Cheney and Seyfarth, 2013). Social bonds are mostly stable over a longer period of time and therefore a predictable component of a group's social structure (Seyfarth, Silk and Cheney, 2014; Silk, 2002; Silk, Cheney and Seyfarth, 2013). Figure 1.1 summarises the key attributes of social bonds. Interaction partners, whose relationship characteristics can be placed towards the right side of the chart, are assumed to have a high relationship quality or, in other words, a close bond (Silk, Cheney and Seyfarth, 2013).

Frequency	Rare	←→	Common
Diversity	Low	←→	High
Symmetry	One-sided	←→	Balanced
Tenor	Hostile	←→	Friendly
Tension	Tense	←→	Relaxed
Predictability	Unpredictable	←→	Predictable
Stability	Unstable	←→	Stable

Figure 1.1 Schematic characterisation of interaction patterns between closely bonded animals, adapted from Silk et al. (2013): Closely bonded individuals can be placed towards the right side of the chart. They show frequent interactions across a diverse range of behaviours that are mostly bi-directional. Furthermore, they show high rates of affiliative interactions and low tension. Moreover, close bonds are often stable over longer periods of time and promote predictability of the relationship.

Among group-living birds and mammals, different types of bonds have evolved: bonds between parents and their offspring (Kulik, Langos and Widdig, 2016; Mendoza and Mason, 1986; Pittet et al., 2014; Ziegler and Crockford, 2017) or bonds between pair partners (Carter et al., 1997; Emery et al., 2007; Hennessy et al., 2006; Hennessy, Kaiser and Sachser, 2009; Johnson and Young, 2015). Depending on the dispersal patterns and the social organisation of a species, bonds can also be formed among kin, such as (half) sisters or brothers (Cheney, Silk and Seyfarth, 2016; Dal Pesco et al., 2021; Frigerio, Weiss and Kotrschal, 2001; McFarland et al., 2017; Scheiber et al., 2017; Silk et al., 2009; Silk, Seyfarth

and Cheney, 2012, 2014). In matrilinear societies, close social bonds are often formed among female kin (Seyfarth and Cheney, 2012; Silk, Alberts and Altmann, 2006), while in patrilinear societies, these are often formed between related males (Mitani, 2009; Seyfarth, Silk and Cheney, 2012).

Besides bonds linked to reproduction, raising young, or increasing inclusive fitness, individuals of some species also form close bonds with unrelated individuals (Seyfarth, Silk and Cheney, 2012; Ziegler and Crockford, 2017). Such bonds can occur between males and females outside the breeding context (Nguyen et al., 2009; Palombit, Cheney and Seyfarth, 2001; Palombit, Seyfarth and Cheney, 1997), between females (Cameron, Setsaas and Linklater, 2009; Lehmann and Boesch, 2009; Riehl and Strong, 2018), or between males (Feh, 1999; Mitani, 2009; Young et al., 2014a). Bonds between unrelated individuals are also interlinked with dispersal patterns and are more likely to occur in the dispersing sex (Cameron, Setsaas and Linklater, 2009; Feh, 1999; Lehmann and Boesch, 2009; Young et al., 2014a).

Species that are known to form such bonds include humans (*Homo sapiens*) (Brent et al., 2014; Feldman, 2012; Hruschka, Hackman and Macfarlan, 2015), baboons (*Papio* spp.) (Nguyen et al., 2009; Palombit, Cheney and Seyfarth, 2001; Palombit, Seyfarth and Cheney, 1997), chimpanzees (*Pan troglodytes*) (Lehmann and Boesch, 2009; Massen and Koski, 2014; Mitani, 2009), macaques (*Macaca* spp.) (Schülke et al., 2010; Young et al., 2014a), lions (*Panthera leo*) (Packer et al., 1991), bottlenose dolphins (*Tursiops truncatus*) (Connor et al., 2000), horses (*Equus caballus*) (Cameron, Setsaas and Linklater, 2009), giraffes (*Giraffa camelopardalis*) (Carter et al., 2013a; b), and birds such as the greater ani (*Crotophaga major*) (Riehl and Strong, 2018).

As different species form social bonds, bond formations need to provide individuals with certain benefits. This is especially true for bonds that occur between unrelated conspecifics outside a breeding context and do not provide benefits that promote indirect or direct fitness (Seyfarth and Cheney, 2012).

1.1.2 Benefits of social bonds

Previous research has shown that bond formations can provide individuals with versatile benefits. On the one hand, they can provide ultimate, long-term benefits. It has been found that individuals' who are socially well integrated, i.e., they either maintain a few strong social bonds or have many weak bonds, show

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enhanced health, longevity, reproductive success, and infant survival (e.g., Horses: Cameron, Setsaas and Linklater, 2009; Giraffes: Bond et al., 2021; Baboons: McFarland et al., 2017; Nguyen et al., 2009; Silk et al., 2009, 2010; Assamese macaques, *Macaca assamensis*: Schülke et al., 2010). These long-term consequences of bond-formation are beneficial as longevity and health can increase an individual's reproductive success (Ellis et al., 2019; Snyder-Mackler et al., 2020).

On the other hand, social bonds can have immediate benefits that impact an individual's daily life. Such benefits include social support and coalition formation (e.g., Chimpanzees: Samuni, Crockford and Wittig, 2021; Assamese macaques: Schülke et al., 2010), which can help individuals to attain and maintain higher social ranks (e.g., Bottlenose dolphins: Connor, Smolker and Richards, 1992; Assamese macaques: Heesen et al., 2015) and males to gain access to or defend females against competitors (e.g., Bottlenose dolphins: Connor et al., 2000; Horses: Feh, 1999; Linklater et al., 1999; Lions: Packer et al., 1991). Moreover, social bonds can protect females from male harassment (e.g., Horses: Cameron, Setsaas and Linklater, 2009; Yellow baboons, *Papio cynocephalus*: Nguyen et al., 2009). Besides active support during agonistic encounters, bonded individuals can provide passive support, whereby the bonded conspecific's mere presence can increase the chances of winning conflicts (e.g., Greylag geese, *Anser anser*: Scheiber, Kotrschal and Weiß, 2009). Additionally, closely bonded conspecifics can provide reconciliation or consolation after agonistic interactions, which helps to re-establish the relationship or promotes faster recovery of a stress response (e.g., Ravens, *Corvus corax*: Fraser and Bugnyar, 2011; Chimpanzees: Wittig and Boesch, 2003; Barbary macaques, *Macaca sylvanus*: Young et al., 2014b). Moreover, social bonds can enhance tolerance (e.g., Assamese macaques: Haunhorst et al., 2017; Japanese macaques, *Macaca fuscata*: Kawazoe, 2021; Chimpanzees: Lehmann and Boesch, 2009; Primates: Ostner and Schülke, 2014) and promote cooperation to obtain and share food (e.g., Ravens: Asakawa-Haas et al., 2016; Wolves, *Canis lupus*: Dale et al., 2017; Chimpanzees: Wittig and Boesch, 2003). Another direct benefit of sociability can be more effective thermoregulation. For example, in Barbary macaques, individuals with more bonded partners are able to form larger huddles to protect them from cold weather (Campbell et al., 2018). A benefit of social bonds that has immediate effects but also promotes long-term benefits, such as health, is 'social

buffering' (Cohen and Wills, 1985). Thereby, the presence of a social partner during or after a stressful event can facilitate an attenuation of the animals' stress response (Cohen and Wills, 1985; Kikusui, Winslow and Mori, 2006; Kiyokawa et al., 2013). Consequently, social buffering can promote benefits on a physiological, mechanistic level (Tinbergen, 1963).

1.1.3 Social buffering of the stress response

Animals live in dynamic environments and, in order to maintain homeostasis, they need to adapt to different immediate stimuli, such as interactions with conspecifics, unpredictable environmental events, novel aspects in their surroundings, or predator attacks (Cannon, 1929; DeVries, Glasper and Detillion, 2003; McEwen, 2008; Romero, Dickens and Cyr, 2009). Some of these stimuli can be defined as 'stressors' if they lack predictability and controllability, or are perceived as threatening (Koolhaas et al., 2011, 2017; Romero, Dickens and Cyr, 2009).

The behavioural and physiological adaptation to a stressor can be described as a 'stress response'. At the behavioural level, the stress response can be indicated by a slight change in behaviour or, in more threatening situations, by a fight, flight, or freeze response (Cannon, 1929; Romero, Dickens and Cyr, 2009). At the physiological level, the stress response is primarily mediated by the activity of the sympathetic-adrenal-medullary (SAM) axis and of the hypothalamic-pituitary-adrenal (HPA) axis (Cannon, 1929; Koolhaas et al., 2011, 2017; Romero, Dickens and Cyr, 2009). In response to a stressor, activity in the SAM-axis and the HPA-axis increases beyond the regular activity level – the 'predictive homeostasis' range – and enters a 'reactive homeostasis' range (based on the Reactive Scope Model, Romero, Dickens and Cyr, 2009; Figure 1.2). Specifically, the SAM-axis is responsible for the rapid stress response. The increased activity in the sympathetic branch of the autonomous nervous system facilitates a release of catecholamines into the bloodstream. These circulating catecholamines initiate an increase in heart rate, vasoconstriction, blood pressure, and respiratory frequency (Godoy et al., 2018; Penzlin, 2005; Uchino, Cacioppo and Kiecolt-Glaser, 1996). The HPA-axis mediates the slow stress response, resulting in the release of glucocorticoids from the adrenal medulla into the bloodstream. Glucocorticoids facilitate, among other things, gluconeogenesis, initiating the metabolic processes to access stored energy which is necessary to maintain the

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behavioural stress response (Adkins-Regan, 2005; Hostinar, Sullivan and Gunnar, 2014; Möstl and Palme, 2002; Penzlin, 2005; Figure 1.2).

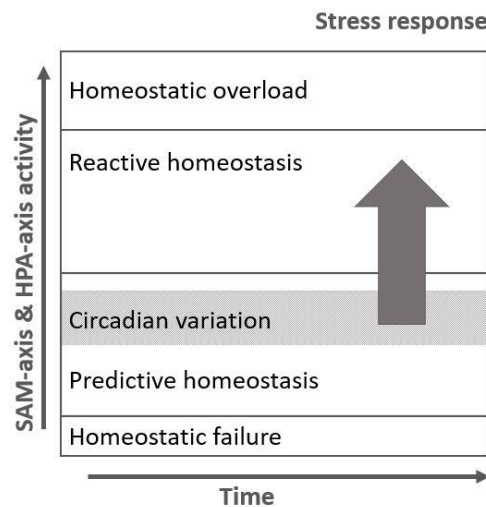


Figure 1.2 The physiological stress response. Following a stimulus that is perceived as a stressor, the SAM-axis and HPA-axis activity levels increase beyond the regular activity level, the 'predictive homeostasis' range, and enter the 'reactive homeostasis' range. This change in physiological activity allows the organism to adapt to potentially threatening situations and ensures that increased metabolic demands are met (based on the Reactive Scope Model, Romero, Dickens and Cyr, 2009).

Fight, flight, or freeze behaviour and the increase in the SAM-axis and HPA-axis activity are adaptive and can ensure survival in case of life-threatening stressors. However, the physiological stress response is also costly. Maintaining the state of reactive homeostasis requires the mobilisation and consumption of energy that could otherwise be used for other purposes, such as tissue maintenance. The activity of both axes inhibits the parasympathetic branch of the autonomous nervous system and mechanisms of growth, repair, digestion, reproduction, and the storage of energy (McEwen, 2008; Moberg, 2000; Sapolsky, 2004). Thus, the physiological stress response causes wear and tear on the organism (Romero, Dickens and Cyr, 2009). If the stress response is chronic, and the SAM-axis and HPA-axis activity levels remain in the reactive homeostasis range, these costs can accumulate and negatively impact health (Sapolsky, 2005). For example, a prolonged increase in SAM-axis activity levels has been found to lead to heightened blood pressure which can, if persisting, result in cardiovascular damage (Sgoifo, Carnevali and Grippo, 2014). Furthermore, a persistent increase in HPA-axis activity levels has been found to suppress the immune system (McEwen, 2008; Moberg, 2000; Sapolsky, 2004),

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making an organism more prone to infection and disease (McEwen, 2008; Moberg, 2000; Sapolsky, 2004).

Previous research has shown that the behavioural and physiological stress response can depend on an animal's social context. The presence or support of social partners during or after exposure to a stressor (Kiyokawa, Takeuchi and Mori, 2007) can buffer the stress response, a mechanism that is termed 'social buffering' (Cohen and Wills, 1985). Social buffering is indicated by a reduced behavioural stress response (e.g., Kiyokawa, Kawai and Takeuchi, 2018) and by an attenuation of the physiological stress response on both stress response axes: Social buffering effects on the SAM-axis activity level are characterised by a weaker increase in heart rate, greater heart rate variability, and lower blood pressure (Kanthak et al., 2016; Lepore, Mata Allen and Evans, 1993; Teoh and Hilmert, 2018; Thorsteinsson and James, 1999). On the HPA-axis activity level, social buffering corresponds to smaller amounts of glucocorticoids released into the bloodstream (Hennessy et al., 2006; Hostinar, Sullivan and Gunnar, 2014; Stanton, Patterson and Levine, 1985; Wittig et al., 2016). Furthermore, social buffering can enhance recovery following a stress response or even prevent a stress response from occurring (Beery et al., 2020; Kiyokawa and Takeuchi, 2017; Moberg, 2000; Figure 1.3). This is beneficial as it allows the individual to adapt its stress response to the given situation and thereby saving energetic costs in less threatening situations (Hostinar, Sullivan and Gunnar, 2014; Romero, Dickens and Cyr, 2009).

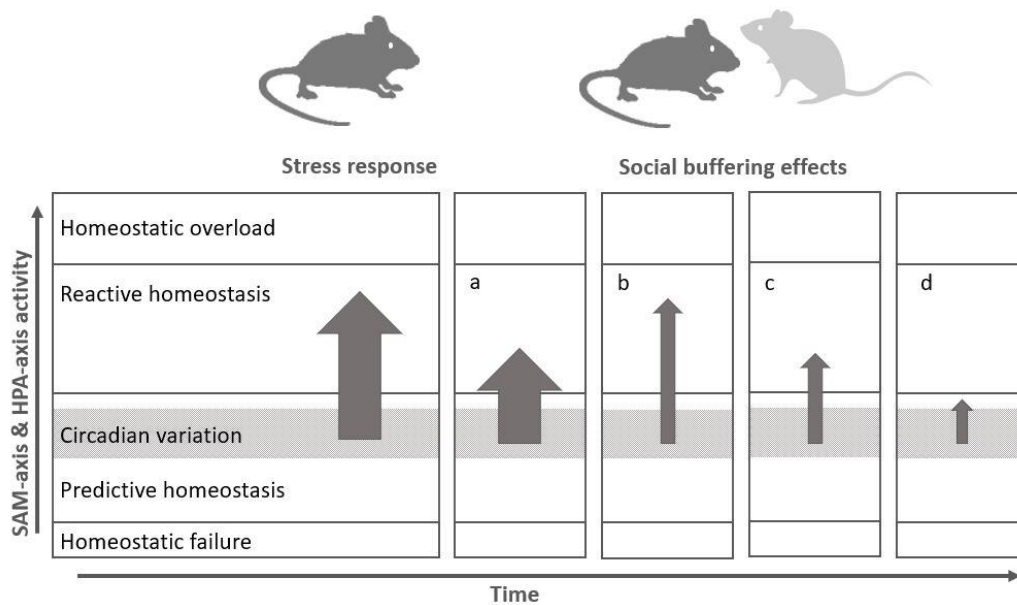


Figure 1.3 Social buffering of the physiological stress response. In response to a stressor, animals increase their SAM-axis and HPA-axis activity. Thereby activity levels enter the reactive homeostasis range (left panel). This physiological arousal has a certain amplitude and duration (indicated by the height and width of the grey arrow). Social buffering can impact the physiological stress response in four different ways: (a) it can decrease the arousal, (b) it can shorten the duration of the arousal, (c) it can decrease and shorten the duration of the arousal, or (d) prevent the physiological stress response from occurring (based on the Reactive Scope Model by Romero, Dickens and Cyr, 2009; mouse shapes adapted from www.phylopic.org).

Two main pathways are proposed to facilitate social buffering on a physiological level:

Firstly, the proximity to or the presence of a conspecific (Kiyokawa et al., 2009; Kiyokawa et al., 2013; Kiyokawa et al., 2014a), or their cues, such as calls (Rukstalis and French, 2005) or odour (Kiyokawa et al., 2014; Takahashi et al., 2013), can induce buffering effects. This is possibly mediated by an altered perception and appraisal of a potentially stressful stimulus (Cohen and Wills, 1985; Hostinar, Sullivan and Gunnar, 2014; Moberg, 2000) which can lead to a suppression of the fear-related activation of the amygdala (Fuzzo et al., 2015) and consequently to a decrease of the physiological stress response (Hostinar, Sullivan and Gunnar, 2014; Sanders and Shekhar, 1991; Young and Leaton, 1996).

Secondly, it was found that physical contact, for example, touch or grooming, can facilitate a buffering effect of the physiological stress response (reviewed in Beery and Kaufer, 2015; Burkett et al., 2016). Thereby, the hormone oxytocin likely plays a role. Oxytocin is synthesised in two sites of the

hypothalamus, the paraventricular nucleus and the supraoptic nucleus (Kleine and Rossmanith, 2014; Landgraf and Neumann, 2004; Ross and Young, 2009) and is released upon physical contact, sex, warmth, and mother-infant interactions such as labour and breastfeeding (Uvnäs-Moberg, 1997a;b). Oxytocin that is released into the bloodstream acts upon the baroreflex, leading to a decreased heart rate and an increase in heart rate variability (Higa et al., 2002; Kanthak et al., 2016; Kemp et al., 2012) and inhibits the release of hormones that promote the HPA-axis activity (Hostinar, Sullivan and Gunnar, 2014).

1.1.4 Social buffering and its link to social bonds

The presence of unfamiliar conspecifics can facilitate buffering effects, and even more so the presence of familiar conspecifics (Hodges et al., 2014; Kiyokawa et al., 2014b; Mommer and Bell, 2013; Terranova, Cirulli and Laviola, 1999). Additionally, the effectiveness of social buffering can depend on different characteristics of the support provider and the relationship between the individuals. For example, older conspecifics (Horses: Rørvang and Christensen, 2018), or calmer individuals have been found to provide a more effective buffer (Chicken, *Gallus gallus domesticus*: Edgar et al., 2015; Horses: Christensen et al., 2008; Rørvang, Ahrendt and Christensen, 2015). Moreover, previous research has shown that buffering effects were greater when buffering was provided by a closely bonded conspecific (Guinea pigs, *Cavia porcellus*: Hennessy, Kaiser and Sachser, 2009; Hennessy, Zate and Maken, 2008; Kaiser et al., 2003; Hennessy et al., 2006; Sachser, Dürschlag and Hirzel, 1998; Humans: Diamond, 2001; Julius et al., 2013). This bonding partner could be the mother for her offspring (Chicken: Edgar et al., 2015; Guinea pigs: Hennessy et al., 2006), a pair partner in a reproductive pair or harem (Guinea pigs: Kaiser et al., 2003; Sachser, Dürschlag and Hirzel, 1998; Wied's black tufted-ear marmosets, *Callithrix kuhlii*: Rukstalis and French, 2005), or a relative in species with strong bonds between related individuals (Baboons: Wittig et al., 2008).

Therefore, one benefit individuals can gain from engaging in social bonds is bond-dependent social buffering (Cohen and Wills, 1985; Sachser, Dürschlag and Hirzel, 1998), which was suggested to increase the individual's capacity to cope with a stressor (Koolhaas et al., 1999; Koolhaas, 2008). A better capacity to cope with stressors can reduce the wear and tear on the organism that is

otherwise linked to the physiological stress response and its maintenance (Ostner and Schülke, 2018; Romero, Dickens and Cyr, 2009). Consequently, social buffering can decrease the risk of health problems caused by chronically elevated stress levels (Cohen and Wills, 1985; Beery and Kaufer, 2015; McEwen, 2008; Moberg, 2000; Sapolsky, 2004, 2005). This indicates that social buffering might be an important mechanism for some group-living animals, especially for species that form social bonds. Studying bond-dependent social buffering effects can provide a better understanding regarding the links between sociality and bond formation on the one hand, and stress physiology and health on the other (Snyder-Mackler et al., 2020).

1.2 Approaches in the study of social buffering and gaps in current research

By reviewing existing literature on social buffering, four topics became evident that are not fully understood or researched. These topics relate to the type of bonds that have been studied, the model species that have been subjected to social buffering research, the physiological measures used to assess buffering effects, and the contexts in which social buffering has been studied. Those four areas will be described in the subsequent sections, followed by an outline of how the studies that are presented in this thesis provide a novel contribution to existing knowledge.

1.2.1 The study of social buffering: bond types

Previous studies that investigated bond-related social buffering effects have primarily focused on certain bond types: the bond between parents and their offspring (Sachser, Dürschlag and Hirzel, 1998), between kin (Wittig et al., 2008), between pair-bond partners (Kaiser et al., 2003; Rukstalis and French, 2005; Sachser, Dürschlag and Hirzel, 1998), or when transitioning from the mother as the main bonding partner to female pair partners in maturing males (Hennessy et al., 2006; Hennessy, Kaiser and Sachser, 2009).

However, little is known whether bonds between unrelated conspecifics can provide benefits such as social buffering. While research has shown that social buffering effects can be facilitated by individuals of the same sex (Coe et al., 1982; Hennessy, Zate and Maken, 2008; Hodges et al., 2014; Ishii et al., 2016;

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Kiyokawa et al., 2014a; Mikami et al., 2016; Terranova, Cirulli and Laviola, 1999), so far only a few studies have considered the relationship quality between the test subjects and their social supporters:

(1) In human studies that assessed social buffering among peers, social support was either provided by a colleague, a roommate or a randomly matched person (Gerin et al., 1995, 1992; Lepore, Mata Allen and Evans, 1993; Uchino and Garvey, 1997). Only a few studies addressed the relationship quality between the test subject and the support provider and asked participants to bring their friend (Gramer and Reitbauer, 2010; Gramer and Supp, 2014; Riem, Kunst and Kop, 2021), or their best or close friend (Heinrichs et al., 2003; Uno, Uchino and Smith, 2002). However, these studies have primarily assessed different support styles such as ‘being supportive’ or ‘ambivalent’ during mental stress tests rather than comparing buffering effects depending on the relationship quality as such.

(2) In animal studies on social buffering among conspecifics of the same sex, social support was provided by either unfamiliar conspecifics (Kiyokawa, Takeuchi and Mori, 2007; Mikami et al., 2016, 2020) or familiar conspecifics, such as cage mates (Coe et al., 1982; Hennessy, Zate and Maken, 2008; Hodges et al., 2014; Kiyokawa et al., 2014a;b; Terranova, Cirulli and Laviola, 1999). Several of these studies showed that familiar conspecifics induced a greater buffering effect than unfamiliar conspecifics (Hodges et al., 2014; Kiyokawa et al., 2014b). However, the actual relationship quality between the two test partners has not been addressed in these studies. This is surprising for two reasons: (a) some group-living species have evolved to form bonds between unrelated individuals of the same sex (Seyfarth and Cheney, 2012), and (b) social bonds between support providers were found to potentially increase buffering effects (Hennessy et al., 2006; Hennessy, Kaiser and Sachser, 2009; Sachser, Dürschlag and Hirzel, 1998). Therefore, it is a meaningful next step to assess bond-related buffering effects between closely bonded but unrelated individuals, specifically of the same sex. Studying social buffering among such bond partners can provide more information about whether such bonds provide benefits on a mechanistic level (Tinbergen, 1963); and, whether such bond formations contribute to the benefits of social integration and long-term health (Snyder-Mackler et al., 2020).

1.2.2 The study of social buffering: species

Previous studies on social buffering have focussed on a small number of species, including humans (*Homo sapiens*) (e.g., Gerin et al., 1992, 1995; Jakubiak and Feeney, 2016; Uchino, Cacioppo and Kiecolt-Glaser, 1996; reviewed in Teoh and Hilmert, 2018), primates, such as chimpanzees (*Pan troglodytes*) (Wittig et al., 2016), baboons (*Papio spp.*) (Morbach, 2020; Wittig et al., 2008), macaques (*Macaca spp.*) (Gilbert and Baker, 2012; Young et al., 2014b), squirrel (*Saimiri sciureus*) and titi monkeys (*Plecturocebus donacophilus*) (Coe et al., 1982; Hoffman et al., 1995; Stanton, Patterson and Levine, 1985). Another body of social buffering research stems from studies in rodents, including guinea pigs (*Cavia porcellus*) (e.g., Hennessy et al., 2006; Kaiser et al., 2003; Sachser, Dürschlag and Hirzel, 1998), rats (*Rattus spp.*) (e.g., Hodges et al., 2014; Insana and Wilson, 2008; Kiyokawa et al., 2013; Kiyokawa, Kawai and Takeuchi, 2018; Machatschke et al., 2004; Mikami et al., 2016; Nakamura et al., 2016; Takahashi et al., 2013), prairie voles (*Microtus ochrogaster*) (Hostetler and Ryabinin, 2014), and mice (*Mus musculus*) (Colnaghi et al., 2016).

This focus on certain species is possibly due to the types of bonds that were investigated in social buffering research. Mother-offspring bonds and bonds among kin were often studied in humans and other primate species (e.g., Coe et al., 1982; Hoffman et al., 1995; Stanton, Patterson and Levine, 1985), whereas buffering effects among pair partners have been investigated in guinea pigs and prairie voles, species that are known to form strong bonds between females and males (e.g., Hennessy et al., 2006; Hostetler and Ryabinin, 2014; Kaiser et al., 2003; Sachser, Dürschlag and Hirzel, 1998). Rats and mice are species that are in general widely used in experimental research (e.g., Home Office UK, 2015; Koolhaas, 2010). Comparatively, fewer studies have addressed social buffering in other species such as pigs (*Sus scrofa*) (Kanitz et al., 2014), sheep (*Ovis aries*) (da Costa et al., 2004), horses (Christensen et al., 2008; Rørvang, Ahrendt and Christensen, 2015; Rørvang and Christensen, 2018), birds (Domestic hens, *Gallus gallus domesticus*: Edgar et al., 2015; Greylag geese, *Anser anser*: Scheiber, Kotrschal and Weiß, 2009; Zebra finch, *Taeniopygia guttata*: Apfelbeck and Raess, 2008), or fish (*Teleost spp.*) (Culbert, Gilmour and Balshine, 2019; Faustino, Tacão-Monteiro and Oliveira, 2017; Galhardo, Vitorino and Oliveira, 2012).

When investigating buffering effects between bonded but unrelated conspecifics, it is necessary to study species that are known to form such bonds, for example, horses (Cameron, Setsaas and Linklater, 2009; Feh, 1999; Linklater et al., 1999), giraffes (Carter et al., 2013a; b), or lions (Packer et al., 1991). The study of social buffering effects across different species that engage in different types of social bonds can extend the knowledge about social buffering effects beyond current evidence that was primarily gained from primate and rodent species. This allows a broader comparative perspective on how sociality impacts physiological mechanisms and potential benefits that arise from it (Aureli et al., 2012; Snyder-Mackler et al., 2020).

1.2.3 The study of social buffering: physiological measures

Besides behavioural indicators of stress, social buffering studies often include physiological stress measures. Thereby, studies have focused on the activity of the two physiological stress response systems: the SAM-axis and the HPA-axis. In studies of social buffering effects, the SAM-axis activity has mostly been measured via heart rate or systolic and diastolic blood pressure (e.g., reviewed in Teoh and Hilmert, 2018), while the HPA-axis activity has mostly been assessed by measuring glucocorticoid concentrations (cortisol in mammals, corticosterone in rodents or birds) or glucocorticoid metabolite concentrations (Machatschke et al., 2004; Möstl and Palme, 2002; Wittig et al., 2016). Furthermore, studies have assessed the expression of transcription factors, such as c-Fos, in the paraventricular nucleus, a brain area that is part of the HPA-axis (e.g., Kiyokawa, Li and Takeuchi, 2019; Mikami et al., 2016; Takahashi et al., 2013). Although physiological mechanisms of social buffering have been extensively studied, the types of measures used vary greatly between taxa. Research in humans has largely assessed social buffering effects on SAM-axis activity (Gerin et al., 1992, 1995; Teoh and Hilmert, 2018; Uchino, 2006; Uchino, Cacioppo and Kiecolt-Glaser, 1996). Consequently, evidence of social buffering effects on SAM-axis activity level primarily stems from human studies. Within animal research, most studies have focused on social buffering effects on the HPA-axis activity, oftentimes due to practical constraints (e.g., DeVries, Glasper and Detillion, 2003; Hennessy, Kaiser and Sachser, 2009; Hostinar, Sullivan and

Gunnar, 2014; Kikusui, Winslow and Mori, 2006; Kiyokawa et al., 2014a; Mikami et al., 2020; Morbach, 2020; Sachser, Dürschlag and Hirzel, 1998; Takahashi et al., 2013).

Comparatively, few social buffering studies in animals have assessed SAM-axis activity levels, for example, via heart rate measurements. Findings from these studies suggest that social support can reduce cardiovascular response to different stressors in animals. The presence of a calm conspecific was found to induce a decrease in heart rate in domestic hens (Edgar et al., 2015), sheep (da Costa et al., 2004), and horses (Christensen et al., 2008; Rørvang and Christensen, 2018). However, compared to the corpus of studies of the HPA-axis, SAM-axis activity levels and social buffering effects remain comparatively understudied in animals and thus less well understood (Hostinar, Sullivan and Gunnar, 2014; Romero et al., 2015). Gathering more evidence for social buffering effects on SAM-axis level is crucial for two reasons: (1) it enables us to assess whether effects that were found in humans are shared across different species, thus allowing us to assess evolutionary trajectories of social buffering mechanisms; and (2) as the release of glucocorticoids is time-delayed to the onset of a stressor (Hostinar, Sullivan and Gunnar, 2014), the measurement of the HPA-axis activity is also time-delayed and therefore provides a more integrative measure to assess the effects of stress and social buffering (DeVries, Glasper and Detillion, 2003; Hostinar, Sullivan and Gunnar, 2014). In contrast, measuring the immediate response to stress and effects of social buffering on SAM-axis activity allows us to directly assess the link between a potentially stressful situation, an animal's physiological response, and social buffering effects.

1.2.4 The study of social buffering: study conditions and types of experiments

Studies in humans and other animals assessed social buffering effects under different test conditions. In humans, social buffering effects have been primarily studied experimentally, and study participants were often subjected to psychological stressors (Teoh and Hilmert, 2018). Examples of such test paradigms are speech tasks (Uchino and Garvey, 1997), involvement in debates or verbal attacks (Gerin et al., 1992), playing computer games (Gerin et al., 1995), or imagining a stressful situation and being instructed to think of different social

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support conditions (Jakubiak and Feeney, 2016; Uchino, Cacioppo and Kiecolt-Glaser, 1996). Thus, much evidence for buffering effects on SAM-axis activity level stems from experiments applying mental stressors.

In contrast, social buffering effects in animals have been studied experimentally in the laboratory or among free-ranging groups of animals, thereby utilising mostly physical stressors. In experimental studies, social buffering effects were assessed during the exposure to stressful stimuli, such as exposure to fear-conditioned stimuli (e.g., Kikusui, Winslow and Mori, 2006; Kiyokawa et al., 2014a; Kiyokawa, Kawai and Takeuchi, 2018; Mikami et al., 2020), during fear-inducing situations such as simulated predation (Coe et al., 1982; Kikusui, Winslow and Mori, 2006), or the elevated plus maze (Colnaghi et al., 2016). Moreover, social buffering effects were studied during exposures to novel environments (Apfelbeck and Raess, 2008; Banerjee and Adkins-Regan, 2011; Coe et al., 1982; Giacomini et al., 2015; Kanitz et al., 2014; Kikusui, Winslow and Mori, 2006; Sachser, Dürschlag and Hirzel, 1998). The experimental conditions under which social buffering effects have been studied in animals were often extreme conditions, applying high-intensity stressors, which animals are probably unlikely to encounter in their daily lives. For example, the induction of repeated and unavoidable pain or shock (Kikusui, Winslow and Mori, 2006; Kiyokawa et al., 2014a; Kiyokawa, Kawai and Takeuchi, 2018; Mikami et al., 2020), or a sudden transfer to an entirely new environment (Apfelbeck and Raess, 2008; Banerjee and Adkins-Regan, 2011; Coe et al., 1982; Giacomini et al., 2015; Kanitz et al., 2014; Kikusui, Winslow and Mori, 2006; Sachser, Dürschlag and Hirzel, 1998). Consequently, little is known about how social buffering influences the behavioural and physiological response to a weaker stressor, such as novelty within a familiar environment; a scenario that free-roaming animals are likely to encounter more often than extreme disturbances in their environment. As animals need to adjust to stressors of different types or intensities in their social and physical environment (Emery Thompson et al., 2010), it seems to be meaningful to assess social buffering effects under different experimental test conditions.

Moreover, animals that were used for laboratory experiments on social buffering effects were mostly housed in rather barren environments and rarely within a social structure that resembles or mimics their natural social environment. For example, Beery et al. (2020) pointed out that laboratory rodents such as mice

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and rats are mostly housed alone or in pairs. However, both species naturally live in bigger colonies where they maintain differentiated relationships with their group members. Therefore, laboratory animals used in social buffering experiments are often not able to establish differentiated social relationships, even though, as described before, it has been shown that bond relationships can impact social buffering effects (Hennessy et al., 2006; Kaiser et al., 2003; Sachser, Dürschlag and Hirzel, 1998). Thus, an important factor that can influence social buffering has often remained excluded from experimental research.

So far, only a few studies have investigated social buffering in free-ranging animals, which were embedded in their (natural) social environment, and thus were able to engage in differentiated relationships. In these studies, buffering effects on HPA-axis activity level were assessed using naturally occurring, potentially stressful situations, such as group instability (Wittig et al., 2008), encounters with other groups of conspecifics (Wittig et al., 2008), agonistic interactions (Scheiber, Kotrschal and Weiß, 2009; Young et al., 2014a), or during periods of environmental stress, such as cold temperatures (Campbell et al., 2018), or predation events (Morbach, 2020). However, there remains a paucity of evidence about social buffering effects on SAM-axis level within study contexts that resemble or mimic types of stressors that animals are more likely to encounter in their daily lives and where social buffering is provided by conspecifics which the test subjects maintain differentiated relationships with.

Based on these four gaps in social buffering research, it can be summarised:

- 1) Although some species form social bonds between unrelated conspecifics (of the same sex), little is known whether these bonds affect social buffering effects in a similar way as found for pair-bonds or bonds among kin. Studying social buffering between closely bonded and unrelated individuals can therefore provide novel insight about the potential benefits of such bond formations.
- 2) Investigating the benefits of social bonds among unrelated conspecifics requires studying species that engage in such bond formations. Addressing different species in the study of social buffering can extend knowledge that was so far primarily gathered from primate and rodent species.

- 3) Besides studying social buffering effects on HPA-axis activity level, social buffering should also be investigated on SAM-axis activity level. This allows the assessment of a more immediate effect of social buffering.
- 4) Studies of social buffering should also be conducted under conditions that reflect or mimic scenarios that can be of daily relevance to the test subjects. Furthermore, as buffering effects have been found to depend on the relationship quality between conspecifics, animals under study should have been able to establish differentiated relationships with their test partners and buffering providers.

1.3 Aims of this study and research questions

The aim of this thesis was to address these knowledge gaps by investigating whether domestic horses (*Equus caballus*) benefit from social buffering provided by closely bonded but unrelated conspecifics, especially of the same sex. Thereby, social buffering effects on SAM-axis activity level were studied in three contexts: (1) during social interactions with members of the horses' social group, (2) while being separated from their social group, and (3) while being exposed to novel stimuli in their familiar physical environment.

1.3.1 Domestic horses as a model species for the study of social buffering

Domestic horses are an ideal study species to investigate social buffering effects between unrelated conspecifics. Horses are among the few species that form long and durable social bonds between unrelated adult individuals and between individuals of the same sex (Cameron, Setsaas and Linklater, 2009; Seyfarth and Cheney, 2012). Social bonds between horses are characterised by spatial proximity and affiliative behaviours such as touches and mutual grooming (Cameron, Setsaas and Linklater, 2009; Feh, 2005). Although the domestication of horses began at least 5000 years ago (Levine, 2005), much of their natural biology, including social behaviour, has been retained (Feh, 2005; Mills and McDonnell, 2005; Zeitler-Feicht, 2008). Additionally, the study of domestic horses allows the use of mobile heart rate monitors to assess the immediate physiological response to different stimuli on the SAM-axis activity level. As most horses are used to being handled or wearing equipment such as saddles or vaulting girths,

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they can be fitted with monitors such as the Polar® V800 Equine system, which are placed in the saddle or vaulting grith area, without causing a great handling-related stress response. The Polar® V800 Equine system has been successfully used in previous studies to measure cardiac activity in domestic horses (e.g., Hendrix et al., 2017; Mendonça et al., 2019; Schmidt et al., 2017).

Horses are highly social, group-living herbivores (Boyd and Keiper, 2005; Gersick and Rubenstein, 2017; Khalil and Kaseda, 1998; Linklater et al., 1999). In the wild, their group size depends on predation pressure and resource availability (Gersick and Rubenstein, 2017; Rubenstein, 1978) and ranges between 4 and 18 members on average (reviewed in Boyd and Keiper, 2005). Horses form female defence polygamous mating systems (Boyd and Keiper, 2005; Khalil and Kaseda, 1998; Linklater et al., 1999). They live in bands that consist of one or more stallions, several mares, and their offspring of different ages. Offspring of both sexes disperse with fillies joining other bands and colts joining all-male units ('bachelor groups') (Khalil and Kaseda, 1997; Stanley and Shultz, 2012). These groups are formed by young stallions that have not yet required their own harem or older stallions that have lost theirs (Feh, 2005a; Khalil and Kaseda, 1998, 1997; Linklater et al., 1999). Recent research has confirmed that horses also form complex multi-level societies, with individual groups being embedded in a wider multi-group context, thereby forming big herds (Maeda et al., 2021).

Besides social bonds, differentiated rank relationships characterise the social structure of horse groups that can be durable over long periods of time, for example, up to several years (Cameron, Setsaas and Linklater, 2009; Feh, 2005; Kaseda and Khalil, 1996). Rank relationships are established by winning and losing agonistic interactions, have been found to be mostly linear, and can determine access to resources (Van Dierendonck, Schilder and De Vries, 1995; Sigurjónsdóttir et al., 2003; Feh, 2005).

Research on horses' social cognition showed that they can distinguish between unfamiliar and familiar conspecifics (Briefer et al., 2017; Proops, McComb and Reby, 2009), a prerequisite for a species to engage in social relationships (Young et al., 2014b). This is further shown by horses' ability to recognise the social status of a familiar horse in relation to its own (Krueger and Heinze, 2008). Horses are also known to show post-conflict consolation (Cozzi et

al., 2010) and third-party intervention in agonistic and affiliative interactions (Krueger et al., 2015; Schneider and Krueger, 2012). These behavioural and cognitive predispositions make horses a suitable study species to investigate social buffering effects provided by unrelated bonded conspecifics, especially by bonded horses of the same sex.

1.3.2 Further implications of this research

As of 2015, the estimated number of Equidae (including horses, donkeys, and their crosses) living under human care in the European Union ranged between 4.16 and 7.74 million (Eurogroup4animals, 2015). As of 2019, 847.000 horses were registered in the United Kingdom (BETA UK, 2019). Many horses are individually stabled, with limited or no physical contact with conspecifics (Christensen et al., 2002; Hartmann, Christensen and Keeling, 2011; Van Dierendonck and Spruijt, 2012), which prevents them from expressing their full repertoire of (social) behaviours and was found to increase the development of behavioural stereotypies (Cooper and Albentosa, 2005; Mills, 2005). However, group housing became more popular in recent years as it allows the horses to express more of their natural behaviours (Christensen et al., 2002; Hartmann, Christensen and Keeling, 2011; Van Dierendonck and Spruijt, 2012; Zeitler-Feicht, 2008).

Studying social bonds and social buffering in horses contributes to the greater context of behavioural ecology. However, by providing new insights into the link between social behaviour, physiology, and social buffering in horses, this study also contributes to equine science and equine welfare. Despite horses being a widely studied species, little is known about the physiological mechanisms underlying their social behaviour – subsequently referred to as socio-physiology. A great number of papers have focussed on describing the social behaviour of horses (e.g., McDonnell and Haviland, 1995; McDonnell and Poulin, 2002; McDonnell, 2003) or on studying specific aspects of horses' social behaviour such as rank (Heitor, do Mar Oom and Vicente, 2006a), affiliation (Heitor, do Mar Oom and Vicente, 2006b), social support (Krueger et al., 2015), or third-party interventions (Schneider and Krueger, 2012). Furthermore, their social behaviour was studied in the context of stabling, thereby linking behavioural studies to animal welfare (e.g., Benhajali et al., 2008; Hartmann, Søndergaard and Keeling,

2012). These papers focused on the social behaviour of horses but did not include physiological measures. Studies that have linked horses' behaviour with underlying physiological mechanisms primarily aimed at assessing the impact of human handling and training on the horses' physiology (e.g., Aerts et al., 2008; Christensen et al., 2021b; Keeling, Jonare and Lanneborn, 2009; von Lewinski et al., 2013; Lindner, Wäschle and Sasse, 2012; Piccione et al., 2013; Rietmann et al., 2004; Schmidt et al., 2010a; b; Sung et al., 2015; Visser et al., 2002). Moreover, some previous studies investigated the horses' behavioural and physiological responses to stressors in their physical environment (Ali, Gutwein and Heleski, 2016; Christensen, Keeling and Nielsen, 2005; Safryghin, Hebesberger and Wascher, 2019). However, to my current knowledge, only two studies also addressed the horses socio-physiology and assessed whether the horses' social context affected their stress response to stressors in their physical environment (Christensen et al., 2008; Rørvang and Christensen, 2018). Whether such effects can be dependent upon relationship quality between horses is still unknown. Moreover, some studies have assessed the link between horses' social cognition and physiology, showing that horses showed an increase in heart rate when presented with a picture showing a threatening conspecific (Wathan et al., 2016) or when hearing whinnies of unfamiliar horses (Briefer et al., 2017). However, it is not yet understood how social interactions among horses influence their (stress) physiology, and whether these effects depend on the rank and bond relationships between the interacting horses. Besides studies that have assessed horses' heart rate responses to grooming or massages provided by humans (Feh and de Mazières, 1993; Kowalik et al., 2017; Lynch et al., 1974; McBride, Hemmings and Robinson, 2004), horses' socio-physiology has, to the author's current knowledge, not been addressed.

Gaining a better understanding of horses' socio-physiology can be relevant to equine welfare, as it highlights potential sources of (social) stress and possible mechanisms to provide horses with social support in potentially stressful situations. The research presented in this thesis aimed at interlinking horses' social interactions with their underlying physiological mechanisms. This can provide valuable insight regarding social stress in group-housed horses. Furthermore, it was assessed whether horses benefit from the companionship of closely bonded conspecifics during different, potentially stressful situations. Studying the socio-physiology of domestic horses and the probable benefits they

gain from engaging in social bonds are relevant to horse welfare. Thereby, this thesis touches upon two pillars of animal welfare: (1), 'natural living' which should allow animals to live as naturally as possible and express their species-specific behaviour, which includes their social behaviour, and (2) 'affective states', which aims at facilitating positive states and reducing or preventing negative states, including stress (Boissy et al., 2007; Dalla Costa et al., 2014; Fraser, 2008).

1.4 Study outline and thesis structure

To study social buffering effects on SAM-axis activity level in domestic horses, a combination of behavioural observations and experiments with time-matched heart rate measurements was applied. Data were collected from horses that were group-housed for the majority of the time and that were embedded in groups that were established for at least a year before the onset of data collection. This made it possible to assess the impact of social bonds on social buffering based on their individual social preferences within their groups (Beery et al., 2020; Gutmann, Spinka and Winckler, 2015). Social buffering was studied in three contexts that were found to facilitate a behavioural or physiological stress response, and that can be of daily relevance to domestic horses.

In **Chapter 2**, the effect of spontaneously occurring social interactions on heart rate in domestic horses was investigated. Social life was found to be a potent stressor in group-living birds and mammals (Koolhaas et al., 2017; Marchant et al., 1995; Meerlo et al., 1999; Sgoifo et al., 1999; Sgoifo, Carnevali and Grippo, 2014; Viblanc et al., 2012; Wascher, Arnold and Kotrschal, 2008). Therefore, stress responses that arise from the animals' social context are of primary relevance to group-living animals (Romero et al., 2015). For this study, horses' heart rate was monitored whilst interacting freely, either in their home field or in the test arena for the experiments. To compile a comprehensive understanding of how social interactions affect heart rate in horses during social interactions, the following questions were addressed:

1. Do social interactions affect heart rate in domestic horses?
2. Does heart rate differ between initiators and receivers of social interactions?
3. Does heart rate during social interactions depend on the relationship quality (bond, rank) between the interacting horses?

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In **Chapter 2**, the focus was on interactions among all members of the groups, considering their bond and rank relationships. This should make it possible to reflect the overall group dynamics. The experiments presented in **Chapter 3 and 4**, specifically addressed social buffering between unrelated horses of the same sex.

In **Chapter 3**, the effect of the horses' social context on their cardiac activity during separation from their social group was assessed. The separation of an individual from its social group can be a stressor (Boissy and Le Neindre, 1997; Lansade, Bouissou and Erhard, 2008a; Ludwig et al., 2017; McNeal et al., 2014; Rault, Boissy and Boivin, 2011; Tuber et al., 1996). Previous research has shown that animals' behavioural and physiological stress responses to separation can be dampened by the presence of a familiar conspecific (Bolt et al., 2017; Erber et al., 2012; Pollard and Littlejohn, 1995). However, little is known with regards to the relationship quality between the separated animals and its potential influence on social buffering effects. Therefore, focal horses were separated from their social group and observed under three test conditions: alone, with a closely bonded field companion, and with a less closely bonded field companion. Moreover, it was investigated potential mechanisms that can facilitate social buffering such as affiliative interactions or spatial proximity (as reviewed in Kiyokawa et al., 2013; Burkett et al., 2016; Edgar et al., 2015; Fürtbauer et al., 2014; Kiyokawa, Li and Takeuchi, 2019). Using this experimental approach, the aim was to answer the following questions:

1. Does the presence of a closely bonded conspecific buffer the behavioural and physiological stress response during separation from the social group?
2. Does the rate of affiliative interactions depend on the social bond between the horses?
3. Does the rate of affiliative interactions between horses facilitate a bond-related buffering effect on cardiac activity level?
4. Does close spatial proximity between horses depend on the social bond between the horses?
5. Does the spatial proximity between horses facilitate a bond-related buffering effect on cardiac activity level?

Chapter 4 investigated the effect of social context on heart rate, fear, and exploratory behaviour during a novel object test. Exposure to novelty is a paradigm that has been used in social buffering research. Thereby, novelty has been mostly operationalised as a sudden transfer into a new environment (e.g., Banerjee and Adkins-Regan, 2011; Coe et al., 1982; Kanitz et al., 2014; Sachser, Dürschlag and Hirzel, 1998). However, it is probably more likely that animals encounter a novel feature within their familiar environment. Therefore, this study aimed at assessing social buffering effects by exposing horses to novel objects within their familiar environment. To account for the potential buffering effects horses gain from closely bonded horses but also from their whole social group (Faustino, Tacão-Monteiro and Oliveira, 2017; Giacomini et al., 2015; Kiyokawa and Hennessy, 2018; Mommer and Bell, 2013; Stanton, Patterson and Levine, 1985; Yusishen et al., 2020) horses were exposed to novel objects in four test conditions: when horses were alone, when they were together with a closely bonded other horse, or together with a less closely bonded horse, and when they were with their group. Using this approach, the aim was to answer the following research questions:

1. Does the behavioural and physiological stress response of horses to a novel object exposure depend on their social context?
2. Does the exploratory behaviour that horses show towards the novel object depend on their social context?

A note to the reader

The three data chapters in this thesis are conceptualised as stand-alone chapters. However, to avoid redundancies in the material and methods sections, certain information is not repeated in detail in all chapters, for example, information about the sample of horses or the calculation of the bond indices. In these cases, cross-referenced between the chapters are provided. Chapter 2 and Chapter 4 link to an appendix which provides additional information and analyses. Future directions and limitations of the studies presented in the data chapters are considered within the single discussion points rather than in separate sections.

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CHAPTER 2

Heart rate during social interactions in domestic horses (*Equus caballus*): effects of role, rank, and bond relationships.



© Denise V Hebesberger

Abstract

This chapter aimed at assessing whether social interactions among domestic horses (*Equus caballus*) affected their cardiac activity and whether the effect was depending on the horses' roles as initiators or receivers of interactions as well as their rank and bond relationships. Furthermore, it was assessed whether social interactions impacted the horses' emotional state. Therefore, the heart rate of 15 group-housed horses was measured during spontaneously occurring agonistic and affiliative interactions and, for comparisons, during behaviours of similar physical activity, such as standing, grazing, and locomotion. The recorded agonistic interactions were predominantly of low intensity and did not have a strong effect on the horses' heart rates. Receiving a threat facilitated an increase in heart rate, and there was a small effect that this increase was higher, the higher the initiator in rank relative to the receiver. The bond strength between the horses did not affect heart rate during agonistic interactions, providing no evidence for a social buffering effect in this context. Mutual grooming corresponded to a lower heart rate than standing, an activity of similar physical activity. This finding indicates a relaxation effect and possibly a positive emotional state. However, this effect was not dependent on the bond strength between the grooming partners. Short affiliative interactions such as sniffs, touches, or head rubs facilitated a slightly but significantly higher heart rate in receivers, the stronger the bond to the initiator. This result opposed the proposed effect that interactions with bonded conspecifics would facilitate relaxation and alleviate the horses' heart rate. However, the slightly higher heart rate potentially reflects a positive emotional state that could be related to excitement when receiving affiliative interactions from a closely bonded group member. These findings contribute to a better understanding of socio-physiology in horses, and, in the wider research context, contribute to the understanding of possible costs and benefits of sociality. Furthermore, they are of relevance for equine welfare, as they demonstrate that group housing is not necessarily a source of social stress and that allowing horses to engage in affiliative interaction can contribute to positive welfare.

Keywords: Social behaviour, socio-physiology, cardiac activity, agonistic interactions, affiliative interactions, mutual grooming.

2.1 Introduction

Repeated agonistic and affiliative interactions are central components of animals' social environments (Beery and Kaufer, 2015). Agonistic interactions are described as aggression, physical conflict, defence, and submission (Briffa et al., 2013) and constitute the basis of rank relationships (Hinde, 1976). Affiliative interactions refer to friendly behaviour such as (mutual) grooming (Feh and de Mazières, 1993; Wittig et al., 2008), licking (Laister et al., 2011), short touches, and prolonged body contact (Burkett et al., 2016), and are the basis of bond relationships (Hinde, 1976). Both agonistic and affiliative interactions have been found to impact the individuals' physiological activity and can have long-term consequences for their health.

Agonistic interactions can be potent stressors, as they can constitute a (physical) threat (Viblanco et al., 2012) and comprise a level of unpredictability or uncontrollability (Koolhaas et al., 2017; Wascher et al., 2009). Engaging in agonistic social interactions can induce a physiological stress response which is indicated by an increase in sympathetic-adrenal-medullary (SAM) axis and hypothalamic-pituitary-adrenal (HPA) axis activity (DeVries, Glasper and Detillion, 2003; Wascher et al., 2009; Wascher, Arnold and Kotrschal, 2008; Yamanashi et al., 2018). The activation of the physiological stress response is adaptive and enables the organism to maintain homeostasis in challenging or dangerous situations and provides the energy needed to perform the behaviour (McEwen and Sapolsky, 1995; McEwen and Wingfield, 2003).

Nevertheless, when individuals receive a high amount of aggression (Abbott et al., 2003; Aureli, Preston and de Waal, 1999; Yamanashi et al., 2018), when they have to actively defend their rank position (Creel et al., 2013), or when they are exposed to high density, competition (Beery and Kaufer, 2015), or group instability (Crockford et al., 2008; Emery Thompson et al., 2010; Noller et al., 2013; Wittig et al., 2008), the stress response can become chronic. A chronic stress response can be costly, as the increased physiological activity needed to maintain the stress response requires the mobilisation of stored energy and causes wear and tear on the organism (Romero, Dickens and Cyr, 2009). Chronic upregulation of SAM-axis activity can be indicated by increased cardiac activity and enhanced blood pressure (hypertension), as well as a shift in cholesterol profile. These changes can become pathological and cause atherosclerotic

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plaque formation and cardiovascular damage (Sgoifo et al., 2001; Sgoifo, Carnevali and Grippo, 2014; Viblanc et al., 2012). A chronically enhanced activity of the HPA-axis results in increased glucocorticoid levels. These increased glucocorticoid levels have been found to suppress the parasympathetic system, mechanisms of growth, and repair, and suppress the immune system, which makes an organism more prone to infection and disease (McEwen, 2008; Sapolsky, 2004, 2005; Sapolsky, Romero and Munck, 2000; Snyder-Mackler et al., 2020). The link between the social environment and reduced health has been found to further translate into an increased mortality risk and a reduction in lifespan (Snyder-Mackler et al., 2020).

Affiliative interactions such as grooming, social licking, or body contact have been found to facilitate the opposite effect of agonistic interactions. They were found to correspond to a decrease in SAM-axis activity (Aureli, Preston and de Waal, 1999; Briefer, Oxley and McElligott, 2015; Laister et al., 2011) and in HPA-axis activity (Wittig et al., 2008). Closely bonded individuals can especially benefit from the described physiological effect of affiliative interactions as they engage in a higher frequency of such interactions (Silk, Cheney and Seyfarth, 2013; Puehringer-Sturmayer et al., 2018). Moreover, bonded conspecifics can show reconciliation (Cozzi et al., 2010; Fraser and Bugnyar, 2011; de Waal and van Roosmalen, 2016), which is described as affiliative interactions between former opponents following an agonistic encounter (de Waal and van Roosmalen, 2016), or conciliation behaviour, described as affiliative interactions directed towards a distressed party (Burkett et al., 2016; de Waal and van Roosmalen, 2016). Both behaviours can also facilitate an alleviation of the physiological stress response and thereby buffer against the effects of (social) stress (Aureli, Preston and de Waal, 1999; Crockford et al., 2008; DeVries, Glasper and Detillion, 2003; Kaiser et al., 2003; Wittig et al., 2016). Furthermore, affiliative interactions between bonded pair partners have been found to buffer their HPA-axis activity, especially during stressful life-history stages such as reproduction (Fürtbauer et al., 2014; Puehringer-Sturmayer et al., 2018). Such buffering effects can be greater the stronger the bond between the interacting individuals (Wittig et al., 2016).

Over time, socially well-integrated individuals that engage in social bonds, or are part of grooming networks, can show an alleviated or less pronounced HPA-axis activity (Fürtbauer et al., 2014; Puehringer-Sturmayer et al., 2018; Wittig

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et al., 2016; Yamanashi et al., 2018; Young et al., 2014a). A less pronounced activation of the physiological stress-axis has been linked to enhanced resistance to infections and better mental wellbeing (Cohen and Janicki-Deverts, 2009; Lakey and Orehek, 2011; Snyder-Mackler et al., 2020; Thoits, 2011). This link between social bond formations, grooming networks, and increased health was also found to correspond to a longer life expectancy (Holt-Lunstad, Smith and Layton, 2010; House et al., 1988; Nuñez, Adelman and Rubenstein, 2015; Snyder-Mackler et al., 2020).

The physiological activity in the course of social interactions reflects the physical activity involved and can also be influenced by characteristics of the interaction and of the interaction partners:

In the context of agonistic interactions, previous research has shown that the intensity of the interaction can affect the corresponding physiological activity level. For example, in pigs (*Sus scrofa domestica*), heart rate was higher when the agonistic interactions led to physical contact compared to threats (Marchant et al., 1995). In king penguins (*Aptenodytes patagonicus*) and greylag geese (*Anser anser*) heart rate was higher during attacks than during low-intensity aggression (Viblanco et al., 2011; Wascher et al., 2009). Furthermore, the duration of the agonistic interaction can affect the physiological response, with longer durations facilitating a higher SAM-axis activity (Viblanco et al., 2012; Wascher et al., 2009). Moreover, the physiological response can be role dependent. Some studies have shown greater physiological arousal in receivers (Abbott et al., 2003; Crockford et al., 2008; Emery Thompson et al., 2010; Yamanashi et al., 2016, 2018); possibly due to reduced controllability or predictability (Koolhaas et al., 2017). Other studies, however, found a greater physiological activity in initiators (Wascher et al., 2009; Wascher, Arnold and Kotrschal, 2008); possibly reflecting a higher motivation to defend resources or rank (Creel et al., 2013; Viblanco et al., 2012; Wascher et al., 2009).

Besides these characteristics of the interaction, the identity and social relationship between the interactors have been found to affect physiological modulation during agonistic interactions. On the one hand, the identity of the interaction partner can facilitate an increase in SAM-axis activity. The approach (Rhesus macaques, *Macaca mulatta*: Aureli, Preston and de Waal, 1999; Herring gulls, *Larus argentatus*: Kanwisher et al., 1978) or received attack (Greylag

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geese: Wascher et al., 2009) from a higher-ranked group member can result in a higher physiological activity than from a lower-ranked conspecific. Even observing agonistic interactions among higher-ranking conspecifics elicited a higher heart rate than among lower-ranking individuals (Greylag geese: Wascher, Scheiber and Kotrschal, 2008). On the other hand, the interaction partner's identity can facilitate a less pronounced increase in physiological activity. In greylag geese, heart rates in initiators have been lower when the target of agonistic interactions was a female as compared to a male (Wascher et al., 2009). Furthermore, in rhesus macaques, the approach of a higher-ranking conspecific caused an increase in heart rate, whereas the approach of a lower-ranked or related group member did not (Aureli, Preston and de Waal, 1999).

A possible explanation as to why patterns of cardiac activity levels differ between agonistic and affiliative interactions (Aureli, Preston and de Waal, 1999), between initiators and receivers (Aureli, Preston and de Waal, 1999; Wascher et al., 2009; Wascher, Arnold and Kotrschal, 2008), or why rank and bond relationships can facilitate different levels of physiological activity during interactions (Aureli, Preston and de Waal, 1999; Wittig et al., 2016) can be the emotional component of an interaction. The level of physiological activation during social interactions does not just reflect the physical activity involved in exhibiting the behaviour. Previous research has shown that heart rate is more strongly affected by social interactions than expected solely based on the physical activity involved (Reefmann, Wechsler and Gygax, 2009; Wascher, Scheiber and Weiß, 2009). This indicates that heart rate also reflects the psychological and emotional component of the interaction (Aureli, Preston and de Waal, 1999; Jansen et al., 2009; Mendl, Burman and Paul, 2010; Wascher, Arnold and Kotrschal, 2008; Wascher, Scheiber and Kotrschal, 2008). An emotion is a short-lived response of an animal to an event or stimulus of importance to the individual (Kremer et al., 2020; Paul and Mendl, 2018). The framework for studying animal emotion proposed by Mendl, Burman, and Paul (2010) allows operationalising emotions by their physiological arousal and positive and negative content (valence). According to this framework, a high heart rate (high arousal) can indicate affective states such as fear or anxiety (negative valence), or excitement and happiness (positive valence). Low heart rate (low arousal) can indicate affective states such as sadness or depression (negative valence), or calmness and relaxation

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(positive valence). Emotions are adaptive as they are the basis for decision making and can motivate behaviour depending on the cognitive, but not necessarily conscious (Lazarus, 1991), appraisal of a stimulus. They promote approach behaviour when stimuli instil positive emotions and avoidance or defence behaviour when stimuli instil negative emotions (Bradley et al., 2001; Mendl et al., 2009; Mendl, Burman and Paul, 2010).

The difference between the physiological activity necessary to maintain a particular behaviour and the physiological activity level measured during this behaviour can reflect the situation's emotional content (Reefmann, Wechsler and Gygax, 2009). Therefore, a comparison between the physiological activity during the behaviour of interest and another behaviour of similar physical activity, for example, during grooming and standing, can inform about the emotional component of a situation (Hall et al., 2018; Jansen et al., 2009; Wascher, Arnold and Kotrschal, 2008).

Although it has been shown that the extent of the physiological activation during agonistic interactions can depend on the sex, rank, or biological relatedness of the interaction partners, it is not well understood whether the bond relationship between the interactors affects their physiological activity. Per definition, social bonds are characterised by a high rate of affiliative interactions (Hinde, 1976; Silk, Cheney and Seyfarth, 2013). Nevertheless, agonistic interactions also occur between bonded conspecifics, such as mothers and their offspring (Negayama, 1981), bonded pair partners (Bookwala, 2002; Brown, 1963), and friends (Stauffer and DeHart, 2006). Moreover, animals live in multi-dimensional social structures and simultaneously maintain rank and bond relationships (Barrett, Henzi and Lusseau, 2012; Hinde, 1976). As both relationship dimensions are not independent of each other (Barrett, Henzi and Lusseau, 2012; Flack et al., 2006), both may affect the underlying physiological mechanisms of agonistic interactions. Therefore, it could be possible that the bond strength between the interacting individuals, besides sex and relatedness, also affects the underlying physiological mechanisms in the context of aggression. Assessing the relationship between bond strength among interacting individuals and their physiological response to agonistic interactions helps to investigate whether social bonds can directly buffer the physiological (stress) response of these interactions (DeVries, Glasper and Detillion, 2003; Wascher et al., 2009;

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Wascher, Arnold and Kotrschal, 2008; Yamanashi et al., 2018). Such knowledge can aid in understanding how sociality and the formation of social bonds can provide benefits and promote long-term health in group-living animals (Snyder-Mackler et al., 2020).

In the context of affiliative interactions, it has been found that the identity of the grooming partner can also have a significant effect on the underlying physiological mechanisms. Wittig et al. (2016) have found that grooming facilitated a decrease in HPA-axis activity in chimpanzees (*Pan troglodytes schweinfurthii*) and that this effect was greater the stronger the bond between the grooming partners. Furthermore, a recent study has demonstrated that the function or purpose of grooming behaviour can affect HPA-axis activity levels in rhesus macaques (*Macaca mulatta*). Grooming with bonded conspecifics in an affiliative context facilitated a decreased HPA-axis activity and inflammation levels, whereas grooming in a 'political context', i.e., to obtain food from a conspecific, did not facilitate an alleviation of the HPA-axis activity (Wooddell et al., 2019).

To date, most knowledge about the effect of affiliative interactions on physiological activity stems from studies that have focused on grooming behaviour (e.g., Aureli, Preston and de Waal, 1999; Wittig et al., 2008, 2016; Yamanashi et al., 2018). This focus is probably due to the fact that a large body of research on the effects of sociality, social interactions, and stress physiology stems from primate species (Aureli, Preston and de Waal, 1999; Crockford et al., 2008; Wittig et al., 2008, 2016; Wooddell et al., 2019; Yamanashi et al., 2018; Young et al., 2014a) in which grooming is the primary affiliative interaction (Lehmann, Korstjens and Dunbar, 2007; Sussman, Garber and Cheverud, 2005). Some primate species spend five to 20 per cent of their time budget performing this behaviour (Silk 2002; Sussman, Garber and Cheverud, 2005). However, there are species in which grooming is less frequent, such as horses (*Equus przewalskii*, *Equus caballus*) (Benhajali et al., 2008; Boyd, 1998; Raspa et al., 2020). In horses, mutual grooming is less frequent and occupies only around 0 – 1.7% of the time budget (Benhajali et al., 2008; Boyd, 1998; Raspa et al., 2020); thereby showing seasonal peaks in spring and autumn (Keiper, 1985; Kimura, 1998). Horses tend to engage more frequently in other affiliative behaviours such as touching, sniffing, and head rubbing (McDonnell and Haviland, 1995). Previous

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studies showed that in addition to grooming, other types of affiliative interactions can affect physiological modulation. For example, cattle (*Bos taurus*) engage in social licking. This unidirectional affiliative behaviour was found to have a role dependant effect on heart rate. It facilitates a decrease of heart rate in the receivers, however not in the performing initiators (Laister et al., 2011). Goats (*Capra aegagrus hircus*) were described to rub their horns or rest side by side with their affiliated conspecifics. These behaviours were also found to correspond with a lower heart rate and a higher heart rate variability, indicating a decreased sympathetic and increased parasympathetic influence on heart rate (Briefer, Oxley and McElligott, 2015). Otherwise, little is known whether different types of affiliative interactions have a differentiated effect on SAM-axis and HPA-axis activity; and whether these effects depend on the relationship quality between the interacting animals as found in chimpanzees (Wittig et al., 2016), or on their role as found in cattle (Laister et al., 2011). Assessing the effect of different types of affiliative interactions on physiological modulation can provide further insight into the underlying physiological mechanisms of social interactions. This can furthermore indicate whether different types of affiliative interactions contribute to the positive health effect that was described for socially well-embedded individuals, especially in species where grooming is not very frequent (Lahey and Orehek, 2011; Wittig et al., 2008, 2016; Snyder-Mackler et al., 2020). Moreover, studying the underlying physiological mechanisms and emotional components of social interactions can enhance our understanding of the potential costs and benefits of group living and the formation of rank and bond relationships (Snyder-Mackler et al., 2020; Viblanc et al., 2012).

Domestic horses are an ideal species to study the effect of social interactions on physiological activity. Horses are group-living animals and engage in agonistic interactions such as threats, bites, attacks, and affiliative interactions such as mutual grooming, sniffs, touches, and body contact (McDonnell and Haviland, 1995). Horses show the ability to recognise familiar conspecifics and can distinguish between cues, such as whinnies, from familiar and unfamiliar horses (Basile et al., 2009; Briefer et al., 2017; Proops, McComb and Reby, 2009). Previous research has shown that horses' heart rate is affected by social cues. Wathan et al. (2016) have found that horses' heart rates increased more during the exposure to pictures of conspecifics with aggressive facial expressions

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(flattened ears, narrowed eyes and nostrils) than when exposed to other facial expressions. Furthermore, horses' heart rates were found to decrease when humans groomed them, indicating a relaxation effect (Feh and de Mazières, 1993; Kędzierski et al., 2017; Lynch et al., 1974; McBride, Hemmings and Robinson, 2004; Normando et al., 2006; Scopa et al., 2020).

The social organisation of horses is structured along linear rank (Van Dierendonck and De Vries, 1995; Granquist, Thorhallsdottir and Sigurjonsdottir, 2012; Sigurjónsdóttir et al., 2003) and bond relationships (Cameron, Setsaas and Linklater, 2009; Nuñez et al., 2014). Thereby, horses are among the few species that form close social bonds between unrelated conspecifics of the same sex (Seyfarth and Cheney, 2012). These characteristics allow assessing the impact of rank and bond relationships on cardiac activity during social interactions. Moreover, horses are used to being handled and wearing tack such as saddles or vaulting girths. Consequently, it is possible to measure their heart rate non-invasively, using mobile heart rate monitors without causing too much handling-related stress (e.g., Christensen et al., 2021; Rørvang and Christensen, 2018; Schmidt et al., 2017).

Generating knowledge about whether agonistic interactions among group-housed horses facilitate social stress and whether domestic horses can benefit from affiliative interactions with bonded group members is also relevant for equine welfare. It can help detect potential social stressors and potential sources of positive emotional states (Boissy et al., 2007; Fraser, 2008).

The aim of this study was to assess whether spontaneously occurring agonistic and affiliative interactions affect the SAM-axis activity in group-housed domestic horses (*Equus caballus*). Thereby, the specific interest was to investigate whether the heart rate of interacting horses depends on their role (initiator, receiver) and their rank and bond relationships. Furthermore, it was assessed whether social interactions affected the horses' emotional state. For this purpose, the heart rate of horses was recorded during social interactions when ranging freely in their field. The measurement of heart rate makes it possible to determine the immediate physiological response to single social interactions. Therefore, the link between the cardiac response, the identity and role of the interacting horses, and their rank and bond relationship can be drawn directly. This study addressed the following questions:

Chapter 2 – Social interactions

1. Do social interactions affect heart rate in domestic horses?

Hypothesis A: Agonistic interactions constitute a potent stressor and facilitate a physiological stress response on the SAM-axis activity level.

Prediction A1: The heart rate of horses will be significantly higher during and after agonistic interactions than before.

Prediction A2: The heart rate during agonistic interactions will be significantly higher than during behaviours of similar physical activity, thus indicating emotional arousal.

Hypothesis B: Short affiliative interactions and mutual grooming facilitate relaxation and correspond to lower cardiac activity.

Prediction B1: The heart rate of horses will be significantly lower during and after affiliative interactions such as sniffs, touches, and head rubs than before.

Prediction B2: The heart rate of horses will be significantly lower during and after mutual grooming than before.

Prediction B3: The heart rate during affiliative interactions such as sniffs, touches, and head rubs will be significantly lower than in behaviours of similar physical activity, thus indicating low arousal and emotional relaxation.

Prediction B4: The heart rate during mutual grooming will be significantly lower than in behaviours of similar physical activity, thus indicating low arousal and emotional relaxation.

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2. Does heart rate differ between initiators and receivers of social interactions?

Hypothesis C: The role of the interacting horses has an effect on their heart rate during social interactions.

Prediction C1: Horses will show a higher heart rate when initiating than when receiving an agonistic interaction.

Prediction C2: Horses will show a lower heart rate when receiving than when initiating an affiliative interaction.

3. Does heart rate during social interactions depend on the relationship quality (bonds, rank) between the interacting horses?

Hypothesis D: Social relationships between the interacting horses have an effect on their SAM-axis activity.

Prediction D1: During agonistic interactions, the heart rate of horses will be higher, the greater the rank difference between the two horses.

Prediction D2: During agonistic interactions, the heart rate of horses will be lower, the stronger the bond between the two horses.

Prediction D3: During affiliative interactions such as sniffs, touches, and head rubs, the heart rate will be lower, the stronger the bond between the two horses.

Prediction D4: During mutual grooming, the heart rate will be lower, the stronger the bond between the two horses.

2.2 Material and Methods

Data collection took place between June and September 2018 in England and included two stables, subsequently referred to as Stable A and Stable B. Both stables were selected due to the horses being kept outside in their social groups for a majority of the time. Moreover, the group composition of each stable has remained consistent for a minimum of a year before the onset of data collection.

2.2.1 Study subjects and housing

At Stable A, the horse group comprised eleven horses, three mares and eight geldings (castrated stallions), aged 19.5 ± 4 (mean \pm SD). At Stable B the group comprised eight horses, two mares and six geldings who were 15 ± 8 (mean \pm SD) years of age. The horse groups at each stable were composed of different breeds (refer to Table 2.1). Regarding workload, four horses were not used for riding activities. Eight horses experienced light work, including short rides and groundwork. Five horses were school horses and regularly used for riding lessons, hacks, coaching, and equine-assisted therapy activities. Two horses were not yet trained and spent their time primarily in the field. Further details about the horses are shown per stable in Table 2.1.

Table 2.1 Overview of the horses in Stable A and B, providing information about their sex, age at the time of data collection, breed, and workload (0 = retired, 1 = ridden 1-2 times a week, 2 = ridden 5-7 times a week).

Stable	Name	Sex	Age	Breed	Work
A	BLL	gelding	18	Quarab x Thoroughbred	2
	BLY	gelding	15	Welsh Cob	2
	BRA	gelding	19	Welsh X	0
	BRO	gelding	25	Irish Cob	0
	HHH	gelding	20	Irish Sports Horse	0
	MDN	mare	23	Welsh Cob	2
	OLV	gelding	14	Irish Cob	2
	ROS	mare	13	Irish Thoroughbred	1
	TAR	mare	21	Irish Sports Horse	0
	TIJ	gelding	22	Belgian WB	1
	WOD	gelding	24	Irish Sports Horse	2
B	BAL	gelding	14	Icelandic	1
	CHA	gelding	4	Welsh Cob	0
	FRE	mare	26	Icelandic	1
	GJO	mare	15	Icelandic	1
	GUI	gelding	7	Shire Horse	1
	KIL	gelding	25	Icelandic	1
	OSK	gelding	8	Icelandic	0
	THO	gelding	21	Icelandic	1

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At Stable A the horse group spent around 16-21 hours per day (66-88% of the time) in a nine-acre field. The field was equipped with two watering sites and a hut providing shelter. Around 9 AM the horses were brought into their single stable for supplemental feed (Hi-Fi Light®, Alfa A-oil®, sugar beet, micro feed or fast fibre®) and for riding lessons. The stables were bedded with wood shavings. Water and hay were available *ad libitum*. The midsections of the stables were around chest height and were without palisades or rails on top. This enabled the horses to maintain visual and physical contact with their stable neighbours. Around noon yard staff started to turn the horses out into the field. Depending on the daily riding schedule, the last horse was back in the field by approximately 5 PM, where they stayed overnight.

At Stable B the horse group spent the majority (92-100%) of the time in an eleven-acre field. This field was equipped with two watering stations, salt licks, a wooden barn, and a tree line that the horses could seek out for shelter. When receiving supplemental feed (Honeychop® and fast fiber®), the horses were brought to the stable yard into box stalls, so that they were able to feed undisturbed. The stables were bedded with wood shavings and straw. Water was provided *ad libitum*. After feeding, the horses were turned back out into the field.

2.2.2 Assessing social relationships

To assess and quantify the bond strength and rank relationships among the horses, behavioural observations were carried out twice a day for a week. The observation times were adapted to the husbandry routine of the respective stable. The first session was conducted between 5:30 AM and 9 AM (Stable A) and between 9 AM and 11 AM (Stable B). The second session was between 6 PM and 9:30 PM (Stable A) and between 3 PM and 9:30 PM (Stable B). Observations were only conducted when all horses were present in the field to prevent observation bias by the absence of a potential social partner. Each observation session comprised two scan sampling sessions and a focal observation block (Figure 2.1).

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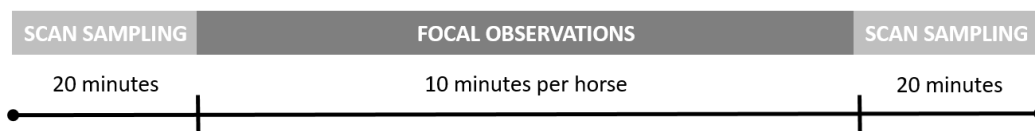


Figure 2.1 Structure of an observational session. One observational session consisted of three parts: (1) an initial scan sampling session (20 minutes) for the nearest neighbour assessment. (2) A block of focal observations comprising 10 minutes for each horse of the group. (3) Lastly, another scan sampling session of 20 minutes was conducted.

Each scan sampling session lasted 20 minutes and was conducted before and after a focal observation block. Thereby, the whole group was observed to identify each horse's nearest neighbour. Based on Cameron, Setsaas, and Linklater's (2009) study, the nearest neighbour assessment was conducted every four minutes, as it was shown that horses are more likely to have changed position after this time interval. Following Kimura (1998), the horse closest to each horse was assigned as its nearest neighbour, independent of how far they were apart. For Stable A 125 and for Stable B 204 nearest neighbour scan samples were obtained. This difference in observation was a consequence of an injury of a horse in Stable A. Consequently, this horse and another gelding from the group were kept stabled at the end of the initial observation period.

In between the scan sampling sessions, focal observations of all group members were conducted. Focal horses were selected in random order and observed for ten minutes. Thereby, all occurrences of initiated and received affiliative and agonistic interactions, as well as approaching and following, were recorded (Altmann, 1974; Cameron, Setsaas and Linklater, 2009). The ethogram with a description of all recorded behaviours is shown in Table 2.2. This resulted in 13 focal observations per horse at Stable A and 14 at Stable B.

For further data processing, the frequencies of all behaviours and interactions were entered in a weighted frequency matrix that accounted for the direction of the interaction (Croft, James and Krause, 2008). Absolute data were transformed into rates per horse and observation bout. These rates were then used to assess the horses' bonding and rank relationships within their group.

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Table 2.2 Ethogram of behaviours recorded during focal observations. All behaviours were recorded as events and were used to assess bond and rank relationships among the horses.

Category	Behaviour	Definition	Source
Proximity behaviour	Approach	A horse seeks proximity of another horse in a straightforward way, which leads to either body contact or spatial proximity within two horse-lengths.	(Heitor, do Mar Oom and Vicente, 2006b; McDonnell, 2003)
	Follow	Moving behind another horse that just initiated locomotion.	(Heitor, do Mar Oom and Vicente, 2006a)
Affiliative interactions	Mutual grooming	Horses mostly in antiparallel stand, scratch the other horse along its neck or back using their teeth.	(Kimura, 1998)
	Body contact	Horse rubs its head on any body part of the other horses, rests its chin or head on another horse, or briefly touches the other horse with its chin or a nostril.	(Feh, 2005b; Jørgensen et al., 2009)
	Sniff	Olfactory investigation nose to nose or nose to body.	(Feh, 2005b; Jørgensen et al., 2009)
Agonistic interactions	Head threat 1	Horse swings its head with pinned ears, sometimes narrowed nostrils towards the target, but does not move towards it.	(Heitor, do Mar Oom and Vicente, 2006a)
	Head threat 2	"Horse moves in the direction of the target with its head lowered and ears pinned, neck stretched or extended toward the target".	(McDonnell and Haviland, 1995)
	Nip	"Similar to bite, but with the mouth less widely opened and the teeth closing on only a small piece of flesh."	(McDonnell and Haviland, 1995)
	Bite	"Opening and rapidly closing the jaws with the teeth grasping the flesh of another" horse.	(McDonnell and Haviland, 1995)
	Kick threat	Horse presents hindquarters towards the other horse or lifts one hind leg towards the other horse "but without sufficient extension or force to make contact with the target".	(McDonnell and Haviland, 1995)
	Kick	"One or both hind legs lift off the ground and rapidly extend backwards towards another" horse.	(McDonnell and Haviland, 1995)
	Strike	"One or both forelegs are rapidly extended forward to contact another" horse.	(McDonnell and Haviland, 1995)
	Push	"Pressing of the head, neck, shoulder, chest, body or rump against another horse in an apparent attempt to displace or pin the target against an object."	(McDonnell and Haviland, 1995)
	Attack	Horse moves with flattened ears in trot or canter towards another horse, with the intention to bite (opened mouth) or kick (hindquarters move towards the other horse).	("chase" in Jørgensen et al., 2009)
	Fight	Including behaviours such as circling, rearing up, boxing, dancing, kneeling, and all forms of bodily contact like grasps, head bumps.	(McDonnell and Haviland, 1995)
	Displace	An agonistic interaction was coded as a displacement when the target responded with an increase in distance (retreat).	(McDonnell and Haviland, 1995)
Submission	Avoid	Increasing spatial distance by moving away in response to an approach of another horse within a three horse-lengths radius.	(adapted from McDonnell and Haviland, 1995)
	Retreat	"Movement that maintains or increases an individual's distance from an approaching and threatening the horse. The head is usually held low, and ears turned back".	(McDonnell and Haviland, 1995)

2.2.2.1 Assessing social bond strength

The bond strength between the horses was quantified by calculating the Dyadic Composite Sociality Index (DSI, or CSI) (Silk, Cheney and Seyfarth, 2013). This index is widely used in social bonds research as it allows for the assessment of bond strength on a dyadic level in relation to the overall bonding structure of the group (Silk, Alberts and Altmann, 2006; Silk, Cheney and Seyfarth, 2013; used in, e.g., Cameron, Setsaas and Linklater, 2009; Kalbitz, Ostner and Schülke, 2016; McFarland et al., 2017; Seyfarth, Silk and Cheney, 2014; Silk et al., 2009). The DSI is based on the relative frequency of affiliative interactions and spatial proximity between dyads of a social group and accounts for intercorrelation of these behaviours. The DSI was calculated based on the rates of behaviours that can be indicative of a social bond in horses (Cameron, Setsaas and Linklater, 2009): nearest neighbour frequency (total number: 329), approaching/following (total number: 633), and body contact, including affiliative behaviours such as touches, sniffs, and head rubs (total number: 633), (Table 2.2). Only behaviours that were intercorrelated, with a correlation coefficient $r \geq 0.5$ (Field, Miles and Field, 2012), were considered as required for the DSI (Silk, Cheney and Seyfarth, 2013). The tests for intercorrelations between the candidate behaviours for the DSI are shown in **Appendix A**, page 225. To calculate the DSI for each possible dyad of the group the Equation 2.1 was used. High values indicate a stronger bond, whilst lower values indicate a weaker bond between two horses.

$$DSI_{xy} = \frac{\sum_{i=1}^d \frac{f_{ixy}}{F_i}}{d}$$

Equation 2.1 The Dyadic Composite Sociality Index (DSI). Calculating the DSI for the dyad x, y : the rate of each behaviour i for dyad $x-y$ (f_{ixy}) is divided by the mean rate of the behaviour i across all dyads (F_i), these fractions are summed for all included behaviours and then the total sum is divided by the number of behaviours that are included in the index (d) (Silk, Cheney and Seyfarth, 2013).

2.2.2.2 Rank relationships: won and lost conflicts

The David Score (DS) was calculated to quantify the relationship between won and lost agonistic interactions between all horses in the two groups (David, 1987). The DS is an appropriate score for small sample sizes (Gammell et al., 2003) and has the advantage that it considers the rate of conflicts won by the

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interaction partner (De Vries, Stevens and Vervaecke, 2006). To calculate this index, the rates of the following behaviours were included: head threats 1 and 2, bites, attacks, displacements, and retreats. Kick threats, hindquarter threats, and kicks were included when they were used offensively rather than as a defensive reaction to a received agonistic interaction (Van Dierendonck and De Vries, 1995). A description of the behaviours is given in the ethogram in Table 2.2, page 42. In total, 308 agonistic interactions and 158 displacements were included in the DS. The following equation (2.2) was used:

$$DS = w + w_2 - l - l_2$$

Equation 2.2 The David Score (DS). Calculating the DS for the dyad x, y: w) sum of P_{xy} ; P_{xy}) the number of times that x defeats y divided by the total number of interactions between x and y; w_2) weighted sum of x's P_{xy} values, weighted by the w values of y; l) sum of P_{yx} ; l_2) weighted sum of x's P_{yx} values, weighted by the l values of its interactants (David, 1987; De Vries, Stevens and Vervaecke, 2006).

To obtain a dyadic measure of the relative success rate between the interaction partners, the Difference in David Score (DS_{Diff}) between the two interaction partners was calculated, following Dales et al.'s (2017) approach. As the David Score can include negative numbers, a constant (100) was added to all scores. Then the DS of the interaction partner was subtracted from the DS of the focal individual (Equation 2.3). Positive DS_{Diff} -values indicate that the focal horse is higher in rank; negative values indicate that the focal horse is lower in rank than its interaction partner.

$$DS_{Diff} = (DS_{focal} + 100) - (DS_{interactant} + 100)$$

Equation 2.3 The Difference in David Score (DS_{Diff}). A constant of 100 was added to the individual David Scores. Thereafter, the DS_{Diff} for the dyad x, y was calculated by subtracting the value of the interactant from the value of the focal horse.

2.2.3 Heart rate monitoring during social interactions

Based on daily availability (e.g., owing to riding lessons, owner visits), one to six horses of the groups were chosen in pseudorandom order for the heart rate recording and behavioural observations. The chosen horses were mounted with Polar® Equine V800 mobile heart rate monitors (Polar Electro Oy, Kempele, FI). This heart rate monitor detected the time intervals between consecutive heartbeats (interbeat intervals). The elastic chest belt was mounted so that the

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sensor strip was placed on the horse's left side, reaching from above the sternum to just below the withers. To enhance the signal transmission, the fur in the sensor area was dampened with water, and the surface of the sensor was covered with electrode gel (Compex® professional by chattanooga™). If the signal transmission was low, the horses' fur was additionally clipped in the sensor area. The receiver (wristwatch) was platted into the horse's tail (Figure 2.2, top). Horses were habituated to wear the heart rate monitor in the two weeks prior to the onset of this study. Therefore, each horse was mounted with the monitor when in their stable for three 15-minute sessions on three different days. Habituation was considered as achieved when horses did not show any behavioural stress responses whilst wearing the heart rate monitor and when their heart rate during habituation fell into the range of horses' expected resting heart rate (Art and Lekeux, 1993).



Figure 2.2 Top: mounted heart rate monitor. The chest belt was positioned so that the sensor reached from below the left withers to above the sternum. The fur of this horse was clipped under the sensor area. The monitor was platted into the horse's tail. Bottom: heart rate monitoring in the field. The focal individual (right) is wearing the Polar® heart rate monitor. Here the horse was captured initiating a sniff with one of its field companions.

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For the purpose of this study, the horses were brought to their field after they were mounted with the heart rate monitors, where they were able to move and interact freely. This made it possible to record cardiac activity during spontaneously occurring social interactions (Figure 2.2, bottom). The horses' behaviour was video recorded (Canon® LEGRIA HF R56). Thereby, the horses with a heart rate monitor were focal individuals during the observation. When in the field, the observer (D.V.H) maintained approximately ten to 20 meters distance to the focal horses. If horses spread out, the observer aimed at keeping as many focal individuals in view as possible. In the case that a focal horse moved out of view, extra observation time was added at the end, where the observer specifically followed these horses for the respective duration of their absence. Depending on the day's husbandry routines, recordings were conducted between 15:00 and 20:00 hours and lasted between one and three hours. Recordings were only conducted when all horses of the group were in the field. In addition to these recordings, all social interactions that were recorded during the experiments in **Chapter 3** and **Chapter 4** were added to the data set.

2.2.4 Study sample and exclusion criteria

Heart rate and behavioural recordings were obtained from nine horses at Stable A and six horses at Stable B (total $N = 15$). Based on ethical considerations, two mares in Stable A were not used as focal individuals as they showed a great stress response and aggressive behaviour when handled by humans. Furthermore, two mares in Stable B could not be used as focal individuals. Due to their thick adipose layer, no reliable heart rate signal could be obtained. However, these horses were represented as interaction partners in the analysis.

2.2.5 Data processing

2.2.5.1 Behaviour

Behaviour from the video recordings was coded using the Solomon® Coder version beta 16.06.26 (© András Péter, <https://solomon.andraspeter.com>). Thereby, the coder (D.V.H) was not blind to the test conditions as the three studies presented in this thesis involved working with identified focal horses. To mitigate this circumstance, the following actions were taken: (1) videos were randomly

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chosen across the three data sets presented in **Chapters 2, 3, and 4** of this thesis, and across the different experimental test conditions. (2) All videos were coded twice to double-check the behavioural codes. (3) Intracoder reliability was calculated to assess whether the coder reliably detected behavioural categories. Therefore, six videos from the novel object test (**Chapter 4**) were coded twice, and the output was compared. These videos were chosen as they comprised all behaviours analysed in this thesis, including locomotion (standing, walking, trot, canter), maintenance behaviour (drinking, grazing, urinating, defecating, sniff floor, body shake), stress-related behaviours (flight, back, startle, alert, snort, yawn), exploratory behaviour towards the novel object (calm approach, alert approach, different distances to the object, investigation, exploration), agonistic, and affiliative interactions between the horses (head threat 1, head threat 2, bite, nip, kick threat, kick, push, displace, retreat, mutual grooming, sniff, touching, rub head), and spatial proximity in horse-lengths. The comparison revealed high intracoder reliability (Pearson's product-moment correlation: $t = 186.52$, $df = 462$, $p < 0.001$, $r = 0.99$; Figure 2.3).

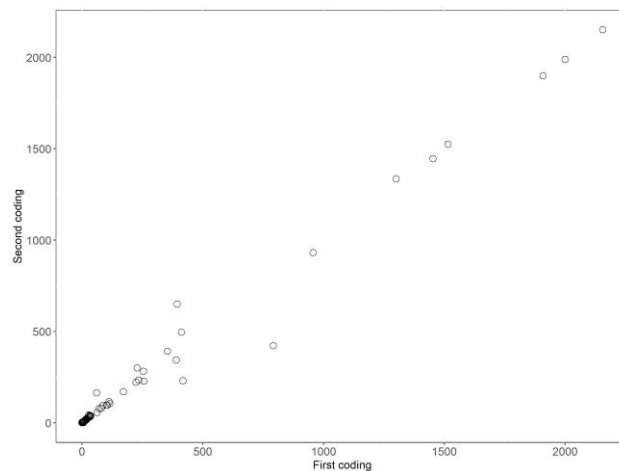


Figure 2.3 Intracoder reliability. The frequency and duration of the first coding session's different behaviours plotted against the frequency and duration of the same behaviours of the repeated second coding session. The coded frequencies and durations were highly correlated ($r = 0.99$) between the two coding sessions.

Lastly, (4) only behaviours that were clearly identifiable were included in further analyses to ensure the accuracy of the behavioural data. The analysis presented in **Chapter 2** focused on affiliative and agonistic interactions and behaviours such as grazing, standing, walking, trotting, and cantering. The recorded behaviours are described in the subsequent ethogram (Table 2.3). As the behaviours were time-matched with heart rate data on a second-to-second basis, they were coded as duration.

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Table 2.3 Ethogram of social interactions and comparative behaviours. The table shows social interactions (affiliative, agonistic, and submissive), locomotion, and other behaviours used to code the behavioural records of horses during turnout. All behaviours were coded as duration.

Behavioural Category	Behaviour	Definition	Source
Affiliative interactions	Mutual grooming	Horses, mostly in antiparallel stand, scratch the other horse along its neck or back using their teeth.	(Kimura, 1998)
	Touch	Horse rests its nose or chin on any body part of the other horse or briefly touching the other horse with its chin or a nostril.	(Feh, 2005; Jørgensen et al., 2009)
	Rub head	Horse rubs his head on any body part of the other horses.	(Feh, 2005; Jørgensen et al., 2009)
	Sniff	Olfactory investigation nose to nose or nose to body.	(Feh, 2005; Jørgensen et al., 2009)
Agonistic interactions	Head threat 1	Horse swings its head with pinned ears, sometimes narrowed nostrils towards the target, but does not move towards it.	(Heitor, do Mar Oom and Vicente, 2006a)
	Head threat 2	"Horse moves in the direction of the target with its head lowered and ears pinned, neck stretched or extended toward the target".	(McDonnell and Haviland, 1995)
	Nip	"Similar to bite, but with the mouth less widely opened and the teeth closing on only a small piece of flesh."	(McDonnell and Haviland, 1995)
	Bite	"Opening and rapidly closing the jaws with the teeth grasping the flesh of another" horse.	(McDonnell and Haviland, 1995)
	Kick threat	Horse presents hindquarters towards the other horse or lifts one hind leg towards the other horse "but without sufficient extension or force to make contact with the target".	(McDonnell and Haviland, 1995)
	Kick	"One or both hind legs lift off the ground and rapidly extend backwards towards another" horse.	(McDonnell and Haviland, 1995)
	Attack	Horse moves with flattened ears in trot or canter towards another horse, with the intention to bite (opened mouth) or kick (hindquarters move towards the other horse).	("chase" in Jørgensen et al., 2009)
	Fight	Including behaviours such as circling, rearing up, boxing, dancing, kneeling, and all forms of bodily contact like grasps, head bumps.	(McDonnell and Haviland, 1995)
Submission	Retreat	"Movement that maintains or increases an individual's distance from an approaching and threatening horse. The head is usually held low and ears turned back".	(McDonnell and Haviland, 1995)
Locomotion	Standing	Horse stands or stand rests with a bent hind leg.	(Malmkvist et al., 2012)
	Walking	The horse moves in a slow four-beat pace, moving a front leg of a side forward, then the hindquarters of the same side and then moving the front leg of the other side, followed by the forward motion of the hind leg of the other side.	(Zeitler-Feicht, 2008)
	Trotting	The horse moves in a faster two-beat pace, moving the diagonal front and hind leg forward at the same time, followed by a short suspension phase before the other diagonal limbs are moved forward.	(Zeitler-Feicht, 2008)
	Cantering	The horse moves in a fast three-beat pace. In the first beat, the horse bares all its weight on a hind leg. The other hind leg and its respective diagonal foreleg take over the weight in the second beat, which is shifted then to the remaining foreleg in a third beat. After that, the horse pushes off the ground into a suspension phase before the circle starts again.	(Zeitler-Feicht, 2008)
Other behaviour	Grazing	Horse feeds from hay or grass, keeps the head to the ground and moves slowly forward. If the horse briefly lifted its head (≤ 5 seconds) and continued chewing, it was also coded as grazing.	(Bulens et al., 2015)
	Drinking	Horse drinks from the water bucket or trough.	(Bulens et al., 2015)
	Urinating	Elimination of urine.	(McDonnell and Haviland, 1995)
	Defecating	Elimination of faeces.	(Malmkvist et al., 2012)

2.2.5.2 Heart rate

The horses' heart rate was calculated from the raw interbeat interval data output provided by the Polar® monitor and obtained via the Polar® Flow software. To eliminate erroneous values that can occur due to a loss of contact between the sensor and the horses' body surface (Giles, Draper and Neil, 2016), all data were visually inspected. Heart rate values outside of the physiological range of horses (Arnold, Ruf and Kuntz, 2006) were deleted. Moreover, data points where the data line's overall pattern was punctually disrupted were also deleted (Schöberl et al., 2015). As horses' heart rates can be higher than 60 beats per minute (bpm), the heart rate records can contain more than one heart rate value per second. Consequently, the heart rate was averaged per second after the error correction was completed. Thereafter, the heart rate and behavioural recordings were matched by merging the timestamps of the heart rate recording with the timestamp of the video recordings on a second-to-second basis.

To assess the impact of social interactions on cardiac activity, heart rates were averaged for the duration of the interaction (subsequently referred to as 'during') as well as ten seconds prior to the interaction (subsequently referred to as 'pre') and ten seconds after the interaction (subsequently referred to as 'post'). The timeframe of ten seconds pre and post-interaction was chosen as a trade-off between capturing the immediate sympathetic response to the interaction and preventing movement of subsequent behaviour to impact the heart rate record (Briefer, Oxley and McElligott, 2015; Reefmann, Wechsler and Gygax, 2009; Safryghin, Hebesberger and Wascher, 2019; Figure 2.4).

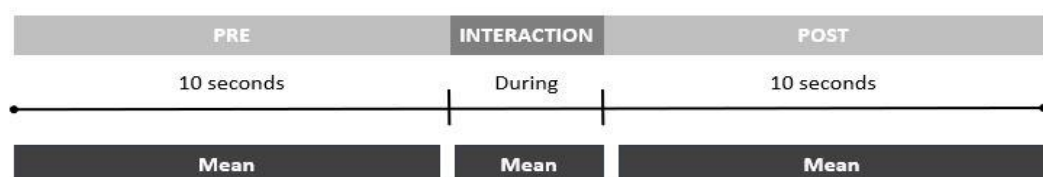


Figure 2.4 Timeframes for calculating mean heart rate for each interaction: pre (ten seconds), during (whole duration), and post (ten seconds) interaction.

If horses engaged in repeated interactions of the same type during an observation session, the respective values for pre, during, and post were averaged to prevent pseudoreplication (Martin and Bateson, 2009). This resulted in a mean heart rate value for each horse and observation day for pre, during, and

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post each interaction type. As heart rate maxima were very variable, the analysis conservatively focused on mean heart rate only.

In total, 1172 (703 initiated, 469 received) time-matched behavioural and physiological records of social interactions were obtained. However, not all horses initiated or received all types of interactions (Table 2.4). The most prevalent interactions were head threats 1 and 2, accounting for ~32 per cent of initiated and 45 per cent of received interactions. This was followed by rubbing their heads (~14 per cent of initiated and 21 per cent of received interactions). Mutual grooming was the least frequent affiliative interaction, followed by touches. Attacks were the least occurring interaction, accounting only for 0.7 per cent of initiated and ~1 per cent of received interactions.

Table 2.4 Total number of social interactions summed across Stable A and B for initiators and receivers, in absolute numbers and in per cent (%). The number of horses contributing to each behaviour is given in column n.

Type	Interaction	Initiated		n	Received		n
		Absolute	%		Absolute	%	
Affiliative	Sniff	85	12	14	27	5.8	7
	Touch	53	7.5	10	50	10.6	8
	Rub head	102	14.5	7	99	21	9
	Grooming	37	5.3	8			
Agonistic	Head threat 1	87	12.4	13	85	18	13
	Head threat 2	140	20	14	128	27	13
	Attack	5	0.7	3	7	1.5	5
	Kick threat	14	2	9	27	5.8	8
	Bite threat	10	1.4	6	10	2	6
	Bites	13	1.8	6	9	2	5
	Nip	34	4.8	9	27	5.8	10
Submissive	Retreat	123	17.5	12			
Total		703			469		

Heart rate was averaged for behaviours such as grazing and locomotion to allow a comparison between heart rate during interactions and behaviour of similar physical activity (e.g., standing without interacting and standing when mutually grooming). For locomotion, it was recorded whether it occurred in the context of agonistic or affiliative interactions and retreat, or in a context that was not tied to a social interaction (e.g., when moving from one feeding site to another, standing when resting).

2.2.6 Statistical analysis

Analyses were carried out using RStudio (Version 1.1.463) in R (Version 3.5.1) (R Core Team, 2019). Normality tests were conducted on all variables using Shapiro-Wilk tests. For variables that were not normally distributed, nonparametric tests were applied. The alpha level was set at 0.05, and test results are given two-tailed. Effect sizes were calculated for significant results based on the z-statistic (Friedman tests and Wilcoxon signed-rank tests) using the following equation: $r = z/\sqrt{n}$ (Rosenthal, 1991).

Wilcoxon signed-rank tests were conducted to determine whether the heart rate of interactions with a comparable biological function such as different types of threats (i.e., head threats, kick threats), or interactions that resulted in physical contact (i.e., nips and bites) was similar and could be pooled for the main analysis.

The effects of social interaction on heart rate were assessed by comparing mean heart rate between pre, during, and post interactions for initiators and receivers, respectively, using Friedman tests (pgirmess, Giraudoux, 2018). Post hoc analyses were carried out with Nemenyi multiple comparison tests (PMCMRPlus, Pohlert, 2020). To assess differences in the horses' heart rate between initiating and receiving interactions Wilcoxon signed-rank tests were conducted. Therefore, mean heart rate pre, during, and post interactions were compared when horses initiated or received interactions.

The emotional component of social interactions was assessed by comparing the heart rate during the interactions with the heart rate of behaviours where the horse showed the same level of physical activity but when they were not involved in a social interaction. For this analysis Friedman tests and Nemenyi multiple comparison tests were conducted.

Linear mixed-effects models (LMMs; Table 2.5) were conducted to explore whether bond strength and rank relationships affected the mean heart rate. Mean heart rate during and post threats (pooling all threat types) as well as during and post short affiliative behaviours (sniffs, touches, rubbing head) were set as response variables. These behaviours were selected as they were the most frequent and performed by most horses. Mean heart rate during grooming was set as response variable in an additional model and analysed separately from the other affiliative behaviours as it is a mutual behaviour and longer in duration. The

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Dyadic Composite Sociality Index (DSI) and the difference in David Score (DS_{Diff}) were set as fixed effects. To account for repeated measures from the same horses, the individual identity was set as a random effect (Zuur et al., 2009; Table 2.5).

Table 2.5 Response variables, fixed, and random effects of the linear mixed-effects models. The response variables were set to the mean heart rate during and after initiating and receiving threats (Models 1-4), the mean heart rate during and after initiating and receiving short affiliative interactions (Models 5-8), and the horses mean heart rate during mutual grooming (Model 9). The Dyadic Composite Sociality index (DSI) and the Difference in David Score (DS_{Diff}) were set as fixed effects. To account for repeated measures, the individual ID was set as random effect.

		Response variables	Fixed effects	Random effect
Model 1		Initiating mean heart rate, during		
Model 2	Threats	Initiator mean heart rate, post		
Model 3		Receiver mean heart rate, during		
Model 4		Receiver mean heart rate, post		
Model 5		Initiator mean heart rate, during		
Model 6	Short	Initiator mean heart rate, post		
Model 7	affiliative	Receiver mean heart rate, during		
Model 8		Receiver mean heart rate, post		
Model 9	Groom	Both horses' mean heart rate, during		

LMMs were carried out using the 'lmer' function in the 'lme4' package (Bates et al., 2015). Outliers in the response variable were assessed via Cleveland dot plots (Zuur et al., 2009). Collinearity between explanatory variables was visually assessed using pairwise scatterplots (Zuur et al., 2009) and tested using the 'vif' function in the 'car' package (Fox and Wisberg, 2011). Vif's of the fixed effects ranged between 1.007 and 1.047 and were below the suggested threshold of 2 (Zuur, Ieno and Elphick, 2010). Consequently, no variable had to be dropped due to collinearity. Normal distribution of residuals was visually assessed using the 'autoplot' function in the 'ggplot' package (Wickham, 2016). Moreover, Cook's distances were computed to determine influential data points. Therefore, the 'stats' package was used (R Core Team, 2019). As all data points had a Cook's distance below 1 (range: 0.0 – 0.97) (Field, Miles and Field, 2012), no data point had to be dropped from the models. To assess the overall model fit, R^2 -values were obtained via the 'r2beta' function in the 'r2glmm' package (Jaeger, 2017). Thereby, the method was set to 'nsj' to calculate marginal R^2 (variation explained by fixed effects) and conditional R^2 (variation explained by the full

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model) as proposed by Nakagawa and Schielzeth (2013). Full models are presented in the results section.

2.2.7 Ethical statement

The experimental procedure was approved by the Departmental Research Ethics Panel under the terms of Anglia Ruskin University's Research Ethics Policy (reference number: A & EB DREP 17-029 and 17-053) and followed the ethics guidelines for the study of animal behaviour provided by the Association for the Study of Animal Behaviour (ASAB, 2020). All applied methods were non-invasive. Two horses were excluded from the study due to ethical considerations, see section 2.2.4, page 46. Consent for all procedures was sought from horse and stable owners.

2.3 Results

2.3.1 Initial analysis and data pooling

The initial analyses showed that the mean heart rates differed between Head threat 1 and Head threat 2 (Wilcoxon signed-rank tests: $p < 0.05$; Table 2.6). Therefore, Head threat 1 was kept as a separate behavioural category. The mean heart rates corresponding to Head threat 2, Bite threat, and Kick threat did not differ (Wilcoxon signed-rank tests: $p > 0.05$; Table 2.6). Consequently, these heart rate data were pooled under the category 'Threat 2'. Similarly, the mean heart rate between Bite and Nip did not differ and was pooled under the category 'Bite' (Wilcoxon signed-rank tests: $p > 0.05$, Table 2.6).

Table 2.6 Comparison of mean heart rate corresponding to agonistic interactions. Results of Wilcoxon signed-rank tests are provided for initiators and receivers, showing the pairwise comparisons of mean heart rate before (pre), during, and after (post) the different types of agonistic interactions. The mean heart rate for receiving Nips and Bites was not compared as no horse performed both behaviours. The respective sample size is given in column *n*. Significant results are marked in bold.

Role	Compared behaviours	<i>n</i>	Phase	Results
Initiator	Head threat 1 - Head threat 2	13	pre	V = 0, $p < 0.001$
			during	V = 2, $p = 0.094$
			post	V = 1, $p = 0.002$
	Head threat 1 - Bite threat	5	pre	V = 3, $p = 0.156$
			during	V = 2, $p = 0.75$
			post	V = 6, $p = 0.813$
	Head threat 2 - Kick threat	8	pre	V = 30, $p = 0.109$
			during	V = 7, $p = 0.625$
			post	V = 30, $p = 0.109$
	Head threat 2 - Bite threat	4	pre	V = 9, $p = 0.813$
			during	V = 12, $p = 0.561$
			post	V = 14, $p = 0.125$
	Kick threat - Bite threat	5	pre	V = 13, $p = 0.188$
			during	V = 8, $p = 0.375$
			post	V = 0, $p = 0.125$
	Nip - Bite	6	pre	V = 9, $p = 0.844$
			during	V = 2, $p = 0.75$
			post	V = 17, $p = 0.688$
Receiver	Head threat 1 - Head threat 2	13	pre	V = 20, $p = 0.080$
			during	V = 14, $p = 0.641$
			post	V = 12, $p = 0.017$
	Head threat 2 - Kick threat	5	pre	V = 22, $p = 0.641$

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Table 2.6 continued.

Head threat 2 - Bite threat	8	during	$V = 14, p = 0.275$
		post	$V = 19, p = 0.945$
		pre	$V = 29, p = 0.148$
		during	$V = 9, p = 0.25$
		post	$V = 28, p = 0.195$
Kick threat - Bite threat	5	pre	$V = 10, p = 0.625$
		during	$V = 5, p = 0.5$
		post	$V = 6, p = 0.556$

The pooled heart rate data for the respective behavioural categories were used for the main analysis (Table 2.7). As the data were not normally distributed, medians, maxima, minima, and interquartile ranges are given.

Table 2.7 Pooled heart rate data for agonistic interactions. The median (\tilde{x}), maximum (Max), minimum (Min), and interquartile range (IQR) of mean heart rate are shown for horses initiating or receiving agonistic social interactions. The number (No.) of horses that exhibited the behaviour and the number of interactions (IAs) per behavioural category are also provided.

Behaviour	Role	Phase	\tilde{x}	Max	Min	IQR	No. horses	No. IAs
Threat 1	Initiator	pre	46.26	65.51	31.97	7.75	13	87
		during	46.99	70.18	36.56	6.93		
		post	46.31	64.89	34.46	6.43		
	Receiver	pre	46.33	62.14	34.48	6.32	13	85
		during	47.59	63.69	35.82	6.81		
		post	46.69	63.87	33.42	6.71		
Threat 2	Initiator	pre	48.45	59.45	40.43	4.06	14	164
		during	49.21	56.49	42.71	4.41		
		post	48.05	60.30	45.11	4.83		
	Receiver	pre	49.35	58.94	41.35	4.89	13	175
		during	49.63	59.04	41.72	6.71		
		post	52.39	60.59	44.32	6.29		
Bite	Initiator	pre	48.09	58.59	36.42	9.62	10	47
		during	46.94	65.50	35.74	7.59		
		post	48.20	55.43	41.37	4.02		
	Receiver	pre	45.63	60.33	37.62	4.57	10	36
		during	47.30	68.81	40.16	6.90		
		post	49.04	76.21	39.27	7.88		
Attack	Initiator	pre	56.84	57.80	40.37	8.72	3	5
		during	82.62	112.53	52.70	29.91		
		post	99.53	127.77	60.44	33.66		
	Receiver	pre	51.73	66.24	47.29	5.61	5	7
		during	51.64	89.55	46.66	9.08		
		post	65.32	80.80	48.79	17.30		

Furthermore, the initial analyses showed that the mean heart rates did not differ between the three categories of affiliative interactions, i.e., Sniffs, Touch, and Rub head (Wilcoxon signed-rank tests: $p > 0.05$; Table 2.8).

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Table 2.8 Comparison of mean heart rate corresponding to affiliative interactions. Results of Wilcoxon signed-rank tests are provided for initiators and receivers, showing the pairwise comparisons of mean heart rate before (pre), during, and after (post) the different types of short affiliative interactions. The respective sample size is given in column *n*.

Role	Compared behaviours	<i>n</i>	Phase	Results
Initiator	Sniff - Touch	10	pre	$V = 38, p = 0.322$
			during	$V = 20, p = 0.375$
			post	$V = 43, p = 0.131$
	Touch - Rub head	6	pre	$V = 10, p = 0.578$
			during	$V = 4, p = 0.219$
			post	$V = 5, p = 0.156$
	Sniff - Rub head	7	pre	$V = 17, p = 0.688$
			during	$V = 10, p = 0.625$
			post	$V = 19, p = 0.469$
Receiver	Sniff - Touch	7	pre	$V = 16, p = 0.813$
			during	$V = 11, p = 0.688$
			post	$V = 13, p = 0.701$
	Touch - Rub head	6	pre	$V = 12, p = 0.813$
			during	$V = 11, p = 0.438$
			post	$V = 10, p = 0.238$
	Sniff - Rub head	7	pre	$V = 17, p = 0.688$
			during	$V = 9, p = 0.823$
			post	$V = 11, p = 0.686$

Consequently, heart rate data were pooled under ‘Short affiliative interactions’. Descriptive statistics for mean heart rate during prior, during, and post Affiliative interactions are shown in Table 2.9. Mutual grooming was kept separate due to its longer duration and as it is a mutual behaviour.

Table 2.9 Heart rate for Short affiliative interactions and Mutual grooming. The median (\tilde{x}), maximum (Max), minimum (Min), and interquartile range (IQR) of mean heart rate are shown for when horses initiated or received short affiliative interactions and grooming. The number (No.) of horses that exhibited the behaviour and the number of interactions (IAs) per behavioural category are also provided.

Behaviour	Role	Phase	\tilde{x}	Max	Min	IQR	No. horses	No. IAs
Short affiliative	Initiator	pre	48.53	55.89	36.58	5.19	14	240
		during	47.18	59.80	36.51	5.16		
		post	47.31	57.43	37.82	5.34		
	Receiver	pre	45.88	50.09	35.83	6.92	13	176
		during	46.82	51.37	29.70	8.94		
		post	47.16	51.10	34.36	6.84		
Grooming	Mutual	pre	43.58	47.99	37.53	6.13	8	37
		during	41.18	46.95	36.89	4.17		
		post	41.68	49.45	36.26	5.71		

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Subsequently, the main results are shown and presented for each behavioural category in the same order: (1) The comparisons of the horses' heart rate pre, during, and post initiating or receiving an interaction. (2) The results of the heart rate comparisons between horses initiating or receiving an interaction, and (3) the results of the comparisons between heart rates of interactions and that of behaviours with similar physical activity.

2.3.2 Agonistic interactions

2.3.2.1 Head threat 1

Initiating and receiving a Head threat 1 did not result in a different mean heart rate pre, during, and post interaction (Friedman tests: $n = 13$: initiating: $X^2 = 1.85$, $df = 2$, $p = 0.397$; receiving: $X^2 = 1.08$, $df = 2$, $p = 0.584$). Furthermore, comparing mean heart rate between when horses initiated or received a Head threat 1, there was also no significant difference pre, during, and post (Wilcoxon signed-rank tests: $n = 11$, pre: $V = 32$, $p = 0.966$, during: $V = 25$, $p = 0.519$; post: $V = 26$, $p = 0.577$). Moreover, when comparing mean heart rate pre, during, and post initiating and receiving a Head threat 1 to the horses' mean heart rate during Standing, a behaviour of similar physical activity, no difference was found (Friedman tests: initiating: $n = 13$, $X^2 = 2.1$, $df = 3$, $p = 0.552$; receiving: $n = 13$, $X^2 = 2.24$, $df = 3$, $p = 0.525$; Figure 2.5).

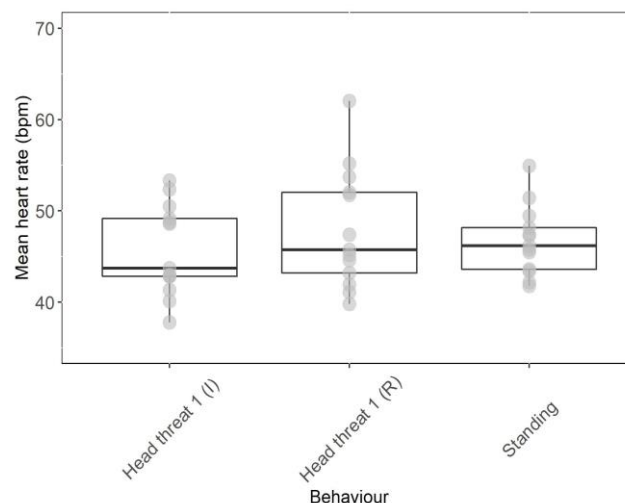


Figure 2.5 Mean heart rate for Head threat 1 and Standing. Tukey whiskers plot showing mean heart rate in beats per minute (bpm) during initiating (I) and receiving (R) a Head threat 1, and Standing. The bold horizontal line indicates the median, the upper and lower hinge of the boxplot correspond to the 25 and 75 percentiles, comprising the interquartile range (IQR). The outwards pointing whiskers extend to the smallest and largest value at most $1.5 * IQR$ from the hinge. There was no difference between mean heart rate when initiating and receiving a Head threat 1 and Standing.

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2.3.2.2 Threat 2

The initiation of a Threat 2 was not related to a difference in mean heart rate pre, during, and post threat (Friedman test: $n = 14$, $X^2 = 1.08$, $df = 2$, $p = 0.584$). However, when horses received a Threat 2 the mean heart rate was significantly higher afterwards than before (Friedman test: $n = 13$, $X^2 = 7$, $df = 2$, $p = 0.032$; Nemenyi multiple comparison test: pre-dur: $p = 0.382$, pre-post: $p = 0.022$, $r = -0.45$, dur-post: $p = 0.382$; Figure 2.6). Thereby, the horses' heart rate was ~6% higher post than pre. The mean heart rate did not differ between initiators and receivers (Wilcoxon signed-rank tests: $n = 13$, pre: $V = 45$, $p = 1$, during: $V = 33$, $p = 0.677$; post: $V = 29$, $p = 0.273$).

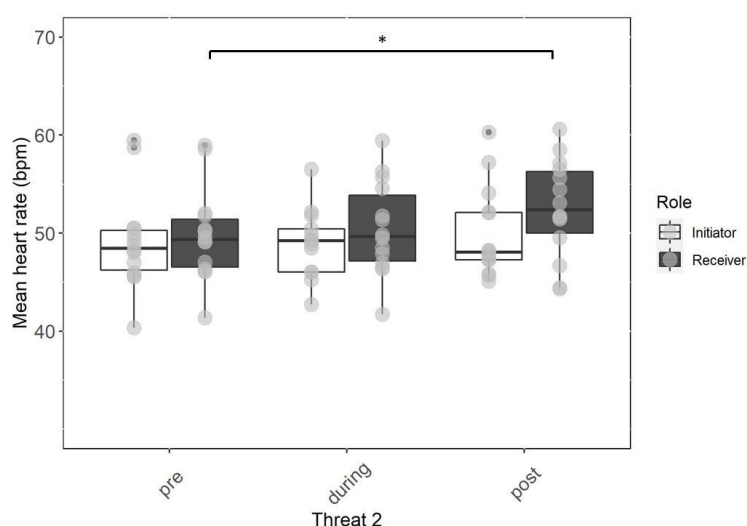


Figure 2.6 Mean heart rate corresponding to Threat 2. Tukey whiskers plot showing medians of mean heart rate in beats per minute (bpm) for pre, during, and post initiating (white) and receiving (grey) threats. The bold horizontal line indicates the median, the upper and lower hinge of the boxplot correspond to the 25 and 75 percentiles, comprising the interquartile range (IQR). The outwards pointing whiskers extend to the smallest and largest value at most $1.5 \times \text{IQR}$ from the hinge. Data points smaller or greater are indicated as single dots. The receivers' heart rate was ~6% higher post Threat 2 than pre. Otherwise, there were no differences. Asterisks: *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

The comparison between the mean heart rate pre, during, and post initiating a Threat 2 with the mean heart rate during Walking, an activity of similar physical activity, did not reveal a significant difference (Friedman test: $n = 14$, $X^2 = 7.11$, $df = 3$, $p = 0.068$). There was no difference in mean heart rate pre, during, and post receiving a Threat 2 and Walking (Friedman test: $n = 13$, $X^2 = 4.89$, $df = 3$, $p = 0.18$). Moreover, there was no difference in heart rate between Walking when not interacting, when initiating a Threat 2, or when Retreating (Friedman test: $n = 9$, $X^2 = 0.889$, $df = 2$, $p = 0.641$; Table 2.10).

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Table 2.10 Mean heart rate in beats per minute for Walking when not interacting, when initiating a Threat 2, or when Retreating. Given are median (\tilde{x}), maximum (Max), and minimum (Min) of mean heart rate, and the interquartile range (IQR). The mean heart rate did not differ between the three walking contexts.

Walking	\tilde{x}	Max	Min	IQR
Not interacting	50.76	58.06	45.74	3.07
Initiating Threat 2	50.25	54.36	40.66	2.82
Retreat	52.50	72.03	43.56	8.69

2.3.2.3 Bites

Initiating Bites did not correspond to a significant difference in heart rate pre, during, and post interaction (Friedman test: $n = 10$, $X^2 = 0.2$, $df = 2$, $p = 0.905$). However, when receiving a Bite, the mean heart rate was significantly higher after Bites than before (Friedman test: $n = 10$, $X^2 = 6.2$, $df = 2$, $p = 0.045$; Nemenyi multiple comparison test: pre-dur: $p = 0.644$, pre-post: $p = 0.037$, $r = -0.67$; dur-post: $p = 0.261$; Figure 2.7). Thereby, the mean heart rate was ~7.5% higher post than pre. Comparing horses' mean heart rate when initiating and receiving Bites revealed no significant difference (Wilcoxon signed-rank test: $n = 9$, pre: $V = 38$, $p = 0.074$, during: $V = 27$, $p = 0.25$, post: $V = 26$, $p = 0.734$).

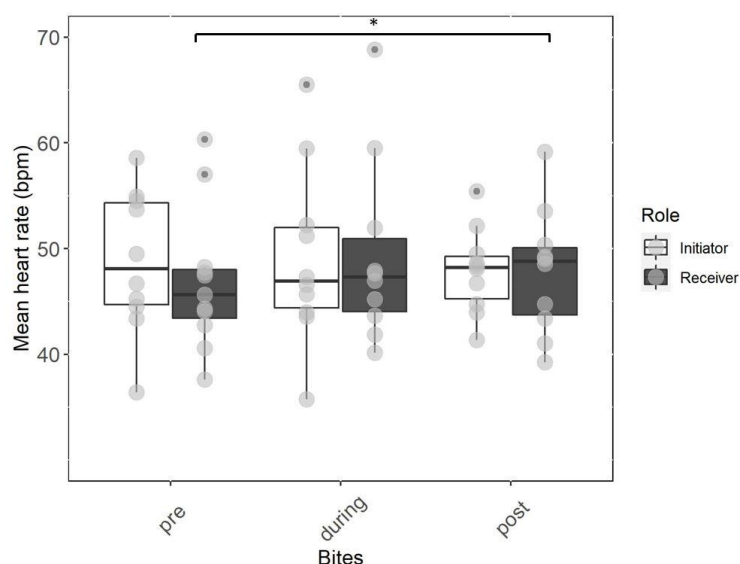


Figure 2.7 Mean heart rate for Bites. Tukey whiskers plot showing medians of mean heart rate in beats per minute (bpm) for pre, during, and post initiating (white) and receiving (grey) Bites. The bold horizontal line indicates the median, the upper and lower hinge of the boxplot correspond to the 25 and 75 percentiles, comprising the interquartile range (IQR). The outwards pointing whiskers extend to the smallest and largest value at most $1.5 \times \text{IQR}$ from the hinge. Data points smaller or greater are indicated as single dots. The mean heart rate of receivers was ~7.5% higher after a Bite than before. Otherwise, there were no significant differences. Asterisks: *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

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Comparing the mean heart rate pre, during, and post initiating a Bite with the mean heart rate during Walking, an activity of similar physical activity, no difference was found (Friedman test: $n = 10$, $X^2 = 8.67$, $df = 3$, $p = 0.315$). However, for receivers of Bites, the heart rate prior to the Bite was slightly (~5%), but significantly lower than during Walking (when not interacting) (Friedman test: $n = 10$, $X^2 = 8.76$, $df = 3$, $p = 0.033$; Nemenyi multiple comparison test: pre-walk: $p = 0.029$, $r = -0.39$, during-walk: $p = 0.23$, post-walk: $p = 0.82$) and resembled that of Standing (Wilcoxon signed-rank test: $n = 10$, $V = 37$, $p = 0.764$).

2.3.2.4 Attacks

Overall, the highest heart rate was measured during (median: 82.62 bpm, IQR: 29.91 bpm) and post (median: 99.53 bpm, IQR: 33.66 bpm) initiating Attacks. However, the occurrence of Attacks (0.7% of all initiated interactions) was low. Due to the low number of initiated Attacks ($n = 3$), it was not possible to analyse the mean heart rate across attacks for initiators or to compare the mean heart rate between initiating and receiving Attacks. Descriptive statistics for heart rate modulation corresponding to attacks are shown in Table 2.7 and in Figure 2.8. The mean heart rate of receivers was significantly higher (~26%) post attack than pre (Friedman test: $n = 5$, $X^2 = 6.2$, $df = 2$, $p = 0.046$; Nemenyi multiple comparison test: pre-dur: $p = 0.42$, pre-post: $p = 0.031$, $r = -0.68$, dur-post: $p = 0.95$).

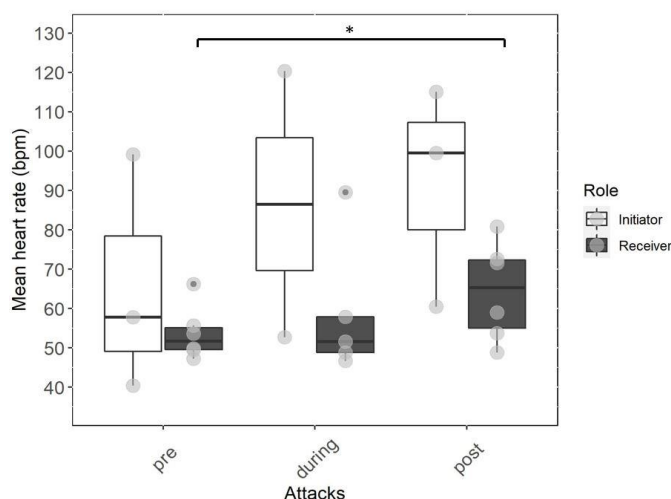


Figure 2.8 Mean heart rate for Attacks. Tukey whiskers plot showing medians of mean heart rate in beats per minute (bpm) for pre, during, and post initiating (white) and receiving (grey) Attacks. The bold horizontal line indicates the median, the upper and lower hinge of the boxplot correspond to the 25 and 75 percentiles, comprising the interquartile range (IQR). The outwards pointing whiskers extend to the smallest and largest value at most $1.5 \times$ IQR from the hinge. Data points smaller or greater are indicated as single dots. The mean heart rate was significantly higher (by ~26%) after receiving an Attack than before. Asterisks: *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

2.3.3 Short affiliative interactions and Mutual grooming

2.3.3.1 Pooled Short affiliative interactions

The mean heart rate did not differ between pre, during, or post Short affiliative interactions; for both, the initiators and the receivers (Friedman tests: initiators: $n = 12$, $X^2 = 4.77$, $df = 2$, $p = 0.092$; receivers: $n = 13$, $X^2 = 0.727$, $df = 2$, $p = 0.695$). The role had no effect on the heart rate (Wilcoxon signed-rank tests: $n = 12$, pre: $V = 63$, $p = 0.064$, during: $V = 33$, $p = 0.625$, post: $p = 0.519$; Figure 2.9).

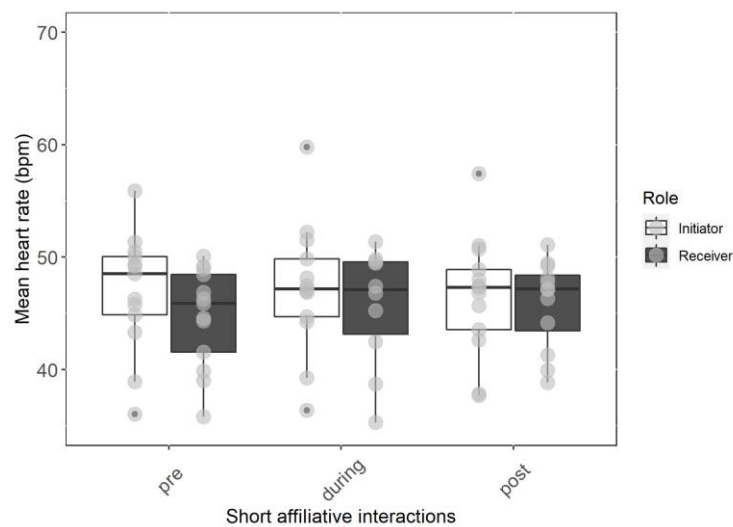


Figure 2.9 Mean heart rate for Short affiliative interactions. Tukey whiskers plot showing medians of mean heart rate in beats per minute (bpm) for pre, during, and post initiating (white) and receiving (grey) Short affiliative interactions. The bold horizontal line indicates the median, the upper and lower hinge of the boxplot correspond to the 25 and 75 percentiles, comprising the interquartile range (IQR). The outwards pointing whiskers extend to the smallest and largest value at most $1.5 * IQR$ from the hinge. Data points smaller or greater are indicated as single dots. There was no significant difference in mean heart rate in the course of the interactions or between initiators and receivers.

Horses' mean heart rate pre, during, and post initiating and receiving Short affiliative interactions did not differ from the mean heart rate during Standing, a behaviour of similar physical activity (Friedman tests: initiators: $n = 12$, $X^2 = 3.7$, $df = 3$, $p = 0.296$; receivers: $n = 13$, $X^2 = 1.44$, $df = 3$, $p = 0.696$).

2.3.3.2 Mutual grooming

Overall, Mutual grooming corresponded to the lowest heart rate measured for all social interactions (median: 41.18 bpm, IQR: 4.17 bpm). The mean heart rate pre, during, and post grooming did not differ (Friedman test: $n = 8$, $X^2 = 1$, df

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= 2, $p = 0.607$). The effect of the role was not assessed as grooming is a mutual interaction in horses. The results provide evidence that the mean heart rate during Grooming was significantly lower (~8%) than during Standing (median: 44.78, IQR: 5.06) (Wilcoxon signed-rank test: $n = 8$, $V = 26$, $p = 0.046$, $r = -0.53$; Figure 2.10).

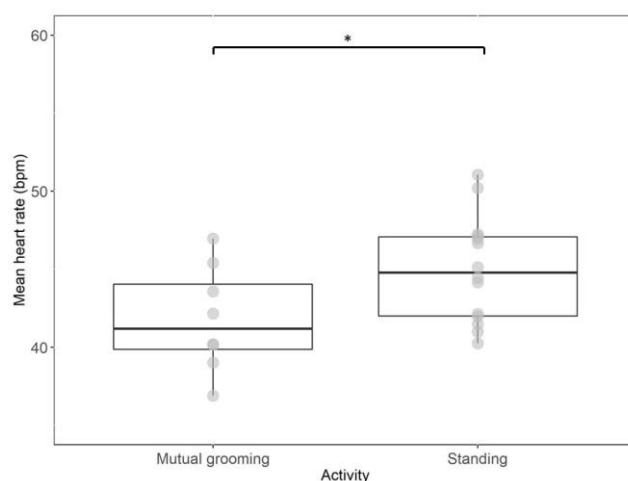


Figure 2.10 Mean heart rate during Mutual grooming and Standing. Tukey whiskers plot showing medians of mean heart rate in beats per minute (bpm) for Mutual grooming and Standing, an activity of similar physical activity. The bold horizontal line indicates the median, the upper and lower hinge of the boxplot correspond to the 25 and 75 percentiles, comprising the interquartile range (IQR). The outwards pointing whiskers extend to the smallest and largest value at most $1.5 \times$ IQR from the hinge. The heart rate during Mutual grooming was significantly lower than during Standing, an activity of similar physical activity. Thereby, the difference in heart rate was ~8%. Asterisks: *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

2.3.4 Impact of relationship quality between interaction partners on heart rate

2.3.4.1 Agonistic interactions

The mean heart rate in receivers post Threat (comprising mean heart rates for Head threat 1 and pooled Threat 2) was slightly higher, the higher the initiator was in rank compared to the receiver (Model 4, LMM: DS_{Diff} Estimate \pm SE = -0.109 ± 0.05 , $t = -2.16$, $p = 0.035$), see Table 2.11 and Figure 2.11. The Dyadic Composite Sociality Index (DSI) did not affect mean heart rate after receiving a threat. Although this result was significant, the effect was small; model 4 accounted for ~7% of the variation in mean heart rate. Thereby, the Difference in David Score (DS_{Diff}) explained 1.7% as indicated by the conditional and marginal

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R^2 . Otherwise, the DS_{Diff} and the DSI had no effect on the horses' mean heart rate during and post Threats (Table 2.11).

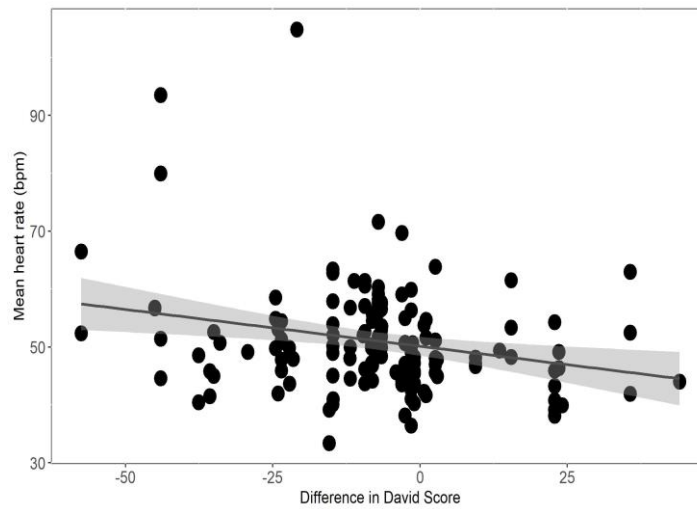


Figure 2.11 Mean heart rate of receivers post Threat and Difference in David Score (DS_{Diff}). The regression line, in dark grey, is based on a simple linear model of mean heart rate in beats per minute (bpm) by DS_{Diff} . The standard error of the fit is depicted in light grey. Negative values of the DS_{Diff} indicate that the receiving horse was lower in rank than the initiator; positive values indicate that the receiver was higher ranked than the initiator. There was a small, but significant effect that the heart after receiving a Threat was higher, the higher the initiator in rank relative to the receiver.

2.3.4.2 Pooled Short affiliative interactions and Mutual grooming

The mean heart rate of receivers during and post Short affiliative interactions was slightly higher the stronger the social bond with the initiator (Model 7, LMM: DSI: Estimate \pm SE = 1.223 ± 0.35 , $t = 3.55$, $p < 0.001$; Model 8, LMM: DSI: 0.758 ± 2.30 , $t = -0.59$, $p = 0.013$; Table 2.11 and Figure 2.12). During short affiliative interactions, the bond strength accounted for 16% of the variation in the heart rate data, post interaction for 8%. The heart rate of initiators during and after affiliative interactions was not affected by the DSI or DS_{Diff} (Models 5 – 6). The heart rate during Mutual grooming was not influenced by the bond strength between the grooming partners (Model 9; Table 2.11).

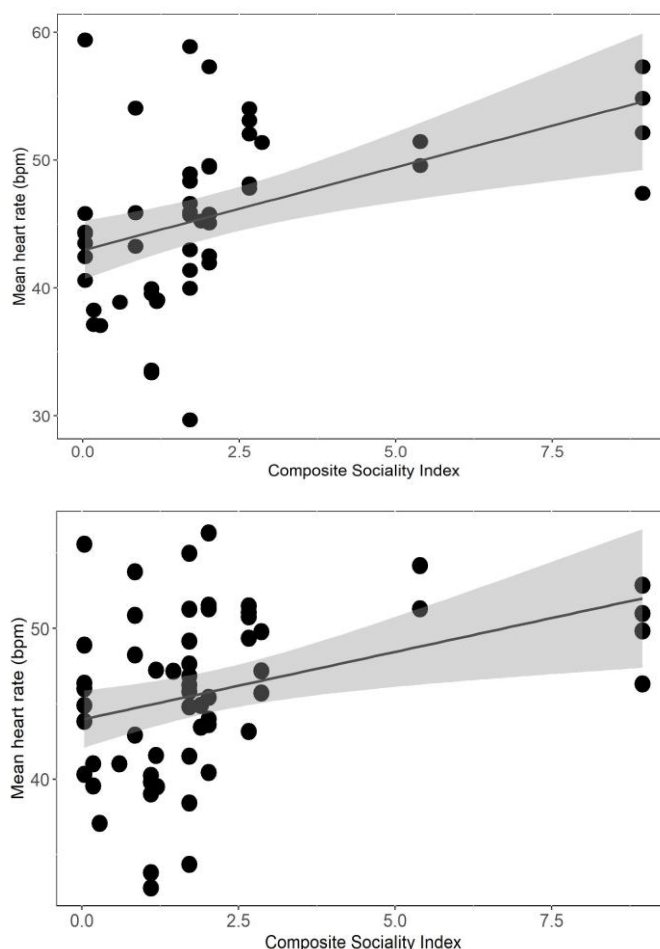


Figure 2.12 Mean heart rate of receivers during and post Affiliative interactions and the Dyadic Composite Sociality Index (DSI). The regression line, in dark grey, is based on a simple linear model of mean heart rate in beats per minute (bpm) by DSI for during (top) and post (bottom) affiliative interactions. The standard error of the fit is depicted in light grey. The higher the DSI, the stronger the bond between the interacting horses. There was a small, but significant effect, that the horses' heart rate during and after receiving a Short affiliative interaction was higher, the stronger the bond to the initiator.

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Table 2.11 Results from linear mixed-effects models (LMM). Response variables: mean heart rate during and post initiating and receiving agonistic interactions (Models 1-4), short affiliative interactions (Models 5-8), and mutual grooming (Model 9). Explanatory variables: Dyadic Composite Sociality Index (DSI) and the Difference in David Score (DS_{Diff}). The individual was set as random effect. Given are model estimates, standard errors (SE), *t*-statistic (*t*), and *p*-values. Significant results are marked in bold. The conditional *R*²-value (*R*²_c) shows the overall model fit, the marginal *R*²-value (*R*²_m) shows the variation explained by the fixed effects.

IA types	Model	Role	Response variables	<i>R</i> ² _c	DSI					DS _{Diff}				
					Estimates	SE	<i>t</i>	<i>p</i>	<i>R</i> ² m.	Estimate	SE	<i>t</i>	<i>p</i>	<i>R</i> ² m.
Threats	Model 1	Initiator	Mean heart rate during	0.021	0.361	0.323	1.115	0.267	0.01	0.049	0.047	1.057	0.295	0.011
	Model 2		Mean heart rate post	0.004	0.158	0.307	0.516	0.607	0.002	0.023	0.044	0.532	0.596	0.003
	Model 3	Receiver	Mean heart rate during	0.031	-0.43	0.428	-1.005	0.318	0.069	-0.068	0.052	-1.289	0.203	0.017
	Model 4		Mean heart rate post	0.069	-0.605	0.409	-1.477	0.142	0.04	-0.109	0.05	-2.16	0.035	0.017
Short Affiliative	Model 5	Initiator	Mean heart rate during	0.021	0.042	0.387	0.108	0.914	0.000	-0.071	0.065	-1.081	0.283	0.021
	Model 6		Mean heart rate post	0.021	-0.105	0.335	-0.315	0.754	0.001	-0.057	0.051	-1.112	0.269	0.019
	Model 7	Receiver	Mean heart rate during	0.164	1.223	0.345	3.547	< 0.001	0.163	-0.029	0.044	-0.673	0.504	0.009
	Model 8		Mean heart rate post	0.086	0.758	0.296	-0.588	0.013	0.084	-0.022	0.037	-0.588	0.559	0.006
Grooming	Model 9	Mutual	Mean heart rate during	0.006	-0.129	0.364	-0.357	0.727	0.005	-0.009	0.052	-1.164	0.872	0.002

2.4 Discussion

This chapter aimed at assessing whether agonistic and affiliative interactions affected SAM-axis activity in domestic horses (*Equus caballus*). A primary assessment was whether cardiac activity during agonistic and affiliative interactions depended on the role (initiator, receiver) and the social rank and bond relationships between the interacting horses. Furthermore, it was investigated whether interactions facilitated emotional arousal or relaxation. For this purpose, the heart rate of 15 horses that were predominantly group-housed, was measured during social interactions and locomotor activity when ranging freely in their home fields.

2.4.1 Agonistic interactions

Previous studies have shown that agonistic interactions can be a potent stressor for group-living animals (DeVries, Glasper and Detillion, 2003; Wascher et al., 2009; Wascher, Arnold and Kotrschal, 2008; Yamanashi et al., 2018). Therefore, the prediction was that agonistic interactions among horses would correspond to a significantly higher heart rate reflecting a stress response.

From all recorded interactions, the mean heart rate was the highest during attacks. Thereby, the heart rate of receivers increased significantly in response to an attack. The cardiac response of initiators was not analysed due to the low number of recorded initiated attacks. The graphical representation of the data suggested that the mean heart rate could be higher in the initiator than in the receiver. Further research is needed to confirm whether this pattern is significant. Overall, attacks were the least frequent interaction type recorded. Most interactions were of low intensity, comprising threats and less frequently bites. In initiators, such interactions had no significant effect on heart rate. In receivers, the mean heart rate was higher after a threat or a bite than before. Thereby, the heart rate resembled that of walking and reflected their retreat. Overall, the heart rate pre, during, and post agonistic interactions resembled the heart rate of the physical activity necessary to perform the behaviour, for example, standing or walking. This indicates that agonistic interactions did not facilitate an additional physiological activity that could indicate emotional arousal (Hall et al., 2018; Jansen et al., 2009). These results suggest that agonistic interactions of high intensity can significantly increase heart rate in horses. However, as the horses

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predominantly engaged in low-intensity agonistic interactions, their social interactions did not seem to constitute a prevalent stressor.

The analyses did not reveal a role-dependent difference in heart rate during low-intensity agonistic interactions, this applies for both, when horses initiated or received such type of interactions. Therefore, the prediction that initiators of agonistic interactions would show a higher heart rate than receivers was also not confirmed in the context of low-intensity aggression. Whether there is a role-dependent difference in the context of attacks needs to be assessed in future research. Previous studies have shown that the heart rate of king penguins and graylag geese was higher when initiating an attack than when receiving attacks (Wascher, Arnold and Kotrschal, 2008; Wascher et al., 2009). During high-intensity aggression, the initiator might have a specific motivation that could facilitate higher emotional arousal. This could be defending or gaining access to a reproductive partner (Viblanco et al., 2012; Wascher et al., 2009). In a domestic or captive setting, this could be the defence of resources such as high calorie feed (Burla et al., 2016; DeVries, Von Keyserlingk and Weary, 2004; Kotrschal, Hemetsberger and Dittami, 1993).

An aspect that could have promoted the prevalence of low-intensity aggression was the temporal stability of the horses' groups. The composition of both horse groups had been established at least a year before the data collection. Group stability can reduce the rate of agonistic interactions (Christensen et al., 2011; Fureix et al., 2012; Granquist, Thorhallsdottir and Sigurjonsdottir, 2012; Noller et al., 2013). Moreover, it can facilitate social network and rank stability which can enhance the certainty and the predictability of potential outcomes of social interactions (Abbott et al., 2003; Barrett, Henzi and Lusseau, 2012; Heitor, do Mar Oom and Vicente, 2006a). Consequently, group stability can reduce social stress as, for example, indicated by lower basal levels of measured glucocorticoids (Crockford et al., 2008; Koolhaas et al., 2017; Nuñez et al., 2014; Vandeleest et al., 2016). This can reflect an evolutionary adaptive strategy, as lower levels of high-intensity aggression cannot just reduce the risk of injuries, but also lower the physiological costs of maintaining high-intensity aggression (Koolhaas et al., 2017; Romero, Dickens and Cyr, 2009; Viblanco et al., 2012). Thus, an environment that facilitates the prevalence of low-intensity aggression could reduce the short-term and long-term consequences of social stress.

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When assessing the effect of rank and bond relationships between the interacting horses, the presented analysis showed that the mean heart rate of receivers was slightly higher when the initiator's rank was higher than their own. This finding confirms the prediction that the rank difference between the horses affects their heart rate. The presented findings resemble findings from previous studies in other species. For example, in greylag geese, receiving an attack from a higher-ranked group member was shown to result in a higher heart rate than an attack from a conspecific of a similar rank. This is possibly due to the risk of being defeated or reduced controllability for the receivers during the interaction (Wascher et al., 2009). Similarly, in rhesus macaques (*Macaca mulatta*) and herring gulls (*Larus argentatus*), the approach by a higher-ranked conspecific facilitated a higher heart rate than an approach by a lower-ranked member of the group (Aureli, Preston and de Waal, 1999; Kanwisher et al., 1978). Even observing agonistic interactions among higher-ranking geese has been found to elicit a higher heart rate in a bystander than when observing lower-ranking conspecifics (Wascher, Scheiber and Kotrschal, 2008). This seems to be an adaptive response that enables the organism to engage in fight or flight behaviour (Cannon, 1929) under conditions of higher social threat; or retreat, in the case of low-intensity agonistic interaction (Moberg, 2000).

However, the bond strength between interacting horses had no effect on their heart rate while initiating and receiving threats. Consequently, the prediction that a strong bond would provide a buffering effect in the context of agonistic interactions was not confirmed. This was surprising, as the opponent's identity, such as sex, or kin, has been found to affect heart rate modulation in the context of agonistic interactions or approaches (Aureli, Preston and de Waal, 1999; Wascher et al., 2009). Taken together, the findings of this study showed that in the context of agonistic interaction, it was primarily the horses' rank relationship, but not their bond relationship, that affected their heart rate. However, again, these findings apply to low-intensity aggression. Whether they translate to contexts of high-intensity aggression needs to be assessed in future studies. Close bonds were found to facilitate tolerance in competitive contexts and reduce aggression (Asakawa-Haas et al., 2016; Dale et al., 2017; Lehmann and Boesch, 2009; Ostner and Schülke, 2014). Consequently, social bonds could buffer the increased physiological activity necessary to engage in high-intensity aggression.

2.4.2 Affiliative interactions

Based on previous studies that have demonstrated that affiliative interactions correspond to a decrease in physiological activity (Aureli, Preston and de Waal, 1999; Briefer, Oxley and McElligott, 2015; Laister et al., 2011) the prediction was that mutual grooming and short affiliative interactions would facilitate a decrease in heart rate. Furthermore, it was predicted that heart rate would be lower during affiliative interactions than during standing, a behaviour of similar physical activity.

The presented findings confirmed the first part of the prediction. The heart rate was lower during mutual grooming than when standing. This reflects experimental findings that have shown that grooming, provided by humans, corresponded with a low heart rate (Feh and de Mazières, 1993; Kędzierski et al., 2017; Lynch et al., 1974; McBride, Hemmings and Robinson, 2004; Normando et al., 2006). To my current knowledge, this is the first study that assessed heart rate during grooming between two horses. The findings demonstrate that a lower heart rate was detectable despite horses performing the behaviour, which included more physical activity than when standing and being groomed by a human (Feh and de Mazières, 1993; Kędzierski et al., 2017; Lynch et al., 1974; McBride, Hemmings and Robinson, 2004; Normando et al., 2006). Previous research has shown that slight movements can lead to an increase in heart rate (Major, 1998). That the movement during the affiliative behaviour can affect the corresponding heart rate was shown in a study on social licking in cattle. Social licking is a unidirectional behaviour and facilitated a decrease in heart rate in the receiver but not in the initiator, who performed the licking behaviour (Laister et al., 2011). That the heart rate in horses was lower than when standing, despite performing the grooming behaviour, suggests that mutual grooming facilitates a strong relaxation effect. As indicated by the lower heart rate, mutual grooming possibly instils a positive emotional state (Baciadonna, Nawroth and McElligott, 2016; Hall et al., 2018; Lansade et al., 2018; Mendl, Burman and Paul, 2010).

Another prediction was that heart rate during mutual grooming would be lower the stronger the bond between the interacting horses. However, the heart rate during grooming was not affected by the bond strength between the horses. This contrasts with findings from chimpanzees, where HPA-axis activity during grooming was lower, the stronger the bond between the grooming partners (Wittig

et al., 2016). A possible explanation for the difference between these findings could be that within the sample of horses in this study, the grooming behaviour was not strongly correlated with other behaviours that can be indicative for a close social bond, such as approaches, following, body contact, and spatial proximity (see details about intercorrelations between affiliative behaviours in **Appendix A**, page 225) (Silk, Cheney and Seyfarth, 2013). Based on the biological market theory (Noë and Hammerstein, 1994), this could suggest that horses might not primarily choose their grooming partners according to the strength of their bond but by the value of the provided grooming commodity.

In contrast to mutual grooming, short affiliative interactions, including sniffs, touches, and head rubs, did not significantly affect the heart rate of initiators or receivers. Against the prediction, heart rate during affiliative interactions was not lower than when standing but not interacting, thus not indicating the predicted relaxation effect. The different duration of these interactions may explain this discrepancy between the findings on mutual grooming and short affiliative interactions. Grooming lasts longer (up to minutes), whilst sniffs, touches or head rubs are short interactions (only several seconds). Grooming has been found to facilitate the release of oxytocin (Uvnäs-Moberg, 1997a;b). Oxytocin that is released during physical contact into the bloodstream can act upon the baroreflex and facilitate a decrease in heart rate (Higa et al., 2002; Kanthak et al., 2016; Kemp et al., 2012). This can promote relaxation and positively valenced emotion (Lansade et al., 2018). In contrast, the other types of affiliative interactions may not be long enough to trigger this oxytocin-mediated mechanism.

Furthermore, it was predicted that the heart rate during affiliative interactions would be lower, the stronger the bond between the horses. However, surprisingly, the analysis revealed that the heart rate in receivers of affiliative interactions followed an, to this prediction, opposite pattern. The analysis revealed a small but significant effect that the heart rate after receiving an affiliative interaction was slightly higher, the stronger the bond with the initiator. Consequently, this finding cannot be explained in the context of the hypothesis that affiliative interactions facilitate a decrease in physiological activity. However, this finding can potentially be discussed based on the framework for the study of animal emotions by Mendl, Burman, and Paul (2010). Within this framework, an increased SAM-axis activity can, on the one hand, be indicative of stress, fear,

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and anxiety, and on the other hand, for positively valenced emotions, such as excitement or happiness. Previous studies in sheep (*Ovis aries*) have shown that negative and positive test conditions facilitated an increase in heart rate. In comparison with negative test conditions, the heart rate increase during positive test conditions was low but significant (Reefmann et al., 2009; Reefmann, Wechsler and Gygax, 2009). Interpreting the presented findings from this perspective, they could suggest that receiving short affiliative interactions from a closely bonded conspecific potentially facilitates positively valenced emotions that correspond to a slight increase in heart rate. However, as this effect was small, more research is necessary to confirm this argument.

Taken together, the findings of this study show that mutual grooming and short affiliative interactions can affect heart rate in domestic horses. Whilst mutual grooming promoted relaxation and a lower SAM-axis activity level, receiving a short affiliative interaction with a strongly bonded conspecific induced a slight physiological arousal that could indicate positive emotion. This is an interesting finding as it could suggest that different types of affiliative interactions might trigger different physiological pathways that can link social integration with long-term health (Cohen and Janicki-Deverts, 2009; Lakey and Orehek, 2011; Snyder-Mackler et al. 2020; Thoits, 2011). On the one hand, it is proposed that the accumulative effect of affiliative interactions and social support in stressful situations can benefit the individual as it buffers the elevation of the SAM-axis activity or the HPA-axis activity. Consequently, the costs of maintaining a physiological stress response are reduced (Cohen and Wills, 1985; Romero, Dickens and Cyr, 2009; Snyder-Mackler et al., 2020). Such a mechanism could be triggered by grooming behaviour. On the other hand, social integration can provide the individual with regular positive experiences and overall wellbeing. This can be beneficial and promote long-term health; a mechanism described as the 'main effect of social support' (Cohen and Wills, 1985). A mediator of those main effects could be the instalment of positively valenced emotion (Mendl, Burman and Paul, 2010) that is generated and updated during repeated affiliative interactions with bonded conspecifics (Cohen and Wills, 1985).

2.4.3 Implications for equine welfare

As indicated by the presented findings, agonistic interactions in established groups of horses were predominantly of low intensity and had little effect on their SAM-axis activity levels. This goes in line with previous studies that found that group stability promoted low levels of aggression in horse groups (Christensen et al., 2002, 2011; Crockford et al., 2008; Granquist, Thorhallsdottir and Sigurjonsdottir, 2012; Jørgensen et al., 2009; Noller et al., 2013; Nuñez et al., 2014; Vandeleest et al., 2016). This is an important finding, as concerns about stress and injuries resulting from agonistic social interactions are used to argue against group housing of horses (Fureix et al., 2012; Hartmann, Søndergaard and Keeling, 2012).

Moreover, this study showed that mutual grooming was accompanied by a lower heart rate than activities with comparable physical activity, such as standing, indicating relaxation and potentially a positively valenced affective state (Baciadonna, Nawroth and McElligott, 2016; Lansade et al., 2018). Therefore, the findings of this study provide further evidence about the positive effect of mutual grooming. Additionally, they revealed that there was a small effect that short affiliative interactions such as sniffs, touches, and head rubs facilitated a slightly higher heart rate in receivers the stronger their bond to the initiator. This could be indicative of a positive emotional state (Reefmann et al., 2009; Reefmann, Wechsler and Gygax, 2009). Allowing horses to perform these behaviours is, therefore, another step towards positive welfare, by facilitating positive experiences rather than just preventing negative ones (Boissy et al., 2007; Dalla Costa et al., 2014; Fraser et al., 1997; Hall et al., 2018; Laurijs et al., 2021; Yeates and Main, 2008).

Although these findings represent valuable first insights into domestic horses' socio-physiology, they need to be generalised with care. The data presented in this study stemmed from established groups of horses and were recorded under conditions where they had *ad libitum* access to grass, water, and shelter. However, different situations, such as competition over high caloric food, could facilitate higher aggression and SAM-axis activity levels (Beery and Kaufer, 2015; Kotrschal, Hemetsberger and Dittami, 1993). Moreover, the horses in the here presented study sample had enough space to avoid agonistic encounters or to retreat. Other studies showed that horses with greater space allowance showed the lowest number of aggressive interactions (Jørgensen et al., 2009). Moreover,

it was shown that when animals had the opportunity to increase the distance to stressors, their physiological arousal was less pronounced (Levine, 2000; Moberg, 2000) as it increases the controllability over the situation (Koolhaas et al., 2017). Therefore, group-housed domestic horses with less space allowance might react differently to agonistic interactions.

Furthermore, the study sample comprised predominantly ponies and cobs, which were found to be overall calmer (Roberts et al., 2016; Sackman and Houpt, 2019). In experimental studies on social stress in rats, it was found that Wistar rats were less responsive to agonistic interactions and social defeat than wild type rats (Sgoifo et al., 1999). Therefore, the impact of agonistic interactions on heart rate modulation could differ in horses of different breeds and, for example, be higher in more temperamental breeds, such as Arabic horses or thoroughbreds (Lloyd et al., 2008; Sackman and Houpt, 2019).

Consequently, the assessment of whether different types of group housing systems, the presence of limited resources, differences in space allowance, and the horses' breeds affect heart rate modulation during social interactions can be a meaningful next step within equine welfare research.

2.5 Conclusion

This study aimed at investigating the effect of social interactions on horses' heart rate modulation. Overall, the findings showed that agonistic interactions among horses of established groups in a spacious field environment were predominantly of low intensity, including threats and bites, which did not facilitate a pronounced stress response. The rank between the horses, but not their bond relationship, had a slight effect on heart rate during agonistic interactions. Furthermore, the data suggest that horses gain a positive effect from engaging in mutual grooming and potentially when receiving other affiliative interactions such as sniffs, touches, or head rubs. This study provided novel insights into the socio-physiology of domestic horses. Furthermore, these findings are of relevance for equine welfare. Allowing horses to interact with conspecifics in an established group context does not necessarily facilitate stress and can contribute to positive welfare. However, the presented findings need to be generalised with care, as the sample of this study included a small number of different breeds, and the horses were kept in spacious fields. Horses of different breeds or under different housing conditions might react differently to social interactions.

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CHAPTER 3

Isolation and separation in gelded domestic horses (*Equus caballus*): effects of social context on behaviour and cardiac activity.



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Abstract

For individuals of gregarious species, separation from their social group poses a potential threat and can induce a behavioural and physiological stress response. This chapter aimed at assessing whether horse's behavioural and physiological responses to separation were affected by the presence of a closely bonded group member of the same sex. Therefore, ten horses were subjected to separation trials in three different test conditions: alone, with a highly-preferred horse, and with a less-preferred horse of their group. Moreover, it was assessed whether the frequency of affiliative interactions or spatial proximity differed between highly-preferred and less-preferred horses, and whether interaction rates and proximity affected the horses' heart rate and heart rate variability. Overall, the horses' behavioural and physiological stress response to the separation trials was low. The horses' vigilance was reduced when in the company of another horse, independent of their social bond. Furthermore, the rate of short affiliative interactions (sniff, touches, rub head), mutual grooming, and spatial proximity did not differ between highly-preferred or less-preferred horses during the separation trials. The frequency of short affiliative interactions, such as sniffs, touches, and head rubs, and spatial proximity did not affect heart rate or heart rate variability. However, heart rate variability (RMSSD) was higher, the higher the grooming duration. This effect was independent of the horses' social preference. Lastly, the findings revealed that although the horses' cardiac activity did not differ between the three test conditions, they were more vigilant when isolated alone. These findings did not support the idea that horses benefitted from a bond-dependent buffering effect when separated from their group. On the contrary, under low-stress conditions, they showed a generalised response towards highly-preferred and less-preferred separation partners. This generalised behaviour could be an adaptive strategy for prey species. Moreover, the findings suggest that the grooming duration can correspond to a shift to parasympathetic control of the horses' heart rate, indicating a relaxation effect. These results are also of relevance to equine welfare. Providing horses with the company of any familiar horse during a short-term separation can be of benefit, indicated by reduced vigilance and non-bond dependent interaction and proximity patterns.

Keywords: Social buffering, social support, heart rate, heart rate variability, proximity, affiliative interactions.

3.1 Introduction

Isolation and separation from conspecifics, or their social units, are known to be a potent source of stress among animals (Boissy and Le Neindre, 1997; Ferland and Schrader, 2011; Grippo et al., 2007; Hennessy, 1997; Jones and Williams, 1992; Lansade, Bouissou and Erhard, 2008a; Ludwig et al., 2017; McNeal et al., 2014; Rault, Boissy and Boivin, 2011; Tuber et al., 1996). This is especially the case for prey species, as isolation or separation from their group can increase predation risk (Boyd and Keiper, 2005; Feh, Boldsukh and Tourenq, 1994). The terms isolation and separation are used flexibly in the literature to refer to different experimental procedures. Both terms are used to describe scenarios in which animals are taken from their group alone or with conspecifics, or scenarios where animals remain behind whilst their social group is removed (Hennessy, 1997; compare operational definitions: (Hartmann, Christensen and Keeling, 2011; Lansade, Bouissou and Erhard, 2008a; Pollard and Littlejohn, 1995). Consequently, it seems necessary to first determine the use of both terms in the context of this study. Following previous definitions, situations or experiments where single individuals were taken from their group will be referred to as 'isolation' and to situations and experiments where more than one animal was taken together from their social group as 'separation' (McNeal et al., 2014; Rault, Boissy and Boivin, 2011).

Isolation was found to facilitate a behavioural and physiological stress response. Behaviourally, isolated animals can respond with increased vigilance (Ludwig et al., 2017), with increased locomotion (Boissy and Le Neindre, 1997; Pollard and Littlejohn, 1995; Reid et al., 2017; Tuber et al., 1996), agitation (Ali, Gutwein and Heleski, 2016; Boissy and Le Neindre, 1997), immobility (McNeal et al., 2014), decreased food intake (Izadi et al., 2018), and vocalisations (Bolt et al., 2017; Hopster, O'Connell and Blokhuis, 1995; Lansade, Bouissou and Erhard, 2008a; Pérez-Torres et al., 2016). On a physiological level, animals can respond with an increase in sympathetic-adrenal-medullary axis (SAM-axis) activity, indicated by a higher heart rate and lower heart rate variability (Boissy and Le Neindre, 1997; Erber et al., 2012; McNeal et al., 2014; Pollard and Littlejohn, 1995) and an increase in hypothalamic-adrenal-pituitary axis (HPA-axis) activity, indicated by an increased release of glucocorticoids (Hennessy, 1997; Ludwig et

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al., 2017; McNeal et al., 2014; Remage-Healey, Adkins-Regan and Romero, 2003).

The behavioural and physiological response to isolation from conspecifics can depend on an individuals' personality, whereby more sociable and more fearful individuals have been found to react with a stronger stress response (Lansade, Bouissou and Erhard, 2008a; Pérez Manrique et al., 2019; Reefmann, Wechsler and Gygax, 2009). However, an animal's stress response to isolation can also depend on social factors. Firstly, the stress response has been stronger when animals were isolated from an attached or strongly bonded conspecific, for example, from a mother or an offspring, or a bonded pair partner (Hennessy, 1997). Secondly, it has been found that the presence of one or more conspecifics during the separation from the social group can buffer the individual's stress response. Alpacas' (*Lama pacos*) and Marmoset monkeys' (*Callithrix jacchus*) behavioural and physiological stress responses were less pronounced during separation from their groups when they were in the company of a familiar conspecific (Pereira and Barros, 2021; Pollard and Littlejohn, 1995). Similarly, lambs (*Ovis aries*) reacted less strongly to separation from their social group if one or more familiar peers were present (Rault, Boissy and Boivin, 2011). Furthermore, the separation from a bonded pair partner did not elicit a significant stress response on SAM-axis and HPA-axis activity levels when the individuals remained within their social unit or when they were group-housed during the separation period (Greylag geese, *Anser anser*: Ludwig et al., 2017; Prairie voles, *Microtus ochrogaster*: McNeal et al., 2014). Similarly, in the context of weaning, it was found that foals of domestic horses (*Equus caballus*) and calves (*Bos taurus*) showed a less pronounced behavioural and physiological stress response when housed with other young conspecifics than when housed alone (Bolt et al., 2017; Erber et al., 2012). Taken together, these findings show that individuals can gain social support and a buffering effect of their stress response during periods of separation when familiar conspecifics are present.

Despite the evidence for social buffering effects during separation, there is little information about whether the relationship quality between the test subject and its separation partner influences such buffering effects. Group-living animals engage in differentiated relationships with their group members, such as social bonds, which are characterised by a preference towards specific members of the

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individual's social unit indicated by a high rate of affiliative interactions and frequent spatial proximity (Silk, Cheney and Seyfarth, 2013). Bonding partners can be parents (Kulik, Langos and Widdig, 2016; Mendoza and Mason, 1986; Ziegler and Crockford, 2017), related conspecifics such as (half) sisters or brothers (Cheney, Silk and Seyfarth, 2016; McFarland et al., 2017; Silk et al., 2009; Silk, Seyfarth and Cheney, 2012, 2018), or unrelated individuals of the same or opposite sex (Seyfarth, Silk and Cheney, 2012; Ziegler and Crockford, 2017).

Previous studies have found evidence that individuals can benefit from a bond-specific buffering effect during separation. One study had shown that the presence of titi monkey fathers (*Callicebus donacophilus*) buffered their offspring's stress response when they were separated from their mothers. In this species, fathers are strongly involved in the caretaking of the young and form a close bond with their offspring (Mendoza and Mason, 1986). Studies that combined social separation with the placement of the separated animals in a novel environment have shown that the presence of familiar conspecifics reduced the animals' stress response. This effect was stronger when the separated animals were closely bonded (Hennessy et al., 2006; Hennessy, Zate and Maken, 2008; Kaiser et al., 2003). Therefore, the behavioural and physiological response to separation from the social group might not only depend on the bonds that are disrupted, but also on the bonds between the study-subject and its support provider.

Based on experiments that combined separation and novelty exposure with the assessment of bond-dependent buffering effects, it was not possible to differentiate between the stress response induced by the separation or by the novel environment (Hennessy, 1997). Consequently, it is not fully understood to what extent the presence of a closely bonded conspecific buffers the behavioural and physiological response to separation from the social group. Furthermore, the named studies focused on buffering effects provided by mothers or bonded pair partners (e.g., Hennessy et al., 2006; Kaiser et al., 2003). However, some species have evolved to form close bonds among unrelated conspecifics of the same sex, such as chimpanzees (Lehmann and Boesch, 2009), Barbary macaques (Young et al., 2014a), and horses (Cameron, Setsaas and Linklater, 2009; Feh, 1999). Whether bonds among unrelated individuals, specifically of the same sex, also provide individuals with social buffering is not well understood. Investigating social

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buffering among such bond-partners can provide insight into whether such bonds provide individuals with benefits regarding the alleviation of their physiological stress response (Young et al., 2014a). Such research can enhance the understanding of the probable benefits of such bond formations on a mechanistic level (Tinbergen, 1963) and can, in the wider research context, provide more insight into why such bonds have evolved (Seyfarth and Cheney, 2012).

Additionally, an experimental separation from the group can serve the purpose to investigate the mechanisms that can facilitate bond-specific buffering effects. Under stressful situations, some species have been found to show consolation behaviour, i.e., by prolonged body contact or affiliative interactions during or following a stressor, such as pawing and grooming (as reviewed in Kiyokawa et al., 2013; Prairie voles, *Microtus ochrogaster*: Burkett et al., 2016; Horses, *Equus caballus*: Cozzi et al., 2010; Chicken, *Gallus gallus domesticus* Edgar et al., 2015; Assamese macaques, *Macaca assamensis*: Fürtbauer et al., 2014; Rats, *Rattus norvegicus*: Kiyokawa, Li and Takeuchi, 2019; Bald ibis, *Geronticus eremita*: Puehringer-Sturmayr et al., 2018). The familiarity or relationship quality between the animals can affect these behaviours. Beery et al. (2020) and Burkett et al. (2016) described that prairie voles provided consolation behaviour to familiar conspecifics such as cage mates, siblings, and pair partners, but not to strangers.

The suggested underlying physiological mechanism that links affiliative consolation behaviour and the buffering of the stress response is a release of oxytocin into the brain and bloodstream upon physical contact with conspecifics, especially when they are closely bonded (Uvnäs-Moberg, 1997a;b). Oxytocin inhibits hormones of the HPA-axis that mediate the stress response (Hostinar, Sullivan and Gunnar, 2014). Furthermore, oxytocin can act upon the baroreflex, facilitating a decreased heart rate and increased heart rate variability (Higa et al., 2002; Kanthak et al., 2016; Kemp et al., 2012). Additionally, it was found that grooming elicits the release of opioids and dopamine in the brain (reviewed in VanDierendonck and Spruijt, 2012), which both mediate gratification and thus can facilitate a positive affective state (Kremer et al., 2020; Lansade et al., 2018; Mendl, Burman and Paul, 2010; Panksepp, 2005).

However, Kiyokawa et al. (2009, 2013) have found that physical contact is not always necessary to facilitate social buffering effects. Already the presence

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or spatial proximity to a conspecific either during or after exposure to a stressor effectively buffered the stress response in rats (*Rattus norvegicus*). This was possibly mediated by an altered perception and appraisal of a potentially stressful situation (Cohen and Wills, 1985; Hostinar, Sullivan and Gunnar, 2014; Moberg, 2000). This altered perception can lead to a suppression of the fear-related activation of the amygdala (Fuzzo et al., 2015) and as a consequence, the physiological stress response can be buffered (Hostinar, Sullivan and Gunnar, 2014; Sanders and Shekhar, 1991; Young and Leaton, 1996;). Similar to affiliative interactions, spatial proximity between conspecifics during a stressful situation can depend on their relationship quality. Seeking proximity during or after a stressor is a functional trait of the attachment system and promotes a reduction of fear and the stress response (Bowlby, 1969). Proximity seeking has been initially described for mothers or caregivers and their infants (Bowlby, 1969) and also later between pair partners (Dewitte et al., 2008). Initially, it was argued that a strong attachment bond, such as between a mother and her offspring, is a necessary prerequisite for proximity seeking (Bowlby, 1969; Cassidy and Shaver, 1999; Hay, 1980; Mikulincer and Shaver, 2003). However, later studies found that proximity seeking can occur across species, for example, between dogs (*Canis lupus familiaris*) and their owners (Gácsi et al., 2001; Solomon et al., 2019; Tuber et al., 1996). Furthermore, ravens (*Corvus corax*) spent more time sitting in close proximity to related flock members than to non-related conspecifics (Stöwe and Kotrschal, 2007), suggesting that bonds among kin also facilitate proximity seeking. These findings indicate that strong bonds can also facilitate proximity seeking; or that besides mother-offspring bonds, other bonds also have attachment qualities. Assessing whether closely bonded individuals of the same sex engage in affiliative behaviours or seek proximity to facilitate social buffering can provide further evidence whether such behaviours promote social buffering outside the attachment bond between mother and offspring or pair partners (Bowlby, 1969; Dewitte et al., 2008).

Horses are an ideal species to investigate bond-dependant social buffering. They are highly gregarious group-living herbivores (Boyd and Keiper, 2005; Gersick and Rubenstein, 2017; Khalil and Kaseda, 1998; Linklater et al., 1999). They are among the few species that form close social bonds between unrelated conspecifics and between individuals of the same sex (Cameron,

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Setsaas and Linklater, 2009; Feh, 2005; Linklater et al., 1999; Seyfarth and Cheney, 2012).

Maintaining close proximity with the herd is of immediate importance to the horse as a prey species, and other behaviours such as grazing or resting, are intermitted to maintain proximity to the group (Hall et al., 2018). Horses show a high motivation to re-establish contact with conspecifics (Lansade, Bouissou and Erhard, 2008a) and, when restricted, even work to regain access to conspecifics (Søndergaard, Jensen and Nicol, 2011). Furthermore, they show consolation behaviour towards individuals who receive aggression by increasing affiliative interactions (Cozzi et al., 2010). This indicates that horses do utilise affiliative interactions to provide social support, a mechanism they might also deploy during separation from the herd, and that might affect their physiological activity.

Isolation has been found to induce stress-related behaviours such as increased call rates, vigilance, locomotion, defecation, and an increase in the SAM-axis and the HPA-axis activity (Ali, Gutwein and Heleski, 2016; Lansade, Bouissou and Erhard, 2008a; Momozawa et al., 2007; Pérez-Torres et al., 2016; Reid et al., 2017). Although the response of horses to social isolation was found to be stable over time (Lansade, Bouissou and Erhard, 2008a; Pérez Manrique et al., 2019), other studies have found that the response can depend on the presence of conspecifics. For example, during handling and training following separation from their stable-companions, horses were calmer when in the company of another horse than when alone (Hartmann, Christensen and Keeling, 2011). Nonetheless, it is not yet understood whether a closely bonded horse can be a more effective support provider during a separation from the horses' stable or field companions than a less closely bonded horse. Furthermore, it is unknown whether affiliative interactions and spatial proximity between closely bonded horses can be mechanisms that facilitate bond-dependent buffering effects. As domestic horses are subjected to husbandry or training routines that can require separation from their group or stable companions (Burla, Siegwart and Nawroth, 2018; Esch et al., 2019; Hartmann, Christensen and Keeling, 2011; Lundblad et al., 2020), it is relevant to assess whether the presence of a specific member of their group can provide them with social support and facilitates a decrease in their stress response. Therefore, addressing these open questions can also be relevant from an equine welfare perspective (Dalla Costa et al., 2014; Fraser, 2010).

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This study investigated social buffering effects on SAM-axis activity level in domestic horses (*Equus caballus*) during a separation from their social group. Firstly, it was assessed whether the presence of a closely bonded conspecific during separation could buffer the horses' behavioural and physiological stress response. Therefore, horses were subjected to one individual isolation as well as dyadic separation trials either with a closely bonded or with a less closely bonded conspecific within a familiar environment. Moreover, it was assessed whether horses showed more affiliative interactions and spent more time in close proximity when separated with a closely bonded than with a less closely bonded conspecific. Lastly, this study investigated whether affiliative interactions and spatial proximity facilitated bond-dependent buffering effects.

To address these questions, two measures of SAM-axis activity can be utilised. Firstly, the horses' heart rate (HR) which provides information about the overall net output of the interaction between the sympathetic and parasympathetic branch of the autonomous system. Secondly, heart rate variability (HRV) which can be used to assess the balance between the influence of the sympathetic and the parasympathetic branch of the autonomous nervous system on heart rate (von Borell et al., 2007; Stucke, Große Ruse and Lebelt, 2015). This provides the possibility to measure physiological and emotional components of stress (von Borell et al., 2007; Hall et al., 2018; Katayama et al., 2016; Reefmann et al., 2009; Reefmann, Wechsler and Gygax, 2009; Stucke, Große Ruse and Lebelt, 2015). In horses, HRV was found to be highly repeatable (McDuffee et al., 2019). Therefore, it constitutes a suitable measure to assess the impact of different experimental conditions on the sympathetic and parasympathetic influence on cardiac activity in horses. Two measures of HRV are (1) the *RMSSD (ms)*, the square root of the mean of the summed squares of differences between successive inter-beat intervals. The RMSSD is the primary time domain measure and reflects the high-frequency beat-to-beat variation representing vagal regulatory activity – thus, RMSSD is lower the higher the stress response of an individual (von Borell et al., 2007; Stucke, Große Ruse and Lebelt, 2015). (2) the *LF/HF-ratio*, the ratio between the low (LF) and the high (HF) frequency power, is a measure for the sympathovagal balance. The LF represents sympathetic, whereas the HF represents vagal activity. Consequently, a higher LF/HF-ratio

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indicates a shift towards sympathetic control of the heart rate (von Borell et al., 2007).

This study addressed the following questions:

- 1. Does the presence of a closely bonded conspecific of the same sex buffer the behavioural and physiological stress response during separation from the social group?**

Hypothesis A: The presence of a closely bonded horse of the same sex facilitates social buffering effects on behavioural and SAM-axis activity levels.

Prediction A1: Horses will show a weaker behavioural stress response when separated from the group together with a closely bonded horse than with a less closely bonded horse, or when isolated alone.

Prediction A2: Horses will show a lower mean and maximum heart rate when separated with a closely bonded horse than with a less closely bonded horse, or when isolated alone.

Prediction A3: Horses will show a higher RMSSD and a lower LF/HF-ratio when separated with a closely bonded horse than with a less closely bonded horse, or when isolated alone.

- 2. Does the occurrence of short affiliative interactions depend on the social bond between the horses?**

Hypothesis B: The rate of short affiliative interactions and the duration of mutual grooming depend on the social bond between the horses.

Prediction B: During separation, the rate of short affiliative interactions between closely bonded horses will be higher, and the grooming duration longer than between less closely bonded horses.

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3. Does the occurrence of short affiliative interactions between horses facilitate a bond-related buffering effect on cardiac activity level?

Hypothesis C: Higher rates of short affiliative interactions and longer mutual grooming durations between bonded conspecifics will facilitate social buffering.

Prediction C: During separation, the horses' heart rate and LF/HF-ratio will be lower, whereas their RMSSD will be higher, the higher the rate of short affiliative interactions, or the longer the grooming duration, with their closely bonded conspecific.

4. Does spatial proximity between horses depend on their social bond?

Hypothesis D: The spatial proximity between the horses depends on their social bond.

Prediction D: During the dyadic separation, the horses will spend more time in close proximity to their closely bonded than to their less closely bonded conspecific.

5. Does close spatial proximity between horses facilitate a bond-related buffering effect on cardiac activity level?

Hypothesis E: Close spatial proximity between bonded horses facilitates social buffering.

Prediction E: During the dyadic separation, the horses' heart rate and their LF/HF-ratio will be lower, whereas their RMSSD will be higher when in close proximity to their closely bonded conspecific.

3.2 Material and Methods

Data collection took place between June and September 2018 in England and included the same two stable yards described in **Chapter 2**, section 2.2, page 39. Subsequently, they are referred to as Stable A and Stable B.

3.2.1 Study subjects and housing

At Stable A, the horse group comprised eleven horses, three mares and eight geldings (castrated stallions), aged 19.5 ± 4 (mean \pm SD). At Stable B the group comprised eight horses, two mares and six geldings who were 15 ± 8 (mean \pm SD) years of age. The horses were of different breeds and were predominantly group-housed for 66-100% of their time. Their groups were established for at least a year before the commencement of data collection. More details about their breeds, workload, and housing conditions are provided in **Chapter 2**, section 2.2.1, page 39f.

3.2.2 Assessing social preference

Based on the scan samples and focal observations derived from the initial observations of the horse groups (for a detailed description, see **Chapter 2**, section 2.2.2, page 40f), social bonds were operationalised by calculating a preference index (PI) to determine social preferences among the horses of each group. The PI was calculated for each focal horse in the group and included affiliative interactions that it directed towards each of its field companions and their spatial proximity. The PI is partially based on the Dyadic Composite Sociality Index (DSI) (Silk, Cheney and Seyfarth, 2013). Like the DSI, the PI takes intercorrelations between different types of affiliative behaviours into account (for details on intercorrelations among the behaviours, see **Appendix A**, page 225). To calculate the PI, all intercorrelated behaviours that the focal horse directed towards a specific group member were summarised and then divided by the number of behaviours contributing to the index (Equation 3.1). To calculate the PI, the nearest neighbour rates ($p_{m \rightarrow n}$), rates of approaches/following ($a_{m \rightarrow n}$), and rates of body contact (including sniffs, touches, and rubbing head) ($b_{m \rightarrow n}$) for each horse and each of its field companions were summarised. The sum was then divided by the number of contributing behaviours (three).

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$$PI_{m \rightarrow n} = \frac{\sum p_{m \rightarrow n} + a_{m \rightarrow n} + b_{m \rightarrow n}}{3}$$

Equation 3.1 Preference Index (PI). The PI was calculated for each focal individual (m) and every possible social partner (n) in the group. Thereby, the nearest neighbour rate (p), the approach/following rate (a), and the rate of body contact (b) directed towards a social partner ($\rightarrow n$) were summarised and then divided by the number of included behaviours (3). The PI was calculated for every possible dyad in the group.

The PI differs from the DSI in two regards: (1) the interaction rates were not averaged for the dyad to allow for asymmetries in social preference (Barrett, Peter Henzi and Lusseau, 2012). (2) the interaction rates were not set in relation to the group mean of the behaviours as the PI should reflect each focal individual's social preferences, independent of the strength of the preference in relation to the strength of other bonds within the group. Based on the PI-indices, a directed and weighted interaction matrix was established for all horses of the group. Based on this matrix, all group members of a focal horse were ranked from most-preferred to least-preferred. Based on this matrix, it was possible to assign a highly-preferred and a less-preferred group member of the same sex for each focal individual (Table 3.1, page 89).

Once the dyads were selected, it was assessed whether the two dyad categories (high and low preference) significantly differed in terms of their PI. The analysis showed that the PIs between the focal horses and their highly-preferred field companions were significantly higher than the PIs between the focal horses and their less-preferred field companions (Paired *t*-test: $n = 10$, $t = 4.01$, $df = 9$, $p = 0.003$, $r = 0.801$; Figure 3.1, left pane). Furthermore, it was tested whether the PIs differed between the two stables. A two-sample *t*-test revealed no significant differences between Stable A and B (Two sample *t*-test: $n_A = 10$, $n_B = 10$, $t = 0.026$, $df = 18$, $p = 0.979$; Figure 3.1, right pane). This demonstrated that the assignment of bond category yielded two significantly different dyad types. Moreover, the comparison between the stables showed that the bonding structure in both groups was similar.

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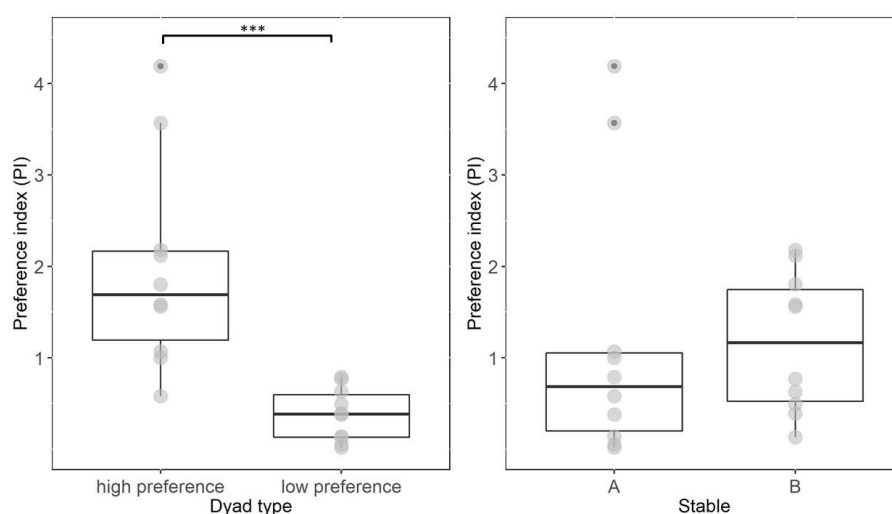


Figure 3.1 Comparisons of the Preference Index (PI). Tukey whiskers plot showing the comparison of the PI between high and low preference dyads (left pane) and between Stable A and Stable B (right pane). The median is indicated by the bold horizontal line, the upper and lower hinge of the boxplot correspond to the 25 and 75 percentiles, comprising the interquartile range (IQR). The outwards pointing whiskers extend to the smallest and largest value at most $1.5 \times \text{IQR}$ from the hinge. Data points smaller or greater are indicated as single dots. Comparing the PI between high and low preference dyads revealed a significant difference between the two dyad types (left pane). There was no difference in the PI between the two stables.

Asterisks: *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

3.2.3 Study sample and exclusion criteria

This study's focus was to assess buffering effects provided by unrelated and closely bonded conspecifics of the same sex. Consequently, female-male bonds or bonds between related individuals were not considered. One gelding at Stable B was excluded as he primarily affiliated with two mares and the interactions with the other group members were predominantly agonistic. Two mares at Stable B were excluded as they were related. Furthermore, horses were excluded based on ethical considerations. At Stable A, two mares reacted aggressively to human contact. One gelding showed a very high-stress response during the first experimental trial and attempted multiple times to jump the enclosure of the test arena. Due to the risk of injury and his owner's concern, he was excluded from the study. Furthermore, one horse was excluded due to technical limitations of the heart rate monitor. Due to his thick adipose layer, no reliable heart rate signal could be obtained. Consequently, ten geldings were selected as focal horses. For each of the focal horses, the social partner with the highest and lowest PI was chosen from the pool of included horses. The focal individuals, with their test partners and respective PI's, are listed in Table 3.1.

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Table 3.1 Focal individuals and their paired test partners with high (highly-preferred) and low (less-preferred) Preference Index (PI).

Stable	Focal individual	Highly-preferred dyads		Less-preferred dyads	
		PI	Horse	PI	Horse
A	BLL	0.58	BLY	0.02	BRA
	BLY	1.00	BLL	0.38	WOD
	BRA	4.19	BRO	0.05	BLL
	BRO	3.57	BRA	0.14	TIJ
	WOD	1.07	BRA	0.79	BLY
B	BAL	1.80	OSK	0.13	THO
	CHA	2.12	OSK	0.63	THO
	GUI	1.58	KIL	0.39	BAL
	KIL	1.56	GUI	0.49	OSK
	OSK	2.18	CHA	0.77	KIL

3.2.4 Isolation and separation tests

3.2.4.1 Study design

In a within-subject design (Martin and Bateson, 2009) data of the behavioural and the physiological response on SAM-axis activity were collected for all focal horses (n = 10) in the following test conditions:

- 1) When isolated alone (ISO)
- 2) When separated with a highly-preferred field companion – high PI (HP)
- 3) When separated with a less preferred field companion – low PI (LP)

The test conditions' order was pseudo-randomised for each focal horse in alignment with husbandry routines in the stables. To better identify whether the rate of affiliative interactions and spatial proximity affected heart rate and heart rate variability, the dyadic trials were repeated three times. Thereby, the order of the HP-trials and LP-trials was again pseudo-randomised for each focal horse. A schematic overview of the isolation and separation trials is given in Figure 3.2.

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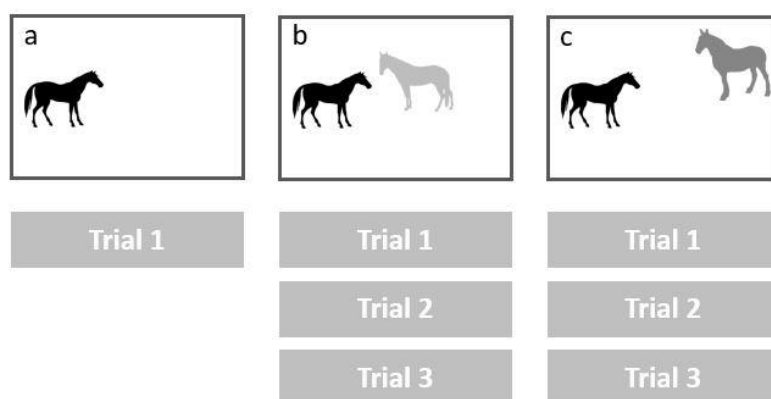


Figure 3.2 Study design for the isolation and dyadic separation trials. Horses were subjected to three different test conditions: (a) alone, (b) with a highly-preferred other horse, and (c) with a less-preferred other horse. The order of the test conditions was pseudo-randomised according to the daily husbandry routines on site. The dyadic trials were repeated three times, again pseudo-randomly varying between the high and low preference condition (horse silhouettes original and adapted from <http://phylopic.org/>).

3.2.4.2 Test procedure

Before any of the experiments, each horse was mounted with a Polar® Equine V800 mobile heart rate monitor (Polar Electro Oy, Kempele, FI). This heart rate monitor detected the time intervals between consecutive heartbeats (interbeat intervals). To enhance signal transmission, the coat in the sensor area was moisturised with water, and the surface of the sensor was covered with electrode gel (Compex® professional by chattanooga™). Individuals with thicker coats were clipped to ensure effective contact with the device. The horses were habituated to the monitors prior to this study (**Chapter 2**, section 2.2.3, page 44f).

After the horses were mounted with the monitors, they were brought into the test arena. The test arenas at Stable A and B were approximately 50m² in size and contained feed such as grass and hay, and a water bucket (Figure 3.3). This should reflect the natural living conditions of a grazing species (Bulens et al., 2015). In Stable A the distance between the test arena and the horses' field was approximately 30 meters, in Stable B, it was approximately 50 meters. The test subjects had auditory but no visual contact with their groups. The horses were familiar with the test arenas, as they were part of their usual stabling environment. For the isolation experiments, the horses were individually brought into the test arena for a duration of 15 minutes. For the dyadic separation (HP and LP), both horses were brought together into the test arena and released there for a duration of 30 minutes. During the experimental trials, the horses were able to move and

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interact freely. Their behaviour was monitored using a Canon® LEGRIA HF R56 camera.



Figure 3.3 Test arenas. Top left and right pane test arena at Stable A; bottom test arena at Stable B.

3.2.5 Data processing

3.2.5.1 Behaviour

Behaviour was coded using the Solomon® Coder version beta 16.06.26 (© András Péter, <https://solomon.andraspeter.com>). Thereby, the coder (D.V.H) was not blind to the test conditions (mitigations were explained in **Chapter 2**, section 2.2.5.1, page 46f). To assess the horses' behavioural stress response, their locomotor activity (standing, grazing, walking, trotting, and catering) and behaviours that can indicate a stress response were recorded, such as alert, startle, snorts, contact calls (whinnies), and defecation. During the dyadic separation trials, all occurrences of social interactions were recorded. A list and description of the recorded behaviours are given in the following ethogram (Table

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3.2). For the analysis of behavioural data, the frequencies of behaviours were transformed into rates per minute and the duration of behaviour was transformed into duration per minute in seconds to account for the different lengths of the experiments (Martin and Bateson, 2009).

Throughout the separation trials, the spatial proximity between the two horses was recorded in one-minute intervals. Thereby, the distance between their heads was assessed in horse-lengths. This resulted in 30 records per trial. To assess whether the horses' distance was depending on the test condition, the proximity records were averaged for each trial. To assess whether the proximity between the horses changed over the course of the experiment, the mean distance for the first, second, and third ten-minute phase of the separation trial (below denoted as beginning, middle, and end of the trial) was calculated for each dyad.

Additionally, it was recorded when the horses were either standing or grazing in close proximity (within one horse-length) or apart (a distance of four or more horse-lengths). These records were then time matched with the interbeat interval records provided from the Polar® heart rate monitor. This made it possible to investigate whether heart rate and heart rate variability (HRV) were depending on the horses' spatial proximity.

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Table 3.2 Ethogram for the behavioural coding during the isolation and separation trials. The record column indicates whether a behaviour was recorded as event (e) or as duration (d). Social interactions were only coded during the dyadic separations.

Behavioural Category	Behaviour	Record	Definition	Source
Affiliative interactions	Mutual grooming	e	Horses, mostly in antiparallel stand, scratch the other horses along its neck or back using their teeth.	(Kimura, 1998)
	Touch	e	Horse leans its head (nostril, forehead, or cheeks) against any body part of another horse.	(McDonnell and Haviland, 1995)
	Sniff	e	Olfactory investigation nose to nose or nose to body.	(Feh, 2005; Jørgensen et al., 2009)
	Rub head	e	A horse moves its head (nose, eye region, ears, or chin) in an up and down or sideways movement on any body part of another horse.	(Feh, 2005; Jørgensen et al., 2009)
Behavioural stress response	Snort	e	Powerful exhalation from nostrils.	(Malmkvist et al., 2012)
	Whinny	e	High pitched call that horse produces with an opened mouth.	(Feh, 2005)
	Alert	s	The horse stands vigilant with elevated neck, head and ears oriented towards the object, chewing is interrupted, and the horse may move up to two steps away from its former position.	(Rørvang and Christensen, 2018)
	Startle	e	Horse shudders suddenly and quickly after the object presentation.	(Lansade, Bouissou and Erhard, 2008b)
	Defecating	e	Elimination of faeces.	(Malmkvist et al., 2012)
Locomotion	Standing	d	Horse stands or stand rests with a bent hind leg.	(Malmkvist et al., 2012)
	Walking	d	The horse moves in a slow four-beat pace, moving a front leg of a side forward, then the hindquarters of the same side and then moving the front leg of the other side, followed by the forward motion of the hind leg of the other side.	(Zeitler-Feicht, 2008)
	Trotting	d	The horse moves in a faster two-beat pace, moving the diagonal front and hind leg forward at the same time, followed by a short suspension phase before the other diagonal limbs are moved forward.	(Zeitler-Feicht, 2008)
	Cantering	d	The horse moves in a fast three-beat pace. In the first beat, the horse bares all its weight on a hind leg. The other hind leg and its respective diagonal foreleg take over the weight in the second beat, which is shifted then to the remaining foreleg in a third beat. Thereafter, the horse pushes off the ground into a suspension phase before the circle starts again.	(Zeitler-Feicht, 2008)
Other behaviour	Grazing	d	Horse feeds from hay or grass.	(Bulens et al., 2015)
	Drinking	d	Horse drinks from the water bucket.	(Bulens et al., 2015)
	Urinating	d	Elimination of urine.	(McDonnell and Haviland, 1995)

3.2.5.2 Heart rate

The heart rate data were processed and error-corrected, as described in **Chapter 2**, section 2.2.5.2, page 49. After the error correction, the mean and maximum heart rate were obtained and averaged for each horse and trial. Eight per cent of the heart rate data collected during all experimental trials had to be discarded due to a high number of erroneous values (one trial KIL: 55% erroneous values, and one trial GUI 78% of erroneous values). Furthermore, the heart rate monitor stopped working during the isolation of BRA. Consequently, no data were available for this trial.

Following recommendations for HRV-analysis, interbeat interval records of similar length were selected (von Borell et al., 2007; Ille et al., 2014; Stucke, Große Ruse and Lebelt, 2015) and where horses showed the same behaviour or behaviours of similar activity levels (von Borell et al., 2007; Physick-Sheard et al., 2010; Stucke, Große Ruse and Lebelt, 2015). Consequently, records were obtained whilst the horses were either standing or grazing uninterruptedly for a duration of 30 seconds.

For the dyadic separation trials, these records were selected when horses were standing or grazing close (within one horse-length) or apart (a distance of four or more horse-lengths). To select records that were representative of the whole trial, three 30-second recordings per behaviour and per horse were selected from the beginning, middle and end of each trial. For the isolation trials, records were selected from the first, second, and third five-minute time intervals. For the dyadic separation experiments, records were chosen from the first, second, and third ten-minute intervals of the trial (Figure 3.4).

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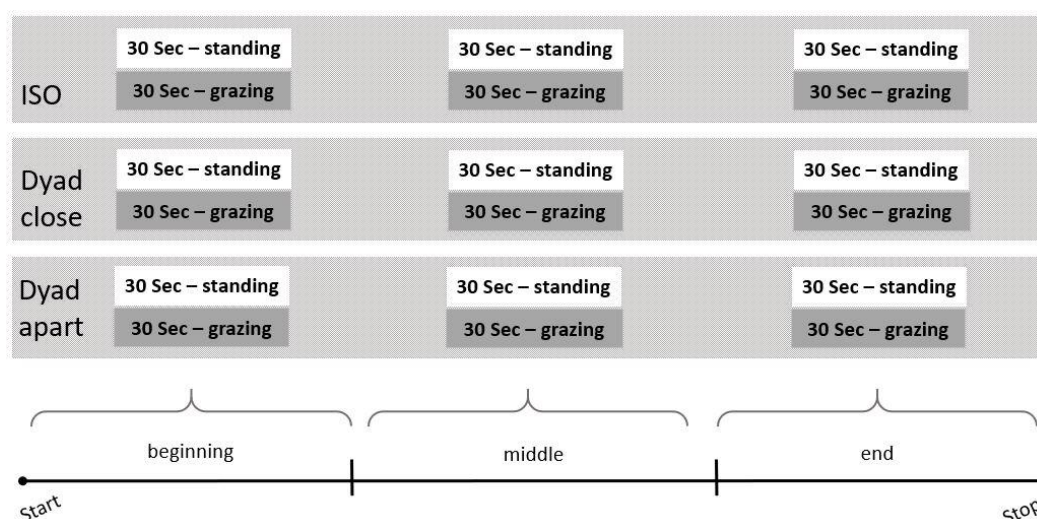


Figure 3.4 Interbeat interval samples for HRV-analysis. 30-second records were selected when horses were grazing and standing during the beginning, middle, and end of the experimental trials. For the individual isolation (ISO) records were selected from the first, second, and third 5-minute interval. For the dyadic separations, records were taken when horses were close and apart from the first, second, and third 10-minute intervals. This ensured that records were representable for the whole trial.

HRV-analyses were conducted using the Kubios® HRV software (Version 3.3; Tarvainen et al. 2019, www.kubios.com). Following previous equine studies (Ille et al., 2014; Lenoir et al., 2017; Schmidt et al., 2010a; d; Squibb et al., 2018), lambda, the prior for detrending, was set at 500 milliseconds (ms). For the artefact correction, the threshold-based custom filter was set at 0.3 seconds. Consequently, interbeat intervals (IBIs) that differed by more than 0.3 seconds from the local average were identified as an error (Ijichi et al., 2018; Squibb et al., 2018). Only data with less than a five per cent error-rate were used for further analysis (von Borell et al., 2007; Stucke, Große Ruse and Lebelt, 2015; Tarvainen et al., 2014). For spectral analysis, the frequency band limits were set according to species-specific recommendations. The low-frequency band (LF) was set at > 0.01 – 0.07 Hertz (Hz), and the high-frequency band (HF) was set at > 0.07 – 0.6 Hz (Kuwahara et al., 1996). For each of the 30-second records, the following HRV-indices were calculated: (1) RMSSD in milliseconds (ms). The RMSSD was selected, as it was found to represent changes in heartbeats over time more reliably in short IBI-samples than SDNN (Ille 2014). (2) LF/HF-ratio, and (3) the mean heart rate in beats per minute (bpm) provided by Kubios® corresponding to each 30-second HRV-sample. For further analyses, each HRV-variable was averaged per horse and trial.

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For the isolation trial, 20 HRV-samples were obtained during grazing (9 horses) and 5 during standing (2 horses). For the high preference dyads, 79 HRV-samples were obtained during grazing (10 horses) and 31 during standing (7 horses), which included 69 within close proximity (10 horses) and 41 when apart (10 horses). For the low preference dyads, 94 HRV-samples were obtained during grazing (10 horses) and 43 HRV-samples during standing (8 horses), which included 79 within close proximity (10 horses) and 58 when apart (10 horses).

3.2.6 Statistical analysis

Analyses were carried out using R (Version 3.5.1) (R Core Team, 2019) in RStudio (Version 1.1.463). Normality tests were carried out using the Shapiro-Wilk tests. Parametric and non-parametric tests were applied, depending on data distribution.

To assess whether the test condition affected the horses' behaviour and cardiac activity, the average duration and frequency for locomotion, stress behaviours, and mean and maximum HR, and the HRV-indices were compared among the isolation and the first trial of the HP-trials and LP-trials, respectively. For normally distributed data, one-way repeated measures ANOVAs in the 'ez' package (Lawrence, 2016) were used with pairwise *t*-tests with adjusted *p*-values after Bonferroni as post hoc tests. For not normally distributed data, Friedman tests in the 'pgirmess' package (Giraudeau, 2018) were applied. Post hoc tests were carried out using Nemenyi multiple comparisons in the 'PMCMR' package (Pohlert, 2018). To assess whether HRV-indices differed between the two behaviours grazing and standing paired *t*-tests were conducted. The interaction rates between horses in the HP and LP test conditions were compared using Wilcoxon signed-rank tests. To assess the influence of trial number on the rate of affiliative interactions, Friedman tests were applied.

To determine whether the rate of affiliative interactions between closely bonded horses facilitated buffering effects on SAM-axis activity level, linear mixed-effects models (LMM) were fitted. The RMSSD, LF/HF-ratio, and mean heart rate of the HRV-samples were averaged across the three trials and set as response variables. The rate of initiated and received affiliative interactions, grooming duration, and their statistical interaction with social preference (HP, LP) were set as explanatory variables. To account for repeated measures from the

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same horses, the individual identity was set as a random factor (Zuur et al., 2009; Table 3.3).

Table 3.3 Response variables, fixed, and random effects of the linear mixed-effects models: Mean RMSSD (square root of the mean of the summed squares of differences between successive interbeat intervals), the mean LF/HF-ratio (ratio between the power of the low and high-frequency bands), and the mean heart rate (HR) of the 30-second records were averaged across trials and set as response variables. Interaction rates (per minute) and grooming duration (seconds per minute) were averaged across trials. Models 1-3 include initiated affiliative interaction rates (Affiliative) and the grooming duration and their statistical interaction (*) with social preference (HP, LP) as fixed effects. Models 4-6 include rates of received affiliative interaction rates (Rec. affiliative) and their statistical interaction (*) with social preference (HP, LP) as fixed effects. The individual identification (ID) was set as a random effect to account for the repeated measures across the three trials.

	Response variables	Fixed effects	Random effect
Model 1	RMSSD	Affiliative*Social preference,	Individual ID
Model 2	LF/HF-ratio	Groom (duration)*Social preference	
Model 3	Mean HR		
Model 4	RMSSD	Rec. affiliative*Social preference	
Model 5	LF/HF-ratio		
Model 6	Mean HR		

LMMs were carried out using the 'lmer' function in the 'lme4' package (Bates et al., 2015). Outliers in the response variable were assessed via Cleveland dot plots (Zuur et al., 2009). Collinearity between the explanatory variables was tested using the 'vif' function in the 'car' package (Fox and Wisberg, 2011). Vif's of the fixed effects ranged between 1.004 and 1.169. Therefore, no variable had to be dropped (Zuur, Ieno & Elphick, 2010). Normal distribution of residuals was visually assessed using the 'autoplot' function in the 'ggplot' package (Wickham, 2016). To assess the overall model fit R^2 -values were obtained via the 'r2beta' function in the 'r2glmm' package (Jaeger, 2017). Thereby, the method was set to 'nsj' to calculate marginal R^2 (variation explained by fixed effects) and conditional R^2 (variation explained by the full model) as proposed by Nakagawa and Schielzeth (2013). Full model outputs are presented in the results section.

To assess whether spatial proximity was dependent on the test condition, the average proximity for HP-trials and LP-trials were compared using Wilcoxon rank-sum tests. To assess whether the proximity between the HP-dyads and LP-

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dyads changed over the course of the separation trial, the average proximity for the beginning, middle, and end phases of the trial were compared. The effect of trial number on spatial proximity was assessed using Friedman tests.

To assess the effect of spatial proximity between the horses on the cardiac activity of the focal horses, the following analysis were run: (1) HRV-indices and the corresponding heart rate of the 30-second records were compared when the horses were either close or apart from their highly-preferred field companions and when they were either close or apart from their less-preferred field companion. (2) HRV-indices and the corresponding heart rate of the 30-second records were compared between the test conditions to assess whether there was a difference when horses were close to either a highly-preferred or less-preferred horse, or when being apart from either a highly-preferred or less-preferred horse.

The alpha level was set at 0.05, and p-values are reported two-tailed. Effect sizes were calculated for significant results based on the z-statistic (Friedman tests and Wilcoxon signed rank-tests) using the following equation:

$$r = z/\sqrt{n} \text{ (Rosenthal, 1991).}$$

3.2.7 Ethical statement

The experimental procedure was approved by the Departmental Research Ethics Panel under the terms of Anglia Ruskin University's Research Ethics Policy (reference number: A & EB DREP 17-029 and 17-053) and followed the ethics guidelines for the study of animal behaviour provided by the Association for the Study of Animal Behaviour (ASAB, 2020). All applied methods were non-invasive. Based on ethical considerations, three horses were excluded from this study. The details are described under section 3.2.3, page 88. Consent for all procedures was sought from horse and stable owners.

3.3 Results

3.3.1 The behavioural and physiological stress response

There was no significant difference in the duration of locomotor activity between the three social test conditions (alone, and the first trial of the HP and LP condition) (Friedman tests: $n = 10$, standing: $X^2 = 0.6$, $df = 2$, $p = 0.741$; grazing: $X^2 = 1.4$, $df = 2$, $p = 0.49$; walking: $X^2 = 2.6$, $df = 2$, $p = 0.273$). Overall, the horses spent most of the time grazing. High locomotor activity was rare. Trot occurred only once in each test condition and canter occurred once in the HP condition (Table 3.4).

Table 3.4 Grazing and locomotion for each test condition. Given are median (\bar{x}), maximum (Max), minimum (Min), and interquartile range (IQR) for the duration of locomotor activity during isolation (ISO) and the first trial of the dyadic separation with the highly-preferred (HP) and less-preferred group member (LP). The duration is given as seconds per minute. There was no difference in grazing and locomotion between the three test conditions.

Condition	ISO				HP				LP			
Behaviour	\bar{x}	Max	Min	IQR	\bar{x}	Max	Min	IQR	\bar{x}	Max	Min	IQR
Grazing	40.73	53.92	10.77	19.56	46.57	56.65	7.54	40.21	38.75	52.83	6.67	29.22
Standing	9.38	38.28	3.00	16.26	8.18	43.76	1.16	33.93	16.26	45.14	1.54	30.48
Walking	6.44	12.77	0.74	6.70	4.22	11.71	0.88	4.49	4.20	7.96	2.49	1.25
Trot	0.00	0.48	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.04	0.00	0.00
Canter	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00

Alert behaviour differed significantly between the test conditions (Friedman test: $n = 10$, $X^2 = 10.4$, $df = 2$, $p = 0.006$). Post hoc analysis indicated that the horses showed more alert postures (~24%) when isolated alone (Median: 5.01 sec/min, IQR: 4.24) than when with their highly-preferred field companion (Median: 1.2 sec/min, IQR: 0.65). There was a tendency, that alert behaviour was higher during the isolation than in the low preference condition (Nemenyi multiple comparison test: ISO-HP: $p = 0.005$, $r = -0.55$; ISO-LP: 0.065; HP-LP: $p = 0.644$; Figure 3.3). Overall, the occurrence of startles differed between the test conditions (Friedman test: $n = 10$, $X^2 = 7$, $df = 2$, $p = 0.030$). However, post hoc analyses were not significant (Nemenyi multiple comparison test: ISO-HP: $p = 0.21$; ISO-LP: $p = 0.94$; HP-LP: $p = 0.37$; Figure 3.5).

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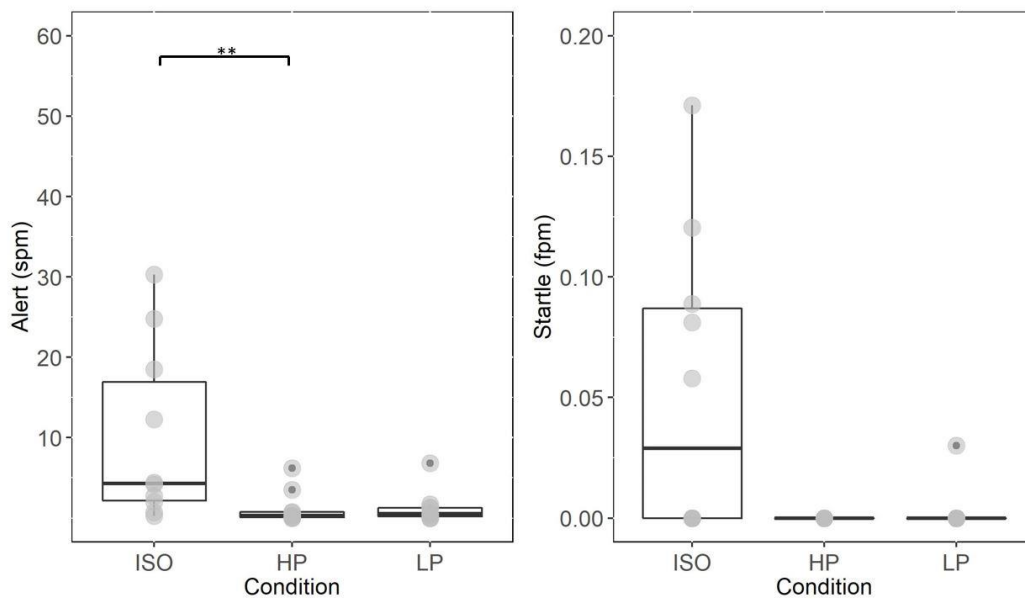


Figure 3.5 Alert and startle per test condition. Tukey whiskers plot showing the duration of alert behaviour in seconds per minute (spm) (left) and the rate of startles per minute (fpm) (right) compared between the three test conditions: alone (ISO), with a highly-preferred (HP) or less-preferred (LP) field companion. The bold horizontal line indicates the median, the upper and lower hinge of the boxplot correspond to the 25 and 75 percentiles, comprising the interquartile range (IQR). The outwards pointing whiskers extend to the smallest and largest value at most $1.5 \times \text{IQR}$ from the hinge. Data points smaller or greater are indicated as single dots. Horses showed ~24% more alert behaviour when isolated alone than when with their highly-preferred conspecific. Asterisks: *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

Only two horses whinnied during the experiments, with a maximum of 11 whinnies occurring during isolation. However, there was no evidence that the test condition influenced the occurrence of whinnies (Friedman test: $n = 10$, $X^2 = 3.71$, $df = 2$, $p = 0.156$) or defecation rate (Friedman test: $n = 10$, $X^2 = 4.45$, $df = 2$, $p = 0.108$). No snorts occurred during the experiments.

The average mean and maximum heart rate for the total trial length did not differ between the three test conditions (Friedman test: $n = 10$, mean: $X^2 = 0.29$, $df = 2$, $p = 0.867$; maximum: $X^2 = 3.71$, $df = 2$, $p = 0.156$; Figure 3.6).

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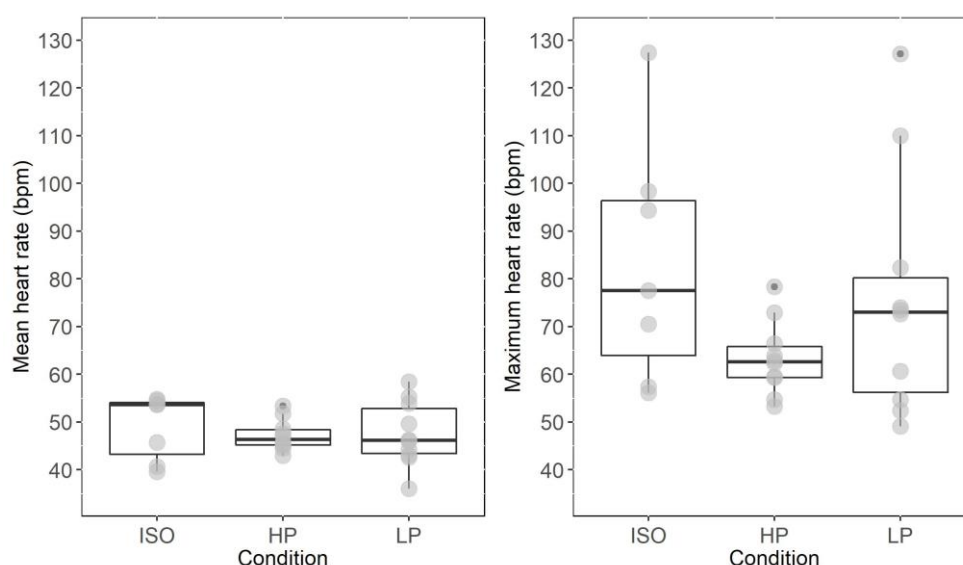


Figure 3.6 Mean and maximum heart rate during isolation and separation. Tukey whiskers plot showing mean (left) and maximum (right) heart rate in beats per minute (bpm) for the alone (ISO) and the first trials of the dyadic separations with the highly-preferred horse (HP) and the less-preferred horse (LP). The bold horizontal line indicates the median, the upper and lower hinge of the boxplot correspond to the 25 and 75 percentiles, comprising the interquartile range (IQR). The outwards pointing whiskers extend to the smallest and largest value at most 1.5 * IQR from the hinge. Data points smaller or greater are indicated as single dots. There was no difference in mean and maximum heart rate between the three test conditions.

The HRV-indices did not differ between the two selected behaviours, grazing and standing (Paired t -test: $n = 7$, RMSSD: $t = 2.158$, $df = 6$, $p = 0.074$; LF/HF: $t = 0.006$; $df = 6$, $p = 0.995$; HR: $t = -0.394$, $df = 6$, $p = 0.707$). Therefore, they were pooled for both behaviours for further analysis. The test condition had no effect on the HRV-indices and the corresponding heart rate (Friedman test: $n = 7$, LF/HF: $\chi^2 = 2$, $df = 2$, $p = 0.368$; One-way repeated measures ANOVAs: $n = 7$, RMSSD: $F_{2,12} = 0.6$, $p = 0.089$, HR: $F_{2,12} = 0.159$, $p = 0.854$; Table 3.5).

Table 3.5 Median (\bar{x}), maximum (Max), minimum (Min), and interquartile range (IQR) for heart rate variability (HRV) measures during isolation (ISO) and the first trial of the dyadic separation with the highly-preferred (HP) and less-preferred (LP) field companion. Given are RMSSD (in milliseconds), the ratio between low frequency and high-frequency power (LF/HF-ratio), and the corresponding mean heart rate (in beats per minute) for the 30-second records. The three measures did not differ between the test conditions.

Condition	ISO				HP				LP			
HRV	\bar{x}	Max	Min	IQR	\bar{x}	Max	Min	IQR	\bar{x}	Max	Min	IQR
RMSSD	65.53	99.63	50.78	33.15	54.49	108.32	14.29	33.33	50.29	71.21	44.19	9.95
LF/HF-ratio	0.49	1.61	0.12	0.89	0.23	1.05	0.11	0.25	0.81	1.10	0.20	0.34
Mean HR	47.58	54.48	40.61	10.82	47.95	51.06	43.76	2.25	45.93	54.11	39.88	5.14

3.3.2 Social interactions and cardiac activity

As the rates of initiated and received short affiliative interactions and the duration of mutual grooming did not depend on the trial number, the interaction rates were pooled per interaction type, horse, and test condition (Friedman tests: $n = 26$, initiated short affiliative: $X^2 = 5.158$, $df = 2$, $p = 0.076$; received short affiliative: $X^2 = 0.929$, $df = 2$, $p = 0.629$; grooming duration: $X^2 = 2.154$, $df = 2$, $p = 0.341$; Table 3.6).

Table 3.6 Affiliative interactions per trial. Initiated (Init.) and received (Rec.) short affiliative interactions in rates per minute as well as grooming duration (dur.) shown as medians (\tilde{x}), maximum (Max), minimum (Min), and interquartile range (IQR) for the three dyadic trials.

Trial	Init. affiliative				Rec. affiliative				Groom (dur.)			
	\tilde{x}	Max	Min	IQR	\tilde{x}	Max	Min	IQR	\tilde{x}	Max	Min	IQR
1	0.01	0.55	0.00	0.02	0.00	1.06	0.00	0.03	0.00	2.48	0.00	0.00
2	0.00	0.32	0.00	0.00	0.00	2.92	0.00	0.00	0.00	0.26	0.00	0.00
3	0.00	0.41	0.00	0.02	0.00	0.43	0.00	0.02	0.00	4.46	0.00	0.00

Comparing interactions between the HP-condition and the LP-condition, did not reveal a difference in the occurrence of initiated and received short affiliative behaviours (including sniffs, touches, and rub head) or mutual grooming (Wilcoxon signed-rank tests: $n = 10$: initiated short affiliative: $V = 20$, $p = 0.475$; received short affiliative: $V = 12$, $p = 0.834$; grooming: $V = 9$, $p = 0.787$; Table 3.7).

Table 3.7 Short affiliative interactions and mutual grooming per test condition. Median (\tilde{x}), maximum (Max), minimum (Min), and interquartile range (IQR) for the rate of short initiated and received affiliative interactions (Affiliative and Rec. affiliative) and the duration of mutual grooming averaged across all three trials of the dyadic separation with the highly-preferred (HP) and less-preferred (LP) field companion. There was no significant difference in these behaviours between the two test conditions.

Condition	HP				LP			
Behaviour	\tilde{x}	Max	Min	IQR	\tilde{x}	Max	Min	IQR
Affiliative	0.01	0.29	0.00	0.02	0.01	0.24	0.00	0.01
Grooming duration	0.00	1.57	0.00	0.00	0.00	0.83	0.00	0.61
Rec. affiliative	0.01	0.64	0.00	0.08	0.00	1.34	0.00	0.02

The trial number did not affect any of the HRV-indices (Friedman test: $n = 10$, LF/HF: $X^2 = 3.2$, $df = 2$, $p = 0.202$; One-way repeated measures ANOVAs: $n = 10$, RMSSD: $F_{2,18} = 0.601$; $p = 0.564$; HR: $F_{2,18} = 0.981$, $p = 0.394$). Therefore, each HRV-index was averaged across the three trials (Table 3.8).

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Table 3.8 HRV-indices per trial number. Depending on the data distribution, the mean (\bar{x}) is shown with the maximum (Max), minimum (Min), and the standard deviation (SD); or the median (\tilde{x}) with the interquartile range (IQR) are presented for the RMSSD (ms), LF/HF-ratio, and corresponding mean heart rate from the 30-second records.

Trial	RMSSD				LF/HF ratio				Mean HR			
	\bar{x}	Max	Min	SD	\tilde{x}	Max	Min	IQR	\bar{x}	Max	Min	SD
1	59.69	71.94	46.81	8.24	0.60	1.29	0.28	0.35	47.44	60.22	40.41	5.70
2	68.31	105.37	41.90	18.92	0.71	1.02	0.29	0.39	44.98	48.51	39.37	2.84
3	60.01	78.22	40.55	10.94	0.61	1.78	0.28	0.32	46.68	56.67	37.46	5.70

The analysis revealed that a higher RMSSD (ms) corresponded with longer grooming durations (Model 1, LMM: Groom: Estimate \pm SE = 13.74 \pm 6.02, $t = 2.28$, $p = 0.010$; Figure 3.7). Thereby, the grooming duration explained around 20% of the variation of the horses' RMSSD, as indicated by the conditional R^2 -value (Table 3.9). There was no effect of the interaction between grooming duration and social preference. The fixed effect affiliative interactions and their statistical interaction with social preference had no effect on mean RMSSD. There was no statistical interaction between any of the fixed effects (rate of affiliative interactions, mutual grooming) with the relationship quality between the horses (high or low preference), see model 1 (Table 3.9).

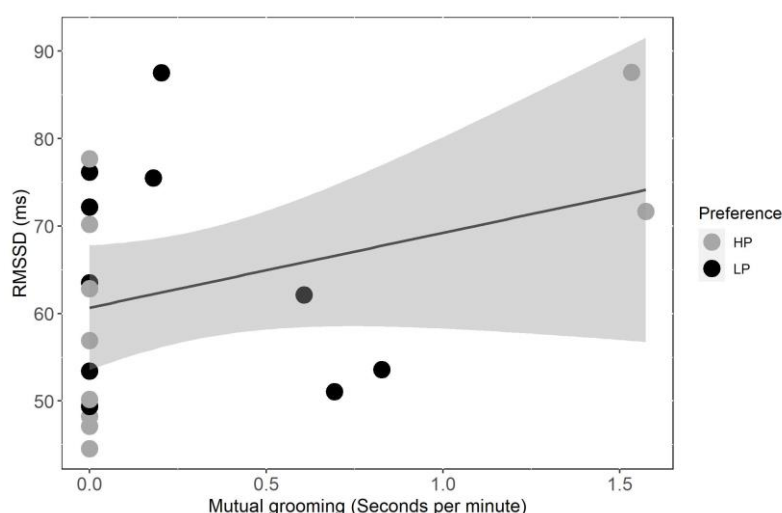


Figure 3.7 Mutual grooming and heart rate variability. The regression line, in dark grey, is based on a simple linear model of RMSSD in milliseconds (ms) by the averaged grooming duration in seconds per minute. The standard error of the fit is depicted in light grey. Data points from the high-preference (HP) condition are depicted in grey, data points from the low-preference (LP) condition are depicted in black. The RMSSD was higher, the longer the horses spent grooming. Thereby, the grooming duration explained ~20% of the variation in the horses' RMSSD. The interaction between grooming and social preference was not significant.

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The LF/HF-ratio and the mean heart rate from the 30-second records did not depend on the rate of initiated and received affiliative interactions or grooming duration and their statistical interaction with social preference, see model 2 to 6 (Table 3.9).

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Table 3.9 Output from linear mixed-effects models testing the relationship between HRV-measures and social interactions. Response variables were set to RMSSD (ms) (Model 1 and 4), LF/HF-ratio (Model 2 and 5), and the corresponding mean HR from the 30-second samples (Model 3 and 6) averaged across trials. Explanatory variables were set as the rate of initiating affiliative interactions and grooming duration (Models 1-3) and receiving affiliative interactions (Rec.aff) (Models 4-6) and their statistical interactions (*) with high or low social preference (SP). Random effect: Individual ID (Name). The conditional R^2 -value (R^2c) shows the overall model fit, the marginal R^2 -value (R^2m) shows the variation explained by the fixed effects. Given are estimates, standard error (SE), degrees of freedom (df), t -statistic (t). Significant results ($p \leq 0.05$) are marked in bold.

Models	Response variables	<i>R</i> ² c.	Fixed effects	Estimates	SE	df	<i>t</i>	<i>p</i>	<i>R</i> ² m.	
Initiating Affiliative behaviour	Model 1	RMSSD	0.349	Affiliative	-55.205	43.664	11.899	-1.264	0.230	0.071
				Groom	13.736	6.020	12.266	2.282	0.041	0.201
				SP	7.601	5.995	6.964	1.268	0.246	0.060
				Affiliative*SP	-65.021	89.778	13.025	-0.724	0.482	0.026
				Groom*SP	-1.635	22.087	11.680	-0.074	0.942	0.000
	Model 2	LF/HF-ratio	0.071	Affiliative	0.390	0.638	8.001	0.612	0.558	0.012
				Groom	0.012	0.088	8.527	0.130	0.899	0.001
				SP	-0.040	0.081	5.651	-0.492	0.641	0.006
				Affiliative*SP	-0.785	1.34	8.855	-0.585	0.573	0.012
				Groom*SP	0.311	0.322	7.876	0.968	0.362	0.030
	Model 3	Mean HR	0.313	Affiliative	-8.660	11.219	11.239	-0.772	0.456	0.028
				Groom	0.584	1.546	11.706	0.378	0.713	0.007
				SP	2.051	1.552	5.335	1.322	0.240	0.066
				Affiliative*SP	21.859	23.027	12.817	0.949	0.360	0.044
				Groom*SP	-12.417	5.677	10.935	-2.187	0.051	0.188
Receiving affiliative behaviour	Model 4	RMSSD	0.135	Rec. aff	126.109	76.899	16.000	1.640	0.121	0.124
				SP	8.078	6.942	16.000	1.164	0.262	0.067
				Rec. aff*SP	-128.868	77.648	16.000	-1.660	0.116	0.127
	Model 5	LF/HF-ratio	0.104	Rec. aff	-1.061	0.749	9.013	-1.415	0.191	0.039
				SP	-0.068	0.056	7.886	-1.221	0.257	0.020
				Rec. aff*SP	1.263	0.761	9.063	1.658	0.131	0.054
	Model 6	Mean HR	0.060	Rec. aff	15.708	18.807	12.887	0.835	0.419	0.029
				SP	0.846	1.489	9.247	0.568	0.583	0.010
				Rec. aff*SP	-18.092	19.051	13.001	-0.950	0.360	0.037

3.3.3 Spatial proximity and cardiac activity

The average proximity between the separated horses did not change over the course of the experiment, that is between the beginning, middle, and end phase (Friedman tests: HP: $n = 6$, $X^2 = 1.14$, $df = 2$, $p = 0.566$; LP: $n = 7$, $X^2 = 1.70$, $df = 2$, $p = 0.428$). Moreover, proximity did not differ between the test conditions (Wilcoxon rank-sum test: $n_{HP} = 6$, $n_{LP} = 7$, $W = 14$, $p = 0.352$; Table 3.10).

Table 3.10 Spatial distance per test condition. The median (\tilde{x}), maximum (Max), minimum (Min), and interquartile range (IQR) of the spatial distance in horse-lengths are shown for highly-preferred (HP) and less-preferred (LP) dyads. The phases refer to the beginning, middle, and end phase of the trial. The average distance for the full trials is shown in the last line. There was no difference in the spatial distance over the course of the trials or between the two test conditions.

Condition	HP				LP			
Phases	\tilde{x}	Max	Min	IQR	\tilde{x}	Max	Min	IQR
Min 1-10	2.8	3.9	1	1.4	2.7	4.3	1.6	0.8
Min 11-20	2.4	4.2	1.3	0.9	2.6	4.4	0.82	0.7
Min 20-30	2.3	3.9	1	1.13	2.4	4	1	1.11
Full trials	2.5	3.1	1.43	0.73	2.87	3.67	1.56	0.83

No difference was found in average HRV and corresponding mean heart rate when focal horses were close or apart from their highly-preferred field companion (Wilcoxon signed-rank test: $n = 10$, LF/HF: $V = 14$, $p = 0.193$; Paired t -tests: $n = 10$, RMSSD: $t = -1.35$, $df = 9$, $p = 0.21$; HR: $t = -0.311$, $df = 9$, $p = 0.763$) or when they were close or apart from their less-preferred field companion (Paired t -tests: $n = 9$, RMSSD: $t = -1.122$, $df = 8$, $p = 0.295$; LF/HF: $t = -1.240$, $df = 8$, $p = 0.816$; HR: $t = -0.134$, $df = 8$, $p = 0.896$; Table 3.11).

Comparing average HRV and corresponding mean heart rate between the two test conditions (HP vs. LP), revealed no difference in HRV and corresponding mean heart rate when focal horses were either close to their highly-preferred or their less-preferred field companion (Wilcoxon signed-rank test: $n = 10$, RMSSD: $V = 23$, $p = 0.695$; LF/HF: $V = 23$, $p = 0.695$; Paired t -test: HR: $n = 10$; $t = 0.201$, $df = 9$, $p = 0.845$). Similarly, there was no difference in HRV or corresponding mean heart rate when focal horses were either apart from their highly-preferred or from their less-preferred field companion (Wilcoxon signed-rank test: $n = 9$, LF/HF: $V = 25$, $p = 0.820$; Paired t -tests: $n = 9$, RMSSD: $t = -0.152$, $df = 8$, $p = 0.882$; HR: $t = 0.069$, $df = 8$, $p = 0.946$; Table 3.11).

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Table 3.11 Heart rate variability (HRV), heart rate (HR), and spatial proximity. Medians (\tilde{x}), maximum (Max), minimum (Min), and interquartile range (IQR) for RMSSD (ms), LF/HF-ratio, and the corresponding mean heart rate (HR) from the 30-second records when horses were close (within one horse-length) or apart (more than four horse-lengths distance) shown for the test conditions high-preference (HP) and low-preference (LP). There was no difference in HRV and HR between different spatial distances and test conditions.

Proximity	HRV	HP				LP			
		\tilde{x}	Max	Min	IQR	\tilde{x}	Max	Min	IQR
Close	RMSSD	57.18	76.69	44.44	24.85	58.92	78.20	45.97	21.40
	LF/HF	0.52	1.00	0.30	0.24	0.59	1.10	0.16	0.48
	HR	47.20	50.16	43.33	2.79	45.91	52.36	41.40	5.16
Apart	RMSSD	64.28	89.15	44.12	17.50	65.26	80.87	44.77	22.09
	LF/HF	0.54	1.92	0.30	0.20	0.52	1.16	0.22	0.57
	HR	45.86	55.74	41.64	6.00	42.92	56.63	38.25	7.84

3.4 Discussion

In this chapter, it was assessed whether the social context affected the behavioural and physiological stress response in domestic horses (*Equus caballus*) during isolation and separation from their social group. Moreover, it was investigated whether affiliative interactions and spatial proximity are mechanisms that can facilitate social buffering effects. First, the findings will be discussed regarding the effect of the test condition on the horses' behavioural and physiological stress responses. Thereafter, the effect of social preference on their affiliative interaction rates, spatial proximity, and cardiac activity will be reviewed.

3.4.1 The behavioural and physiological stress response

Based on previous research, the prediction was that the presence of a closely bonded conspecific would buffer the horses' behavioural and physiological stress response. Overall, the experimental trials carried out for this study elicited a moderate stress response. The locomotor activity was low, and the horses spent most of the time during the trials grazing. Whinnies occurred rarely, and snorts never occurred. These behavioural patterns did not differ between the three test conditions (alone, with a highly-preferred horse, or with a less-preferred horse). However, horses showed significantly more alert postures, defined as standing or walking with their head lifted high (lips above withers height), when isolated alone. Additionally, the analysis revealed an overall difference in startle frequency. Thereby, the highest startle rate occurred when horses were isolated alone, compared to both dyadic separation conditions. However, this effect did not reach significance in the post hoc pairwise comparisons between the test conditions. Taken together, these findings indicate that horses were more vigilant when alone than when separated with another horse. This effect was independent of the social bond between the separated horses. An enhanced vigilance during experimental isolation was reported for different species in previous studies (e.g., European starlings, *Sturnus vulgaris*: Apfelbeck and Raess, 2008; Greylag geese, *Anser anser*: Ludwig et al., 2017). Vigilance can strongly depend on the animals' social context. In prey species, vigilance can decrease with group size (e.g., Bighorn sheep, *Ovis canadensis*: Rieucan and Martin, 2008; Elks, *Cervus elaphus*: Childress and Lung, 2003; Kiangs, *Equus kiang*: Xu et al., 2013), a phenomenon

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described as ‘many eyes effect’ (Pulliam, 1973; Roberts, 1996; Van Der Post, Verbrugge and Hemelrijk, 2015; Xu et al., 2013). Additionally, individuals can benefit from a ‘dilution effect’, which describes a reduced chance of being attacked with an increased number of conspecifics (Foster and Treherne, 1981). Thus, group life can be a protective factor, especially for prey species, such as horses (Feh, Boldsukh and Tourenq, 1994; Krause and Ruxton, 2002; Rubenstein, 1978; Van Der Post, Verbrugge and Hemelrijk, 2015). Therefore, when separated from their group, horses might benefit from a safety-effect provided by any familiar member of their group, independently of the closeness of their bond.

In regard to the proposed effect of the horses’ social context on SAM-axis activity level, no difference in heart rate and heart rate variability between the three test conditions was found: alone, with a highly-preferred or with a less-preferred horse. These findings stand in contrast to previous studies, which had demonstrated that the presence of conspecifics buffers the stress response more than when individuals were isolated alone (Boissy and Le Neindre, 1997; Erber et al., 2012; Pollard and Littlejohn, 1995; Reid et al., 2017; Tuber et al., 1996). However, the isolation and separation trials had overall only a moderate effect on SAM-axis activity levels. As previous research has shown, the effect of social buffering can increase with increased stress intensity. Wild Barbary macaques (*Macaca sylvanus*) showed stronger buffering effects under conditions of higher social and environmental stress than under conditions of low stress (Young et al., 2014a). One aspect that may have contributed to this moderate stress response could be the location of the isolation and separation trials. The isolation and separation trials were conducted in a test arena that was highly familiar to the horses. This is in contrast to previous studies, where animals were separated from their social unit and transferred into a novel environment (Banerjee and Adkins-Regan, 2011; Coe et al., 1982; Hennessy, Zate and Maken, 2008; Kanitz et al., 2014; Sachser, Dürschlag and Hirzel, 1998). However, horses from both stables were housed on site between one to ten years prior to the data collection. This could have promoted habituation to the location itself, which could have facilitated perceived safety and made a strong stress response obsolete. In support of this suggestion, Tuber et al. (1996) found that shelter dogs (*Canis lupus familiaris*) did not show a significant stress response when they were separated from their

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kennel mate but were remaining within their home kennel. Moreover, Hennessy (1997) had collated findings of other studies in his review that had demonstrated that social isolation only provoked a significant stress response when the test subjects were exposed to an additional stressor, such as novelty. In line with this review, Baron (1963) found that the fear response of mice (*Mus musculus*) following electric shocks was more pronounced when they were thereafter transferred into an unfamiliar environment than when transferred into a familiar environment. Taken together, this could suggest that animals might not just gain buffering effects from conspecifics as shown in other studies (Bolt et al., 2017; Erber et al., 2012; Pollard and Littlejohn, 1995; Rault, Boissy and Boivin, 2011). They might also benefit from familiarity with their physical environment. Future research could assess which aspects determine whether the social context or environmental features affect the behavioural and physiological (stress) response of group-living animals. This could help to gain a better understanding regarding aspects that can increase the individual's capacity to cope with stressors (Koolhaas et al., 2017) and how social buffering relates to other stress coping mechanisms (Hennessy, 1997).

Although the horses showed a higher vigilance when they were isolated alone, they did not show a difference in mean heart rate or HRV when isolated than when separated with another horse. This finding seems unexpected, however, a similar disjunction between the behavioural and physiological stress response has been found in other studies. Squibb et al. (2018) found that horses showing greater behavioural resistance in a novel handling task did not show a higher heart rate than compliant horses. Similarly, horses with higher behavioural reactivity during fur clipping did not show a higher heart rate than less reactive horses (Yarnell, Hall and Billett, 2013). In contrast to these findings, other studies have shown the opposite effect. Munsters et al. (2013) found that horses that stood still and did not show behavioural responses when exposed to a strong external stressor, had a significantly increased heart rate. Similarly, Safryghin, Hebesberger, and Wascher (2019) showed that horses that showed less behavioural arousal prior to feeding showed a significantly higher heart rate. Therefore, there seems to be an occasional discrepancy between the behavioural and the physiological stress response. Squibb et al. (2018) have discussed their results, arguing that equine training methods based on negative reinforcement

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could facilitate horses' strategy to obscure stress-related behaviour. Consequently, horses' stress response could be primarily indicated by an increase in their physiological activity. Safryghin, Hebesberger and Wascher (2019) have discussed that the type of stressor might contribute to different responses. When exposed to a novel object, the horses' behavioural stress response was in line with their physiological stress response, whereas in a feeding experiment, where horses were waiting for their hard feed, the behavioural and physiological were not aligned. A similar pattern is found in the studies presented in this thesis. During the isolation/separation, which horses in this study did not perceive as a great stressor, as indicated by low behavioural and physiological arousal, the behavioural and physiological responses were discrepant. However, in the context of the novel object exposure, a higher behavioural arousal corresponded to a higher physiological arousal (**Chapter 4**). Similarly, previous research in starlings (*Sturnus vulgaris*) showed that during exposure to different stressors such as light, music, and different types of human intrusion, their behavioural stress response, and their physiological stress response on the SAM-axis activity level and the HPA-axis activity level did not necessarily correspond. Whilst the physiological stress response on their HPA-axis activity level was similar for the different stressors, their response on SAM-axis activity level was differentiated and lowest during the light stressor. Significant behavioural changes occurred when the birds were exposed to music or a threatening human intrusion. The authors of this study have concluded that the degree of the stressor's novelty could be the most significant factor in determining the stress response on SAM-axis activity level (Nephew, Kahn and Romero, 2003). This suggests that three main components of the stress response, namely behaviour, SAM-axis activity, and HPA-axis activity, may be regulated independently and that their regulation could be sensitive to different contexts.

Responding to different stressors with differentiated behavioural and physiological activity levels could be adaptive. On the one hand, it enables the individual to react to stimuli in its environment. On the other hand, it could also save energy, which otherwise would be needed to elicit and maintain the stress response on the levels of behaviour, SAM-axis activity, and HPA-axis activity (Nephew, Kahn and Romero, 2003; Romero, Dickens and Cyr, 2009). Therefore, future research could investigate the context-specific modulation of animals' stress responses on those three levels. A more differentiated insight into how

animals regulate their stress response could provide more information about underlying mechanisms of behaviour (Tinbergen, 1963) and how those responses potentially influence long-term health (McEwen, 2008).

3.4.2 Social interactions, spatial proximity and SAM-axis activity

The prediction was that horses would show higher affiliation rates and closer spatial proximity when separated with their highly-preferred field companions. However, there was no difference in the frequency of affiliative interactions or spatial proximity between the focal horse and the highly-preferred and less-preferred test partner during the separation trials. This is surprising, as horses showed a significant preference in their social interactions when within their whole group. This finding could indicate that horses generalise their interactions with, or proximity to the available social partner during a separation from their group, independently of their social preference. This observed difference in the rate of affiliative interactions and spatial proximity patterns during group and dyadic test conditions could also be understood based on the biological market theory (Noë and Hammerstein, 1994). This theory describes that individuals chose, for example, a social or sexual partner depending on their relative quality, or the quality of an offered commodity compared to other available conspecifics (Barclay, 2016; Noë and Hammerstein, 1994). This could explain observed preferences among horses within their whole social unit, where different social partners are available to choose from. However, selectiveness is only beneficial if there are several partner options (Barclay, 2016). Being selective in a situation where fewer social partners are available can be costly (Noë and Hammerstein, 1994). For horses, as prey animals, grouping was found to be one anti-predator strategy (Feh, Boldsukh and Tourenq, 1994; Rubenstein, 1978; Van Der Post, Verbrugge and Hemelrijk, 2015). Therefore, selectiveness could be costly when being separated from their social group as it could increase the potential risk of predation. Thus, affiliating and spending time in proximity during separation could be a generalised (i.e., not bond-dependent) strategy and potentially more beneficial when there is a limited number of conspecifics available.

Moreover, it was predicted that higher rates of affiliative interactions with and close proximity to highly-preferred field companions would facilitate an increase in RMSSD, a decrease in LF/HF-ratio and the corresponding mean heart rate. When investigating the effect of affiliative interactions and spatial proximity on cardiac activity, the finding revealed, in line with the prediction, that the average RMSSD was higher, the longer horses were mutually grooming. This suggests that longer a grooming duration corresponds with higher vagal activity and a shift towards parasympathetic control of the horses' heart rate (von Borell et al., 2007; Stucke, Große Ruse and Lebelt, 2015). Similarly to findings presented in **Chapter 2**, this effect did not depend on the relationship quality between the grooming partners. However, as the model fit for this analysis was relatively low, and as mutual grooming did not occur between all dyads, these findings need to be interpreted cautiously. Furthermore, the findings showed that the RMSSD, LF/HF-ratio and the corresponding heart rate did not depend on the frequency of other affiliative interactions, such as touch and head rubs, or the spatial proximity between the horses. Summarising these findings, this study did not provide strong evidence that affiliative interactions between highly-preferred or less-preferred horses affected heart rate and HRV during repeated social separation, as suggested by previous research (reviewed in Kiyokawa et al., 2013; Beery et al., 2020; Burkett et al., 2016; Fürtbauer et al., 2014). However, the findings presented in this chapter apply to a situation that can be described as a mild stressor, as besides vigilance, horses did not show other behaviours that would indicate a significant stress response. Whether affiliative interactions and spatial proximity have a more pronounced effect on cardiac activity during situations of higher stress intensity (Burkett et al., 2016; Kiyokawa et al., 2013; Kiyokawa, Li and Takeuchi, 2019) needs to be assessed in future studies.

3.4.3 Implications for equine welfare

Separation from the group has been reported to provoke a stress response in horses (Ali, Gutwein and Heleski, 2016; Lansade, Bouissou and Erhard, 2008a; Momozawa et al., 2007; Pérez-Torres et al., 2016; Reid et al., 2017). Nevertheless, group-housed horses sometimes need to be separated from their group due to different feeding needs or medicinal purposes and in the course of husbandry routines or training (Burla, Siegwart and Nawroth, 2018; Esch et al., 2019; Lundblad et al., 2020). The findings presented in this chapter suggest that

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the presence of any familiar field companion, independent of the bond relationship, can be beneficial during the separation from the social group. Horses showed less vigilance when together with a familiar horse. This can allow them to spend more time performing other behaviours such as feeding or maintenance behaviours (Pulliam, 1973; Roberts, 1996; Van Der Post, Verbrugge and Hemelrijk, 2015; Xu et al., 2013). Highly-preferred and less-preferred horses showed the same rates of affiliative behaviours and spatial proximity. Moreover, there was no difference in the horses' heart rate or heart rate variability when together with either a highly-preferred or a less-preferred field companion. Lastly, mutual grooming between highly-preferred and less-preferred horses had a positive effect on their HRV. This suggests that, when separated from the group for a short amount of time, the company of any field companion can enable horses to fulfil their social needs.

These findings present novel insights into the effect of horses' social context on their behaviour and cardiac activity during separation. Whether they also apply to more temperamental breeds needs to be assessed in future studies. Previous research on cattle has found that their behavioural and physiological response to social isolation can depend on their breed, with the calmer breed showing a less pronounced stress response (Boissy and Le Neindre, 1997). Wolff, Hausberger and Le Sclan (1997) found that horses' response to social separation had a strong genetic basis, with half-siblings showing a significant correlation in their responses. The analysis of a large-scale study where human handlers rated horses' sociability, which is defined here as a trait for gregariousness and is reflected in the horses' propensity to socialise, has shown that the sociability ratings were breed-dependent (Lloyd et al., 2008). In conjunction with the findings presented in this chapter, the results of these studies could suggest that calmer, less fearful horses can be more independent of their social context in their behavioural and physiological response to social separation. Gaining more insight into the interplay between breeds, stress physiology, and social support could further the understanding of which individuals are more susceptible to perceive situations or stimuli as stressors and which individuals benefit more from social support. Such an understanding can be beneficial from the perspective of equine welfare, as it can guide and promote husbandry and

training strategies that can decrease stress for domestic horses (Dalla Costa et al., 2014; Fraser, 2010).

Another finding relevant for equine welfare is the discrepancy between the behavioural and physiological responses detected in this study. Although the horses showed a significantly higher vigilance when isolated alone, their mean and maximum heart rate were not significantly higher than in the dyadic test conditions. As such discrepancies were reported in other studies, as discussed in section 3.4.1, page 108ff, it seems relevant to increase the awareness of equestrians that a horse that shows a behavioural stress response is not necessarily showing a physiological stress response, and that a horse that seems calm, might experience stress as indicated by a response on a physiological level. Future research could address this phenomenon and assess whether specific contexts promote these discrepancies between the behavioural and physiological stress response. Furthermore, more reliable indicators seem necessary to enable equestrians to distinguish between stress-related behaviours and learnt behaviour (Cooper and McGreevy, 2002) on the one hand, and that can help to detect a stressed horse that does not display obvious behaviour that can be indicative for a stress response, on the other hand. Reliable detection of the horses' stress response is an important aspect to determine factors during housing, handling, and training that constitute stressors for the horses under our care. This is an essential prerequisite to promote practices that can decrease stress for domestic horses (Dalla Costa et al., 2014; Fraser, 2010).

3.5 Conclusion

This study aimed at assessing whether domestic horses benefitted from the presence of closely bonded conspecifics during separation from their group. The horses in the study sample showed a moderate stress response to isolation and separation from their social group. Horses showed the highest vigilance when isolated alone. The presence of both a highly-preferred and a less-preferred field companion of the same sex facilitated a decrease in vigilance. The rates of affiliative interactions and spatial proximity patterns did not differ between highly-preferred and less-preferred horses during the separation trials. These findings suggest that horses generalised their behaviour towards a familiar conspecific, independently of their bond relationships. This is an interesting finding as

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affiliative interactions and spatial proximity differed significantly between focal horses and their highly-preferred or less-preferred field companion when they were observed in the context of their whole group. Moreover, higher vigilance was not accompanied by a higher heart rate or lower heart rate variability. This reflects other studies' findings, indicating that horses' behavioural response and physiological adaptation can be regulated independently.

Regarding possible mechanisms of social buffering, this study showed that the duration of mutual grooming corresponded with a higher average RMSSD, indicating a shift to parasympathetic control of cardiac activity. However, this effect was small and again, not depending on the social bond between the grooming partners. Otherwise, the rate of initiated and received affiliative interactions such as sniffs, touches, and head rubs, or spatial proximity did not affect cardiac activity. Overall, the presented findings suggest that the presence of a highly-preferred conspecific did not facilitate bond-dependent behavioural and physiological responses in the context of separation from their social group. Rather, horses showed a predominantly generalised behavioural and physiological response when separated with either a highly-preferred or less-preferred field companion. This generalisation could be an adaptive strategy for prey animals.

CHAPTER 4

Novelty and social buffering in gelded domestic horses (*Equus caballus*): effects of social context on stress response and exploratory behaviour



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Abstract

Animals, especially those under human care, need to adapt to novel stimuli in their environment. The study presented in this chapter assessed whether domestic horses (*Equus caballus*) benefited from social buffering when exposed to a novel object. For this purpose, ten horses were exposed to a novel object in four different test conditions: alone, with a highly-preferred horse of their group, a less-preferred horse of their group, or their whole group. Furthermore, it was investigated whether the presence of conspecifics facilitated exploratory behaviour. The novel object tests provoked a significant but mild stress response. After the initial object exposure, horses showed closer proximity to their highly-preferred than to a less-preferred group member. The closer proximity did not correspond to a lower heart rate. Otherwise, the horses' stress-related behaviour, cardiac activity, and exploratory behaviour during the novel object tests were not significantly affected by their social context. The presented findings suggest that domestic horses show a bond-dependent proximity-seeking behaviour. Besides this finding, the study did not provide further evidence that the presence of a highly-preferred conspecific of the same sex or the whole group facilitated a social buffering effect in horses during a novel object exposure. However, they provided novel insight into horses' socio-physiology, suggesting that horses deploy individual behavioural and physiological strategies when confronted with a mild stressor rather than adjusting their response to their social environment. Compared with previous research, this could indicate that social buffering is only one possible coping strategy and that its occurrence might depend on the intensity of the stressor.

Keywords: Novel object test, social support, heart rate, fear response, exploration.

4.1 Introduction

Besides potential stress from the social environment (DeVries, Glasper and Detillion, 2003; Sgoifo, Carnevali and Grippo, 2014; Viblanc et al., 2012; Wascher, Arnold and Kotrschal, 2008; Yamanashi et al., 2018; **Chapter 2**), animals are sometimes exposed to novel stimuli in their physical environment that can be perceived as stressors (Emery Thompson et al., 2010). Exposure to novelty can be a consequence of changes within the animals' familiar habitat or when animals expand their habitat to new territory (Russell, McMorland and MacKay, 2010). Due to anthropogenic influences, animals are increasingly exposed to novelty through habitat shifts and fragmentation or through the provision of new resources such as food or shelters (Sih et al., 2012). Domestic species, such as horses, are commonly exposed to novelty in the contexts of housing, handling, and training (Burla, Siegwart and Nawroth, 2018; Esch et al., 2019; Lundblad et al., 2020).

Depending on the perceived threat of a novel stimulus (Moberg, 2000), animals need to adapt their behaviour and physiological response. If the stimulus is perceived as dangerous or threatening, the stimulus represents a stressor, and the subsequent behavioural and physiological changes can be described as a stress response (Cannon, 1929; Koolhaas et al., 2011, 2017; Romero, Dickens and Cyr, 2009). These behavioural and physiological changes are necessary to enable the individual to cope with the stressor and maintain its homeostasis (Cannon, 1929; Romero, Dickens and Cyr, 2009). The behavioural changes can include a fight, flight, or freeze response (Cannon, 1929). Furthermore, animals can seek proximity to a specific conspecific, such as a mother or pair partner (Bowlby, 1969; Cassidy, 1994; Dujardin et al., 2019), or show flocking behaviour to increase group cohesiveness and reduce the potential risk for the individual (Foster and Treherne, 1981; Hamilton, 1971; Iranzo et al., 2018; King et al., 2012). On a physiological level, the stress response is characterised by an increased activity of the sympathetic-adrenal-medullary axis (SAM-axis), as indicated by an increase in heart rate or blood pressure. Moreover, the physiological stress response is characterised by an increase in the hypothalamic-adrenal-pituitary axis (HPA-axis) activity, as indicated by an increased release of glucocorticoids (Cannon, 1929; Koolhaas et al., 2011, 2017; Romero, Dickens and Cyr, 2009).

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These adaptations can increase survival in the case of life-threatening stressors (Cannon, 1929). However, they are also costly as they require the mobilisation of energy to maintain the stress response and are thought to cause wear and tear on the organism (Romero, Dickens and Cyr, 2009).

If a novel stimulus is perceived as less threatening, animals can show exploratory behaviour (Lester, 1968) and approach to investigate or manipulate it (Mettke-Hofmann, Winkler and Leisler, 2002). Exploratory behaviour can be beneficial as it enables the individual to learn about the properties and quality of novel features in the environment such as new sources of food (Beecham, 2001; Stöwe et al., 2006b), escape routes, shelters, or the location of predators, and potential mates (Birke and Archer, 1993; Dingemanse et al., 2002; Mettke-Hofmann, Winkler and Leisler, 2002; Tebbich, Fessl and Blomqvist, 2009). Exploratory behaviour and sampling are an integral part of forming a mental map of the animals' environment (Beecham, 2001) and has been found to benefit survival and reproduction (summarised in Moretti et al., 2015). However, exploratory behaviour can also be costly. Due to a decreased vigilance during exploration or when exploratory behaviour occurs in an exposed location, it can increase the risk of predation (Mettke-Hofmann, Winkler and Leisler, 2002).

Therefore, animals need to adapt their behavioural and physiological response to a novel stimulus depending on the perceived threat. Balancing the response to novelty between flight and exploratory behaviour ensures survival in dangerous situations and the conservation of energy while also enabling the animal to learn and exploit the habitat and new resources in situations that are less dangerous (Birke and Archer, 1993; Christensen et al., 2021a; Dingemanse et al., 2002; Mettke-Hofmann, Winkler and Leisler, 2002; Miller et al., 2015; Stöwe et al., 2006b). Both, the behavioural and physiological stress response and the occurrence of exploratory behaviour, when exposed to novelty, can depend on an individual's social context. During a potentially stressful situation, the presence of conspecifics can alter the perception and appraisal of that stimulus (Cannon, 1929) and reduce the stress response - a phenomenon referred to as social buffering (Cohen and Wills, 1985). On the behavioural level, social buffering has been found to reduce fear reactions, flight or alert behaviour (Christensen et al., 2008), freezing or inactivity (Ishii et al., 2016; Kikusui, Winslow and Mori, 2006b;

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Kiyokawa et al., 2013b; Kiyokawa and Hennessy, 2018b), escape attempts (Kanitz et al., 2014), and contact calls (Kanitz et al., 2014; Kiyokawa and Hennessy, 2018). On the physiological level, social buffering has been found to alleviate the stress response, which is indicated by lower SAM-axis activity levels and lower HPA-axis activity levels (Cohen and Wills, 1985; DeVries, Glasper and Detillion, 2003b).

Although social buffering can be induced by unfamiliar conspecifics (Rats, *Rattus norvegicus*: Kiyokawa et al., 2014a), the buffering effects were greater when the social support was provided by a familiar conspecific (Rats: Hodges et al., 2014; Kiyokawa et al., 2014b; Terranova, Cirulli and Laviola, 1999), and even more so when it was provided by a strongly bonded conspecific (Guinea pigs, *Cavia porcellus*: Hennessy et al., 2006; Hennessy, Kaiser and Sachser, 2009; Kaiser et al., 2003; Sachser, Dürschlag and Hirzel, 1998). This bond partner could be the mother for her offspring (e.g., Chicken, *Gallus gallus domesticus*: Edgar et al., 2015; Guinea pigs: Hennessy et al., 2006; Humans, *Homo sapiens*: Yirmiya et al., 2020), a bonded pair partner (e.g., Guinea pigs: Kaiser et al., 2003; Sachser, Dürschlag and Hirzel, 1998; Wied's black tufted-ear marmosets, *Callithrix kuhlii*: Rukstalis and French, 2005), or a relative in species with strong bonds between kin (e.g., Chacma baboons, *Papio ursinus*: Wittig et al., 2008).

In situations that are not life-threatening, social buffering can be regarded as beneficial. By increasing the perceived safety and reducing the physiological stress response, or preventing it from occurring, it lowers the energetic costs required to elicit or maintain the stress response (Moberg, 2000; Romero, Dickens and Cyr, 2009). Another benefit is that the reduction of the stress response can help individuals to cope with new or challenging situations and shift the motivational conflict between fear and exploration (Bradley et al., 2001) towards exploratory behaviour (Cassidy, 1994; Dujardin et al., 2019; Hayes, 1960; Galhardo, Vitorino and Oliveira, 2012; Jakubiak and Feeney, 2016; Lester, 1969).

Like the modulation of the stress response, exploratory behaviour can depend on an individual's social context. That the presence of conspecifics can facilitate exploratory behaviour has been shown in different species. For example, Capuchin monkeys (*Cebus apella*) (Dindo, Whiten and de Waal, 2009), horses (*Equus caballus*) (Mendonça et al., 2019), wolves (*Canis lupus*), dogs (*Canis*

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Iupus familiaris) (Moretti et al., 2015), birds of the crow family (*Corvidae* spp.) (Miller et al., 2014, 2015; Stöwe et al., 2006b; Stöwe and Kotrschal, 2007), and fish (e.g., Guppies, *Poecilia reticulata*: Croft et al., 2006; Mosquitofish, *Gambusia holbrooki*: Ward, 2012) were exploring faster or longer when with either one or more conspecifics. Similarly to social buffering, the social facilitation of exploratory behaviour can depend on the social bond between the involved individuals. In humans, children explored more when a strongly bonded attachment figure, for example, their mother or primary caregiver, was present than in the presence of a stranger (Jakubiak and Feeney, 2016). Also, goat kids (*Capra aegarus hircus*) explored more when their mother was present (Lyons, Price and Moberg, 1988), and dogs explored longer and faster when with their owners than with strangers (Horn, Huber and Range, 2013; Palmer and Custance, 2008; Payne et al., 2016; Schöberl et al., 2016; Solomon et al., 2019). Furthermore, ravens were found to join siblings faster than non-siblings to approach a novel object (Stöwe et al., 2006b).

Taken together, these findings indicate that the behavioural and physiological response to novelty can depend on the presence of conspecifics that provide social support. Thereby, the relationship quality to the support providers can play an important role in the regulation of the behavioural and physiological stress response and the facilitation of exploratory behaviour. Although the interplay between an individual's social context, social buffering and socially facilitated exploration has been evidenced, this chapter tackles explicitly three knowledge gaps:

- (1) Evidence of social buffering among closely bonded conspecifics of the same sex is scarce. It stands out that most of the evidence for the effect of relationship quality on social buffering or socially facilitated exploration stems from studies investigating bonds between mothers and offspring (e.g., Ainsworth et al., 1978; Edgar et al., 2015; Hennessy et al., 2006), between pair partners (e.g., Kaiser et al., 2003; Rukstalis and French, 2005; Sachser, Dürschlag and Hirzel, 1998), or between kin (e.g., Stöwe et al., 2006b; Wittig et al., 2008). However, some species form close bonds between individuals of the same sex, including humans (*Homo sapiens*) (Brent et al., 2014; Feldman, 2012; Hruschka, Hackman and Macfarlan,

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2015), baboons (*Papio cynosephalus*) (Nguyen et al., 2009; Palombit, Cheney and Seyfarth, 2001; Palombit, Seyfarth and Cheney, 1997), chimpanzees (*Pan troglodytes*) (Lehmann and Boesch, 2009; Massen and Koski, 2014; Mitani, 2009), lions (*Panthera leo*) (Packer et al., 1991), bottlenose dolphins (*Tursiops truncatus*) (Connor et al., 2000), and horses (*Equus caballus*) (Cameron, Setsaas and Linklater, 2009; Feh, 1999). However, little is known about whether closely bonded conspecifics of the same sex also provide social buffering and facilitate exploratory behaviour. Studying social buffering among closely bonded but unrelated animals of the same sex can help to gain a better understanding of whether such bonds provide benefits on a mechanistic level, thereby contributing to the proposed positive effects of social integration regarding long-term health (Snyder-Mackler et al., 2020).

- (2) Evidence for social buffering on group level is scarce. Evidence for social buffering effects has been primarily gathered from studies in dyadic experimental settings (e.g., Hodges et al., 2014; Kiyokawa et al., 2014a;b). However, group-living animals are mostly embedded in a bigger social unit. Therefore, their appraisal of a potentially stressful stimulus and their behavioural and physiological adaptations might depend not only on the presence of one strongly bonded conspecific but also on the presence of the animal's whole social group (Hennessy, Kaiser and Sachser, 2009). Some studies have found that buffering effects were greater in groups than in dyadic situations (Colnaghi et al., 2016; González et al., 2013; Hennessy, Kaiser and Sachser, 2009; Kiyokawa, Kawai and Takeuchi, 2018; Stanton, Patterson and Levine, 1985). Consequently, accounting for a species' social organisation in social buffering research is a biologically meaningful next step when studying social buffering in group-living species (Coe et al., 1982; Hennessy, Kaiser and Sachser, 2009). Studying social buffering effects in group-living animals on dyadic and group level makes it possible to assess how social buffering effects on a dyadic level relate to those received from an individual's whole social unit. Therefore, it can be determined which benefits, within the social buffering framework,

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individuals gain from forming bonds with specific members of their group (Hennessy, Kaiser and Sachser, 2009).

- (3) Social buffering and socially facilitated exploration are rarely studied together. Despite the proposed link between social buffering and socially facilitated exploration, and the relationship quality among the involved conspecifics (Ainsworth et al., 1978; Bowlby, 1969; Horn, Huber and Range, 2013; Ijichi et al., 2018; Moretti et al., 2015; Payne et al., 2016; Solomon et al., 2019), comparatively few studies assessed the effect of social bonds on the behavioural and physiological stress response and exploratory behaviour together. The studies that have assessed social buffering and socially facilitated exploration together have presented different findings. Terranova, Cirulli, and Laviola (1999) assessed the effects of conspecific familiarity on HPA-axis activity level and exploratory behaviour in rats (*Rattus norvegicus domesticus*). Despite finding a significant buffering effect provided by familiar rats, no significant difference in exploratory behaviour was found. Another study that has linked social buffering and exploration found the opposite effect when testing cichlid fish (*Oreochromis mossambicus*). The presence of familiar females resulted in an increase in exploration; however, not in an attenuation in hypothalamic-pituitary-interrenal axis activity, the structure in teleost fish corresponding to the HPA-axis (Galhardo, 2010; Galhardo, Vitorino and Oliveira, 2012). Studying bond-dependent buffering effects and bond-dependent facilitation of exploratory behaviour together can provide more insight into whether social bonds promote social buffering and enable the individual to engage in exploratory behaviour when exposed to novelty. This can help to determine which benefits individuals gain from social bond formations when adapting to novelty.

Domestic horses are an ideal study species to investigate social buffering effects and socially facilitated exploration. They are highly gregarious group-living animals known to form strong bonds between adult horses of the same sex (Seyfarth and Cheney, 2012). These bonds are long-lasting and can be maintained for several years (Feh and de Mazières, 1993; Linklater et al., 1999;

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Zeitler-Feicht, 2008). Studying horses makes it possible to investigate potential social buffering effects of closely bonded adults of the same sex and to integrate social buffering at group level.

As prey animals, horses have developed an adaptive fear and flight response and thus may respond to novel situations, objects, sounds, or smells with neophobia, a fear-related stress response (McGreevy, et al., 2009). Previous studies have shown that a horse's fear response can either depend on its personality (Lansade, Bouissou and Erhard, 2008b; Visser et al., 2002; Wolff, Hausberger and Le Sclan, 1997) or on its social context: The presence of habituated and calm or older conspecifics was found to decrease the horses' fear response when exposed to novelty (Christensen et al., 2008; Rørvang, Ahrendt and Christensen, 2015; Rørvang and Christensen, 2018). Besides their fear response, horses have been found to adjust their exploratory behaviour to their social context. Burke and Whishow (2020) showed that horses utilised familiar conspecifics as a 'secure base' from which to start their exploration of a novel arena. Furthermore, Mendonça et al. (2019) showed that horses explored more when a conspecific was present. However, based on the presented body of research, it remains to be shown whether the relationship quality or bond between horses affects social buffering or their exploratory behaviour when exposed to a novel stimulus. Investigating whether domestic horses benefit from close social bonds when presented with novelty can not only contribute to a better understanding of behavioural mechanisms but also be of relevance from an equine welfare perspective. Domestic horses are frequently exposed to novelty or stressful situations (Burla, Siegwart and Nawroth, 2018; Esch et al., 2019; Lundblad et al., 2020) during handling (Schmidt et al., 2010b;c), training (Fowler, Kennedy and Marlin, 2012; Munsters et al., 2013; Schmidt et al., 2010a), and husbandry routines (Yarnell, Hall and Billett, 2013). Insight into whether horses can benefit from their social context during stressful situations can inform husbandry, handling, and training routines. By facilitating an environment that reduces negative states, such as stress and fear, this work can contribute to equine welfare (Fraser, 2008), and enhance safety for humans and horses during different handling procedures (Drewek and Scofield, 2016; Hartmann, Christensen and Keeling, 2011).

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This study aimed at investigating social buffering effects on SAM-axis activity level and socially facilitated exploration in the context of novelty in domestic horses (*Equus caballus*). Thereby, it was assessed whether the presence of a closely bonded conspecific of the same sex alleviated the horses' behavioural and physiological stress response and whether this effect was stronger when the horses were with their whole social group. Furthermore, it was investigated whether the presence of a closely bonded conspecific and of the whole group facilitated exploratory behaviour. For this purpose, ten horses were exposed to a novel object in four different test conditions: alone, with a closely bonded conspecific of the same sex, with a less closely bonded conspecific, and within their whole group. As a measure for SAM-axis activity, the horses' heart rate was recorded throughout the novel object tests. This made it possible to measure the horses' immediate physiological response to the novel object presentation. Thereby, the following research questions were addressed:

1. Does the horses' behavioural and physiological stress response to a novel object exposure depend on their social context?

Hypothesis A: The presence of a closely bonded conspecific of the same sex buffers the focal horse's behavioural and physiological stress response.

Prediction A1: The horses' behavioural stress response following the object presentation will be less pronounced when in the presence of a closely bonded group member than when in the presence of a less closely bonded horse or when alone.

Prediction A2: Following the object presentation, horses will seek proximity to a closely bonded horse of the same sex.

Prediction A3: The horses' physiological stress response following the object presentation will be less pronounced (lower heart rate) when exposed to a novel object together with a closely bonded group member than with a less closely bonded horse or when alone.

Prediction A4: Proximity seeking will facilitate a calming effect and correspond to a lower heart rate.

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Hypothesis B: The presence of the whole group facilitates a stronger buffering effect than the presence of a closely bonded conspecific.

Prediction B1: The horses' behavioural stress response following the object presentation will be less pronounced when with their group than when alone or in a dyadic test condition.

Prediction B2: The horses' physiological stress response following the object presentation will be lower when with their group than when alone or in a dyadic test condition.

2. Does the exploratory behaviour that horses exhibit towards a novel object depend on their social context?

Hypothesis C: The presence of a closely bonded conspecific of the same sex facilitates exploratory behaviour.

Prediction C: Horses will show more exploratory behaviour when with a closely bonded conspecific than compared to when alone or when with a less closely bonded conspecific. This will be indicated by a shorter latency to approach the object, a higher frequency and a longer duration of investigation and exploration.

Hypothesis D: The group's presence has a stronger facilitatory effect on exploratory behaviour than the presence of a single conspecific.

Prediction D: Horses will show more exploratory behaviour when within their group than when alone or with only one other conspecific. This will be indicated by a shorter latency to approach the object, a higher frequency and a longer duration of investigation and exploration.

4.2 Material and Methods

Data collection took place between June and September 2018 in England and included the same two stable yards (Stable A and Stable B) as described in **Chapter 2**, section 2.2, page 39.

4.2.1 Study subjects and housing

At Stable A, the horse group comprised eleven horses, three mares and eight geldings (castrated stallions), aged 19.5 ± 4 (mean \pm SD). At Stable B the group comprised eight horses, two mares and six geldings who were 15 ± 8 (mean \pm SD) years of age. All horses were group-housed for the majority (66 – 100%) of the time, and the group composition has been established for at least a year before data collection commenced. Details about housing, feeding routines, and the horses' workload are provided in **Chapter 2**, section 2.2.1, page 39f.

4.2.2 Study sample and exclusion criteria

The novel object test was carried out with the same ten focal individuals and their assigned test partners as in the isolation and separation experiment presented in **Chapter 3**. The test partners were assigned as highly-preferred and less-preferred field companions based on the Preference Index (PI) (**Chapter 3**, section 3.2.2, page 86ff) and (ethical) exclusion criteria (**Chapter 3**, section 3.2.3, page 88).

4.2.3 Novel object tests

4.2.3.1 Study design

In a within-subject design (Martin and Bateson, 2009), all focal individuals ($n = 10$) were exposed to novel objects once in each of the four different conditions (Figure 4.1):

- a) alone (A)
- b) with a highly-preferred field companion (HP) – high PI
- c) with a less-preferred field companion (LP) – low PI
- d) together with all horses of its group (G).

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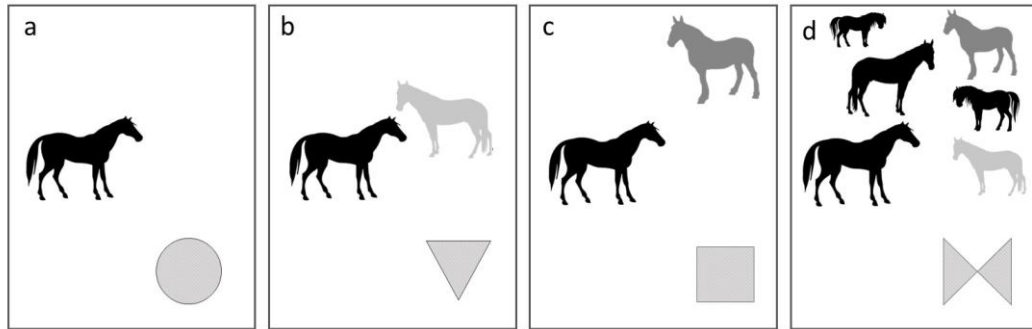


Figure 4.1 Experimental setup novel object tests. The novel object exposure was carried out in four different test conditions: a) alone, b) with a highly-preferred horse, c) with a less-preferred horse, and d) within the group. The order of social test conditions and the novel objects were pseudo-randomly assigned for each focal horse. Different objects were chosen across trials so that horses were exposed to each object only once (horse silhouettes original and adapted from <http://phylopic.org/>).

The order of the test conditions was pseudo-randomised for each focal horse, as aligned with husbandry routines on site. In the group condition, all focal horses of a stable were tested together and within their whole group in one trial. For each trial, a novel object was pseudo-randomly preselected from various inflatable toys, strings with attached bags, balloons, and bands that were similar in size for the alone or dyadic conditions. In the group condition, the horses were exposed to a white tarpaulin (Table 4.1).

Table 4.1 Novel objects descriptions. Description of the objects that were used for the novel object tests, including their form, dimensions, and colours.

Object	Form and dimensions	Colour
Paddling pool	round diameter: 1.2 m height: 35 cm	white ground and yellow sides
Swimming ring	ring diameter: 1.1 m height: 20 cm	yellow
Surfboard	flat length: 1.2 m height 20 cm	blue and white
Inflatable goal	conical and three dimensional diameter: 0.7 m height: 0.8 m	orange and green
Bags with bands	flat and movable length: 1.2 m height 15 cm	black bags and red and white bands
Tarpaulin	dimension: 4 x 4 meters	white

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To prevent habituation (Martin and Bateson, 2009), all focal horses and their test partners were exposed to each object only once. Each focal horse underwent four trials. If they were assigned as a test partner for another member of the herd, they were only subjected to one additional trial but not more, again with the purpose to prevent habituation.

4.2.3.2 Test procedure

The horses that were subject to the novel object test were mounted with Polar® Equine V800 mobile heart rate monitors (Polar Electro Oy, Kempele, FI). This heart rate monitor detected the time intervals between consecutive heartbeats. Details about the mounting procedure and positioning of the monitor, as well as the habituation procedure, are given in **Chapter 2**, section 2.2.3, page 44f. In the alone and dyadic (highly-preferred, less-preferred) test conditions, the focal horses and their test partners were mounted with the heart rate monitor. In the group condition, only the focal horses were equipped with monitors.

After the heart rate monitor was mounted, the horses were brought to the test area. For the alone and dyadic tests, this was a fenced arena of approximately 50m² in size (pictures and details are provided in **Chapter 3**, section 3.2.4.2, page 90f). The horses were familiar with the test arenas, as they were part of their usual stabling environment. Furthermore, they were habituated to spending time in the arena alone or with their highly-preferred or less-preferred conspecifics after the experimental series presented in **Chapter 3**.

For the group test, the horses were brought back to their home field (field sizes: Stable A: 9 acres, Stable B: 11 acres). Horses were released and allowed to move freely. In all cases, horses were provided with *ad libitum* access to fresh water, hay, and grass to reflect the natural living conditions of a grazing species (Bulens et al., 2015).

The experiment started one minute after the experimenter (D.V.H) had left the test area and taken position outside the fence. Ten minutes after the onset of the experiment, the novel object was presented. In the alone and dyadic conditions, the object was dropped across the fence into the arena. In the group condition, the object was dropped on the floor around eight meters from the horse group's periphery. After dropping the object, the experimenter moved back for

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around eight meters, while holding on to a string tied to the object and crouched down. To not influence the horses' behaviour, she lowered my gaze, and neither looked at the object nor the horses.

The experiment itself comprised of different events and phases. As horses' vision is particularly sensitive for detecting movement (Saslow, 2002) and can respond more strongly when they are exposed to a moving object (Malmkvist et al., 2012), the object was moved in two sequences during the experiment following the procedure from Christensen et al. (2008): three minutes after the initial object presentation (Drop 1), the object was pulled up the fencing via the attached string (Move 1). The object was held on the fencing's top edge (approximately chest height of the horses) for another three minutes and was then dropped to the floor (Drop 2). The moves were implemented in a slightly different way for the group trial. Instead of moving the object up a fence and dropping it, it was moved twice (Move 1 and Move 2) on the ground for approximately four meters. Ten minutes after Drop 2/Move 2 the object was removed. The experiment ended ten minutes thereafter (Figure 4.2). To ensure the same timing across trials, a stopwatch was used.

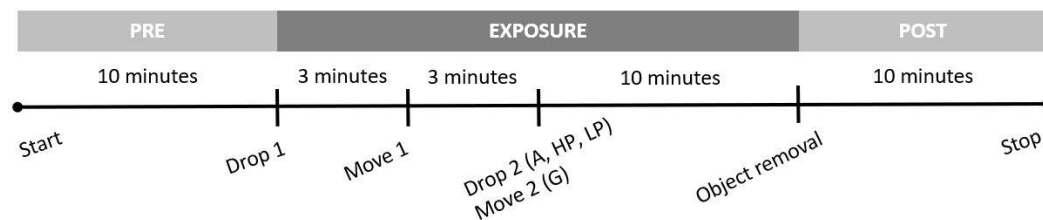


Figure 4.2 Timeline and events of the novel object exposure. Ten minutes after the start of the experiment, the object was presented (Drop 1). After three minutes the object was moved for the first time (Move 1) and after another three minutes for the second time: in the alone (A) and dyadic conditions (HP, LP), this was a second drop from the fence line (Drop 2). In the group (G) condition, this was a second move on the ground (Move 2). Ten minutes later, the object was removed. The experiment stopped after another ten minutes.

To monitor the horses' behaviour during individual and dyadic experiments a Canon® LEGRIA HF R56 camera was used. During the group experiment an additional Apeman® wide-angle camera was used to capture the experiment from a larger angle.

4.2.4 Data processing

4.2.4.1 Behaviour

Behaviour was coded using the Solomon® Coder version beta 16.06.26 (© András Péter, <https://solomon.andraspeter.com>). Thereby, the coder (D.V.H) was not blind to the test conditions. Mitigations that were put in place are described in **Chapter 2**, section 2.2.5.1, page 46f. The behavioural responses upon the presentation and movement of the object, horses' locomotion, the spatial distance between the focal horse and its test partner, and investigation and exploration of the object were coded. The behavioural categories are listed in the ethogram in Table 4.2. Thereafter, the behavioural data were transformed into rates and durations per minute to account for the different lengths of phases in the experiment (Martin and Bateson, 2009).

To assess whether the test condition affected the horses' behavioural reaction to the novel object, their immediate behavioural responses to the object following each of the events in the experiment (Drop 1, Move 1, Drop 2/Move 2) were assigned to the following categories: stress response (including alert, startle, back, and flight), explore, head up, and no response. The behaviours are described in the ethogram in Table 4.2.

To assess whether the horses' spatial proximity during the object test was related to their social preference (HP, LP), the distance between the two horses in the dyadic test conditions was measured throughout the dyadic experiments (i.e., the distance between two horses' heads was measured in horse-lengths). The spatial proximity was not assessed in the group trials, as due to the size of the field (depth of view) and the combination of a normal and wide-angle camera, the proximity assessment can become unreliable.

To investigate whether the horses' exploratory behaviour was affected by their social context, the following variables were recorded (Table 4.2):

- 1) The latency to approach was obtained by measuring the time in seconds from the initial object presentation (Drop 1) until the horse first approached the object resulting in a stop to investigate or explore.
- 2) The frequency and duration of investigatory behaviour (no physical contact with the object).

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- 3) The frequency and duration of exploration (physical contact with the object).

If investigation or exploration was paused for at least five seconds, or interrupted by another behaviour such as grazing, scratching, looking away, or a social interaction, it was recorded as another bout. To account for the different lengths of the experimental phases, behavioural events were transformed into rates per minute and the duration of behaviour were transformed into seconds per minute.

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Table 4.2 Ethogram for the behavioural coding during the novel object tests. Descriptions of the recorded behaviours during the novel object exposure. The record column indicates whether a behaviour was coded as an event (e) or duration (d).

Behavioural Category	Behaviour	Record	Definition	Source
Immediate behavioural response to the object	No response	e	The horse does not react to the test stimulus and continues its current activity.	(Rørvang and Christensen, 2018)
	Head up	e	The horse raises its head from the grass or hay; chewing may be interrupted. Horse remains in an overall relaxed posture and does not move away from its position.	(Rørvang and Christensen, 2018)
	Explore	e	Immediate approach to investigate or explore.	
	Alert	e	The horse stands vigilant with elevated neck, head and ears oriented towards the object, chewing is interrupted, and the horse might move up to two steps away from its former position.	(Rørvang and Christensen, 2018)
	Startle	e	Horse shudders suddenly and quickly after the object presentation.	(Lansade, Bouissou and Erhard, 2008b)
	Back	e	The horse shows alertness and moves more than two steps backwards or sideways from its previous position.	(Rørvang and Christensen, 2018)
	Flight	e	The horse turns/jumps away from its position, followed by trotting or cantering.	(Rørvang and Christensen, 2018)
Vocalisation	Snort	e	Powerful exhalation from nostrils.	(Malmkvist et al., 2012)
Exploratory behaviour	Investigation	d + e	Horse stops (longer than 3 seconds) close to the object, eventually lowers its head towards the object but does not sniff or touch it.	(Malmkvist et al., 2012)
	Exploration	d + e	Sniffing and/or touching and/or mouthing the object.	(Malmkvist et al., 2012)
Locomotion	Standing	d	Horse stands or stand rests with a bent hind leg.	(Malmkvist et al., 2012)
	Walking	d	The horse moves at a slow four-beat pace, moving a front leg of a side forward, then the hindquarters of the same side and then moving the front leg of the other side, followed by the forward motion of the hind leg of the other side.	(Zeitler-Feicht, 2008)
	Trotting	d	The horse moves at a faster two-beat pace, moving the diagonal front and hind leg forward at the same time, followed by a short suspension phase before the other diagonal limbs are moved forward.	(Zeitler-Feicht, 2008)
	Cantering	d	The horse moves at a fast three-beat pace. In the first beat, the horse bares all its weight on a hind leg. In the second beat, the other hind leg and its respective diagonal foreleg take over the weight, which is then, in a third beat, shifted to the remaining foreleg. After that, the horse pushes off the ground into a suspension phase before the circle starts again.	(Malmkvist et al., 2012)
Other behaviour	Grazing	d	Horse feeds from hay or grass.	(Bulens et al., 2015)
	Drinking	d	Horse drinks from the water bucket.	(Bulens et al., 2015)
	Urinating	d	Elimination of urine.	(McDonnell and Haviland, 1995)
	Defecating	d	Elimination of faeces.	(Malmkvist et al., 2012)

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4.2.4.2 Heart rate

The processing and error correction of heart rate data followed the same procedure described in **Chapter 2**, page 2.2.5.2, page 49. In four cases heart rate data had to be discarded as the monitor stopped recording (BLL and BLY group condition) or accumulated too many erroneous values (BLY (HP): 20% erroneous values, CHA (A): 100% erroneous values). Thus, a total of 10.5% of heart rate data could not be used for the analysis.

To assess the overall effect of the novel object exposure on cardiac activity, the mean heart rate was calculated for the different phases of the experiment (Figure 4.3):

Pre:	ten minutes proceeding the initial object exposure.
Phase 1:	from the initial exposure (Drop 1) to the first move (Move 1) of the object.
Phase 2:	from Move 1 to the drop/second move (Drop 2/Move 2).
Phase 3:	from the Drop 2/Move 2 until the removal of the object.
Post:	from the removal to ten minutes after.

To assess whether the novel object exposure resulted in a change of the SAM-axis activity, mean and maximum heart rates were calculated for ten-second timeframes before and after the events of the experiment (Figure 4.3):

Baseline:	five minutes before the object exposure.
Drop 1:	initial object presentation.
Move:	moving the object.
Drop 2/Move2:	dropping the object (alone and dyadic) or second move (group).
Remove:	the object was taken out.
End:	five minutes after the object was removed.

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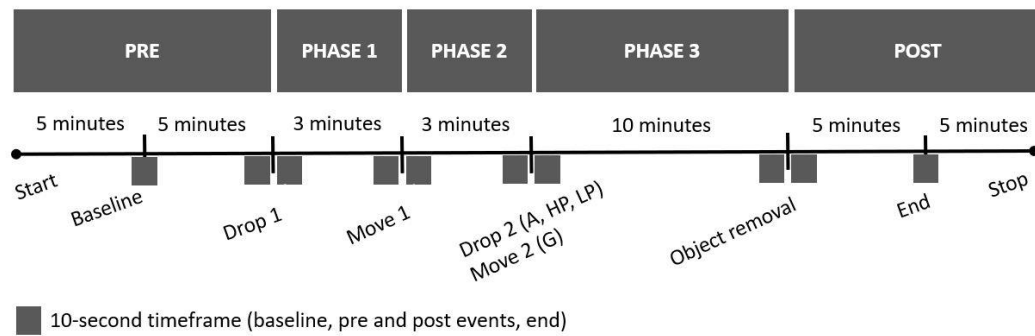


Figure 4.3 Timeframes for the calculation of mean and maximum heart rate. The dark bars alongside the experimental timeline indicate the phases and events for which heart rate variables were calculated.

The ten-second timeframes were chosen following several other studies (Briefer et al., 2015; Reefmann, Wechsler and Gygax, 2009; Safryghin, Hebesberger and Wascher, 2019; Wascher, Scheiber and Kotrschal, 2008) and present a trade-off between capturing the immediate sympathetic response to the events in the experiment and preventing subsequent behaviour from influencing the heart rate measures.

4.2.5 Statistical analysis

Analyses were carried out using R (Version 3.5.1) (R Core Team, 2019) in RStudio (Version 1.1.463). Normality tests were carried out using the Shapiro-Wilk tests. Parametric and non-parametric tests were applied, depending on data distribution.

Pearson's Chi-squared tests were carried out to assess whether the immediate behavioural responses (stress response, explore, head up, no response) differed between the events of the object test (Drop 1, Move 1, Drop 2/Move 2). Post hoc analyses were carried out applying the Bonferroni adjustment using the package 'chisq.posthoc.test' (Ebbert, 2019).

To assess whether the initial behavioural response (following Drop 1) was depending on the test condition, generalised linear mixed-effects models with a logit link function (Hawkins, 2014) were run, using 'glmer' in the 'lme4' package (Bates, Maechler, and Walker, 2015). Each of the behavioural categories (stress response, explore, head up, no response) was transformed into a binary variable, indicating whether the behaviour was shown (1) or not (0), and set as a response variable: Model 1: stress response, Model 2: exploration, Model 3: head up, and Model 4: no response. The test condition (alone, high preference, low preference,

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group) were set as fixed effect. Individual IDs and object type were set as random effects, to account for repeated measures and thus interdependencies within the data set (Harrison et al., 2018; Zuur and Ieno, 2016; Table 4.3). As indicators for the goodness of fit p -values and χ^2 -values (Type III Wald chi square) were obtained using the 'Anova' function in the package 'car' (Fox and Weisberg, 2019). To assess the variation explained by the model the R^2 -values for the models were obtained with the 'r2' function in the package 'performance' (Lüdtke et al. 2020) following Nakagawa, Johnson and Schielzeth (2017).

Table 4.3 Components of the generalised linear mixed-effects models with logit link function.

	Response variables	Fixed effect	Random effects
Model 1	Stress response	Test condition (alone, high preference, low preference, group)	Individual ID, Object
Model 2	Exploration		
Model 3	Head up		
Model 4	No response		

To compare spatial distances between highly-preferred and less-preferred dyads, two sample t -tests were carried out for the whole duration of the experiment. Additionally, Wilcoxon rank-sum tests were carried out to assess differences in spatial proximity for the single experimental phases (Pre, Phase 1, Phase 2, Phase 3, Post). To assess whether proximity seeking corresponded to a lower heart rate, the horses' mean heart rate during the experimental phases was compared between the two dyadic test conditions using Wilcoxon signed-rank tests.

To assess the effect of the novel object tests on cardiac activity, the mean and the maximum heart rate for the ten-second intervals prior and after the initial object presentation (Drop 1) were averaged for all horses across all test conditions and compared using paired t -tests or Wilcoxon signed-rank tests. To assess differences in mean heart rate across experimental phases, one-way repeated measures ANOVAs were conducted using the 'ez' package (Lawrence, 2016).

To assess the effect of the test condition, the averaged mean and maximum heart rate of the 10-second timeframes following the experimental events (Drop 1, Move 1, Drop 2/Move 2) and the averaged mean heart rate for the experimental phases (Phase 1, 2, 3) across the four test conditions were compared using Friedman tests in the 'pgirmess' package (Giraudeau, 2018).

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Post hoc tests were carried out using Nemenyi multiple comparisons in the 'PMCMR' package (Pohlert, 2018).

To assess the effect of the social context on exploratory behaviour, including the latency to approach and the rates and durations of investigation and exploration, Friedman tests and Nemenyi multiple comparisons were conducted. To assess the influence of trial number on heart rate and exploratory behaviour, Friedman tests were applied.

The alpha-level was set at $p = 0.05$, and p -values are reported two-tailed. Effect sizes were calculated for significant results based on the z -statistic (Friedman tests and Wilcoxon signed-rank tests) using the following equation: $r = z/\sqrt{n}$ (Rosenthal, 1991). For t -tests effect sizes were calculated based on the t -statistic using the following equation: $r = \sqrt{t^2/(t^2 + df)}$ (Rosenthal, 1991).

4.2.6 Ethical statement

The experimental procedure was approved by the Departmental Research Ethics Panel under the terms of Anglia Ruskin University's Research Ethics Policy (reference number: A & EB DREP 17-029 and 17-053) and followed the ethics guidelines for the study of animal behaviour provided by the Association for the Study of Animal Behaviour (ASAB, 2020). All applied methods were non-invasive. Based on ethical considerations (exclusion criteria are described in **Chapter 3**, section 3.2.3, page 88), three horses were excluded from the study. Consent for all procedures was sought from horse and stable owners.

4.3 Results

4.3.1 Behavioural stress response

4.3.1.1 Immediate behavioural response to the object exposure

The immediate behavioural response, that is the first behaviour the horse showed upon each of the experimental events (Drop 1, Move 1, Drop 2/Move 2), was the strongest following the initial object presentation (Drop 1). Immediate exploration occurred most often after Drop 1 and was significantly less frequent following the other experimental events (Table 4.4). 'No response' occurred significantly more often after Drop 2 (Pearson's Chi-squared tests: $n = 48$, Drop 1-Move 1: $X^2_3 = 8.851$, $p = 0.031$, Stress response: $p = 1$, Head up: $p = 1$, Explore: $p = 0.058$, No response: $p = 0.53$; Move 1-Drop 2: $X^2_3 = 16.627$, $p < 0.001$, Stress response: $p = 1$, Head up: $p = 1$, Explore: $p = 0.016$, No response: $p = 0.005$; Move 2-Drop 2: Stress response $p = 1$, Head up: $p = 1$, Explore: $p = 1$, No response: $p = 0.798$). Consequently, the analysis regarding the effect of the horses' social context on their immediate behavioural responses was focused on the behaviour following Drop 1. The horses' immediate behavioural responses after Drop 1 were not dependent on their social context. Model outputs are summarised in the Tables 4.5 – 4.8.

Table 4.4 Immediate behavioural responses. Behavioural responses following the experimental events (Drop 1, Move 1, Drop 2) of all focal horses and test partners ($n = 12$) for all test conditions.

Behavioural Response	Drop 1	Move 1	Drop 2
Fear response	11	10	8
Explore	11	2	1
Head up	17	19	14
No response	9	17	25

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Table 4.5 Model 1 – Stress response: Output from the generalised linear mixed-effects model with logit link function testing the relationship between the horses' immediate behavioural response and the test condition (alone, less-preferred (LP), highly-preferred (HP), group). Binary response variable: behavioural stress response (yes, no) upon initial object presentation (Drop 1). Fixed effect: social test condition. Random effects: Individual ID (Name) and object. Par (parameter), Est (estimate), SE (standard error), *z* (z-statistic), *df* (degrees of freedom), *F* (*F*-statistic), *p* (p-value), χ^2 (Wald III), Var (variance), SD (standard deviation).

Effects	Model 1	Par	Est	SE	<i>z</i>	df	<i>F</i>	<i>p</i>	<i>X</i> ²	
Fixed	Condition	Intercept (Alone)	-0.98	1.20	-1.65	3	1.46	0.10	0.33	
		Group	-1.03	1.49	-0.68			0.49		
		LP	-1.03	1.49	-0.68			0.49		
		HP	1.37	1.26	1.09			0.27		
									Var	SD
Random	Name								2.29	1.52
	Object								0.00	0.00

Table 4.6 Model 2 – Exploration: Output from the generalised linear mixed-effects model with logit link function testing the relationship between the horses' immediate behavioural response and the test condition (alone, less-preferred (LP), highly-preferred (HP), group). Binary response variable: exploration (yes, no) upon initial object presentation (Drop 1). Fixed effect: social test condition. Random effects: Individual ID (Name) and object. Par (parameter), Est (estimate), SE (standard error), *z* (z-statistic), *df* (degrees of freedom), *F* (*F*-statistic), *p* (p-value), χ^2 (Wald III), Var (variance), SD (standard deviation).

Effects	Model 2	Par	Est	SE	z	df	F	p	χ²	
Fixed	Condition	Intercept (Alone)	-0.89	0.74	-1.21	3	0.84	0.23	0.47	
		Group	0.48	0.97	0.48			0.63		
		LP	-1.39	1.29	-1.09			0.28		
		HP	-0.56	1.07	-0.53			0.60		
									Var	SD
Random	Name								0.25	0.50
	Object								0.00	0.00

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Table 4.7 Model 3 – Head up: Output from the generalised linear mixed-effects model with logit link function testing the relationship between the horses' immediate behavioural response and the test condition (alone, less-preferred (LP), highly-preferred (HP), group). Binary response variable: head up (yes, no) upon initial object presentation (Drop 1). Fixed effect: social test condition. Random effects: Individual ID (Name) and object. Par (parameter), Est (estimate), SE (standard error), *z* (z-statistic), *df* (degrees of freedom), *F* (*F*-statistic), *p* (p-value), χ^2 (Wald III), Var (variance), SD (standard deviation).

Effects	Model 3	Par	Est	SE	<i>z</i>	df	<i>F</i>	<i>p</i>	<i>X</i> ²	
Fixed	Condition	Intercept (Alone)	-0.41	0.65	-0.63	3	1.16	0.53	0.34	
		Group	-0.98	1.03	-0.95			0.34		
		LP	0.81	0.92	0.89			0.38		
		HP	-0.44	0.96	-0.47			0.64		
									Var	SD
Random	Name								0.00	0.00
	Object								0.01	0.09

Table 4.8 Model 4 – No response: Output from the generalised linear mixed-effects model with logit link function testing the relationship between the horses' immediate behavioural response and the test condition (alone, less-preferred (LP), highly-preferred (HP), group). Binary response variable: no response (yes, no) upon initial object presentation (Drop 1). Fixed effect: social test condition. Random effects: Individual ID (Name) and object. Par (parameter), Est (estimate), SE (standard error), *z* (z-statistic), *df* (degrees of freedom), *F* (*F*-statistic), *p* (p-value), χ^2 (Wald III), Var (variance), SD (standard deviation).

Effects	Model 4	Par	Est	SE	<i>z</i>	df	<i>F</i>	<i>p</i>	χ^2	
Fixed	Condition	Intercept (Alone)	-2.31	1.17	-1.98	3	0.61	0.04	0.62	
		Group	1.41	1.30	1.08			0.28		
		LP	0.84	1.34	0.62			0.53		
		HP	0.00	1.51	0.00			1.00		
									Var	SD
Random	Name								0.29	0.53
	Object								0.00	0.00

4.3.1.2 Proximity

Taken for the whole duration of the dyadic experiments, the mean distance between highly-preferred dyads was 2.06 ± 0.71 horse-lengths (mean \pm SD) and between less-preferred dyads 2.34 ± 0.62 horse-lengths; this difference was not significant (Two sample *t*-test: $n_{HP} = 6$, $n_{LP} = 7$, $t = 0.70$, $df = 8.09$, $p = 0.501$). However, analysed for the single phases of the experiment (Phase 1, Phase 2, Phase 3), highly-preferred dyads were closer (1.36 ± 0.18) during Phase 1, i.e., following Drop 1, than less-preferred dyads (2.09 ± 1.15) (Wilcoxon rank-sum test: $n_{HP} = 6$, $n_{LP} = 7$, $W = 1$, $p = 0.002$, $r = -0.266$; Figure 4.4).

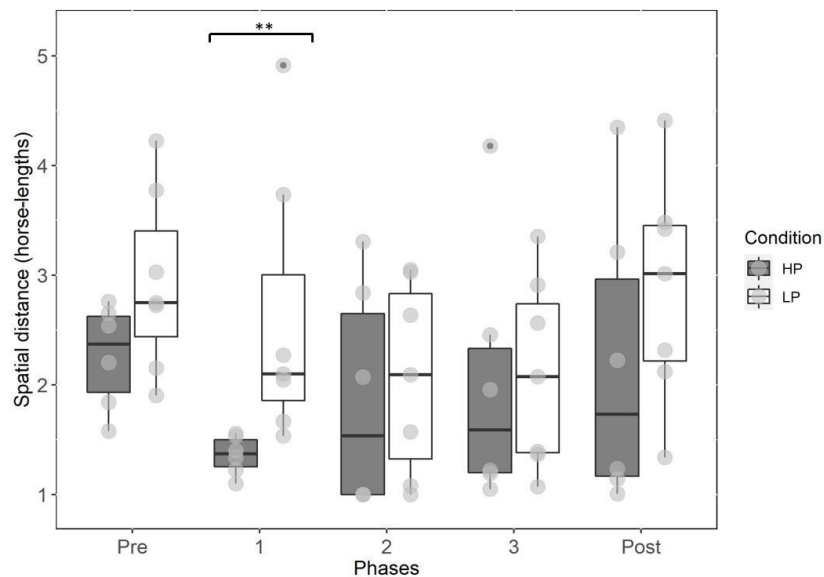


Figure 4.4 Spatial proximity across experimental phases. Tukey whiskers plot for spatial proximity (in horse-lengths) for the different phases of the experiment. The bold horizontal line indicates the group median, the upper and lower hinge of the boxplot correspond to the 25 and 75 percentiles, comprising the interquartile range (IQR). The outwards pointing whiskers extend to the smallest and largest value at most $1.5 \times \text{IQR}$ from the hinge. Data points smaller or greater are indicated as single dots. In Phase 1, following the initial object presentation, highly-preferred (HP) dyads (grey) were significantly (nearly a full horse-length) closer than less-preferred (LP) dyads (white). Asterisks: *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

4.3.2 Physiological stress response

4.3.2.1 Impact of the novel object test on cardiac activity

Comparing the mean and maximum heart rate averaged for each focal horse and its test partner across all trials, the analysis showed that heart rate was significantly higher after Drop 1 (mean heart rate: paired t -test: $n = 12$, $t = -3.48$, $df = 11$, $p = 0.005$, $r = 0.724$; maximum heart rate: Wilcoxon signed-rank test: $n = 12$, $V = 5$, $p = 0.004$, $r = -0.629$; Figure 4.5). Thereby, the mean heart rate increased about 11%; from 47.97 ± 5.96 (mean \pm SD) beats per minute (bpm) to 53.26 ± 7.71 bpm. The maximum heart rate increased about 8.7%; from 51.90 ± 5.59 bpm to 56.40 ± 9.78 bpm.

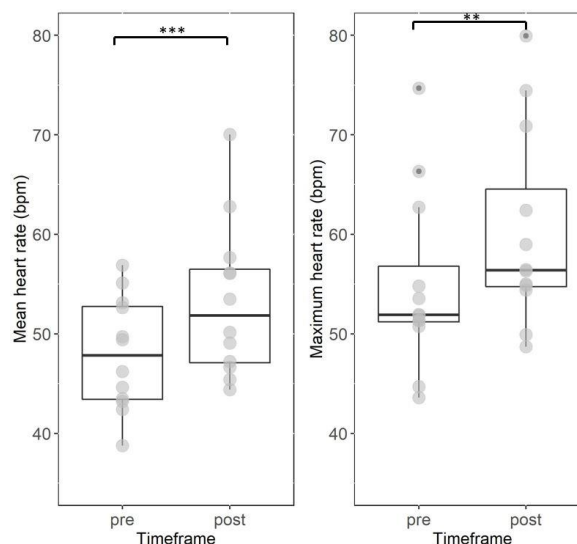


Figure 4.5 Cardiac response to the novel object presentation. Tukey whiskers plot showing averaged mean (left) and maximum (right) heart rates in beats per minutes (bpm) for all horses ten-second timeframe prior (pre) and after (post) the initial object presentation. The bold horizontal line indicates the group median, the upper and lower hinge of the boxplot correspond to the 25 and 75 percentiles, comprising the interquartile range (IQR). The outwards pointing whiskers extend to the smallest and largest value at most $1.5 \times$ IQR from the hinge. Data points smaller or greater are indicated as single dots. Mean and maximum heart rate were significantly higher after the object presentation. Asterisks: *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

The trial number had no effect on the horses' mean and maximum heart rate following Drop 1 (Friedman tests: $n = 11$; mean heart rate: $X^2 = 2.67$, $df = 2$, $p = 0.27$; maximum heart rate: $X^2 = 2.89$, $df = 2$, $p = 0.242$; Table 4.9).

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Table 4.9 Mean and maximum heart rate across trials. As data were not normally distributed the median (\tilde{x}), maximum (Max), minimum (Min), and interquartile range (IQR) are presented. Data for trial 4 are shown, however, they were not included in the analysis due to the small number of horses undergoing an additional trial in the role of a test partner for another focal horse.

	Mean heart rate				Maximum heart rate			
	\tilde{x}	Max	Min	IQR	\tilde{x}	Max	Min	IQR
Trial 1	48.63	91.00	40.81	11.88	52.22	103.81	42.40	11.46
Trial 2	50.69	121.69	39.68	19.76	54.40	129.31	41.07	20.10
Trial 3	48.44	68.37	38.97	10.08	52.59	72.73	40.49	11.78
Trial 4	46.07	63.11	44.34	18.68	52.91	69.93	52.77	17.16

The physiological response to the events (Baseline, Drop 1, Move 1, Drop/Move 2, Remove, Post) of the novel object test differed significantly (Friedman tests: $n = 12$, mean heart rate: $X^2 = 32.56$, $df = 5$, $p < 0.001$; maximum heart rate: $X^2 = 30.09$, $df = 5$, $p < 0.001$). Thereby, the strongest physiological response occurred following Drop 1 (Nemenyi multiple comparison tests: mean heart rate: Baseline-Drop 1: $p < 0.001$, $r = -0.623$; Drop 1-Post: $p < 0.001$, $r = -0.676$; Move 1-Post: $p = 0.03$, $r = -0.658$; Drop 2-Post: $p = 0.004$, $r = -0.676$; maximum heart rate: Baseline-Drop 1: $p = 0.006$, $r = -0.711$; Drop 1-Post: $p < 0.001$, $r = -0.780$; Drop 2- Post: $p = 0.003$, $r = -0.737$; Table 4.10; Figure 4.6).

Table 4.10 Full test results mean and maximum heart rate per event. Based on the Nemenyi multiple comparisons tests, the p -values are shown for the comparison between the different events of the novel object tests. Significant results are marked in bold.

		Baseline	Drop 1	Move	Drop 2	Remove
Mean heart rate	Drop 1	< 0.001	–	–	–	–
	Move	0.301	0.363	–	–	–
	Drop 2	0.093	0.716	0.994	–	–
	Remove	0.716	0.093	0.987	0.837	–
	Post	0.924	< 0.001	0.027	0.004	0.156
Maximum heart rate	Drop 1	0.006	–	–	–	–
	Move	0.924	0.121	–	–	–
	Drop 2	0.246	0.780	0.837	–	–
	Remove	0.974	0.070	1.000	0.716	–
	Post	0.646	< 0.001	0.121	0.003	0.198

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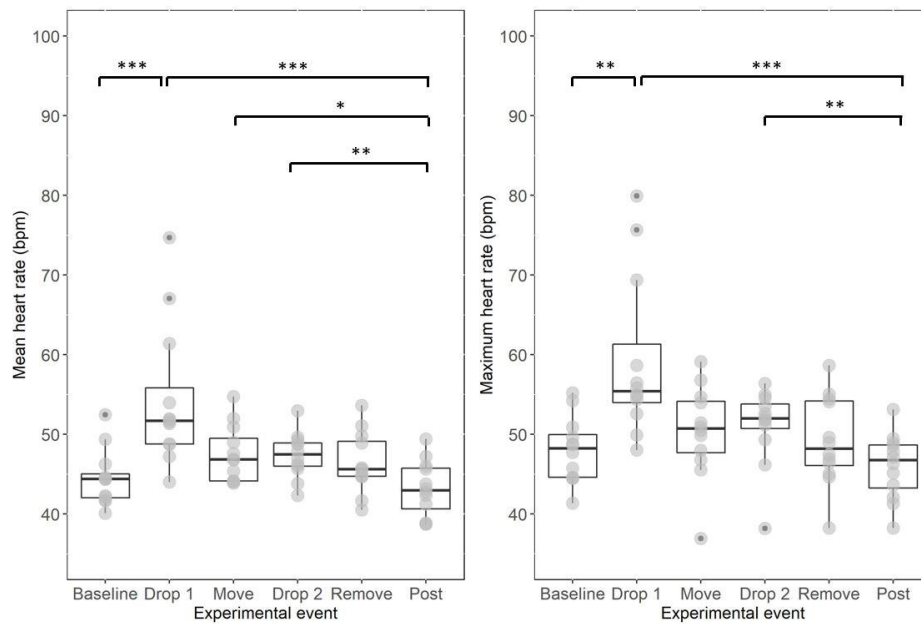


Figure 4.6 Heart rate and experimental events. Tukey whiskers plots for averaged mean and maximum heart rate in beats per minute (bpm) for all horses across all trials shown for the ten seconds after each event. The bold horizontal line indicates the median, the upper and lower hinge of the boxplot correspond to the 25 and 75 percentiles, comprising the interquartile range (IQR). The outwards pointing whiskers extend to the smallest and largest value at most $1.5 \times \text{IQR}$ from the hinge. Data points smaller or greater are indicated as single dots. The mean heart rate after Drop 1 was significantly higher than during the Baseline, and Post. Moreover, the mean heart rate Post was significantly lower than after the Move and Drop 2. The maximum heart rate was significantly higher following Drop 1 than during Baseline and Post. Moreover, the maximum heart rate Post was significantly lower than after Drop 2. Asterisks: *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

Additionally, the averaged mean heart rate for all horses and trials was compared between the experiment's phases (Pre, Phase 1, Phase 2, Phase 3, and Post). The mean heart rate differed significantly (One-way repeated measures ANOVA: $n = 12$, $F_{4,44} = 13.00$, $p_{(\text{HFe})} < 0.001$). Post hoc analysis revealed a significantly higher heart rate in Phase 1 (following Drop 1) than Post (Post hoc pairwise t -test, Bonferroni adjustment: $p = 0.048$, $r = 0.833$; full test results Table 4.11; Figure 4.7).

Table 4.11 Full test results mean heart rate per phase. Based on the ANOVA post hoc analysis (pairwise t -test with Bonferroni adjustment), the p -values are shown for the comparisons between the different phases of the novel object tests. Significant results are marked in bold.

	Pre	Phase 1	Phase 2	Phase 3
Phase 1	0.294	–	–	–
Phase 2	1	0.158	–	–
Phase 3	1	0.205	1	–
Post	1	0.048	1	1

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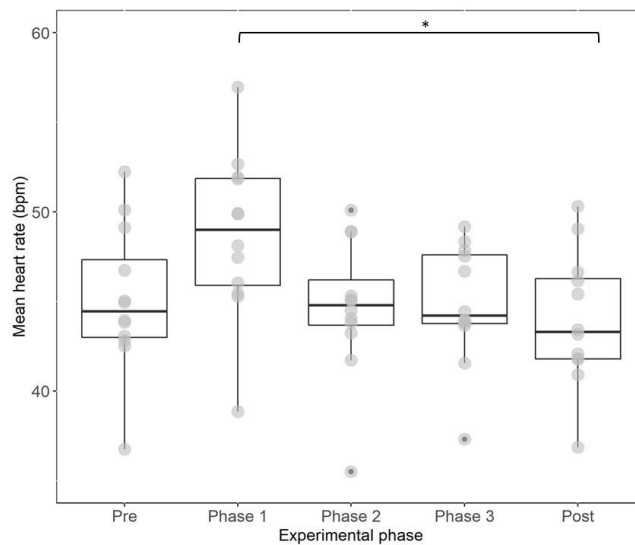


Figure 4.7 Heart rate and phases of the experiment. Tukey whiskers plot for mean heart rates in beats per minute (bpm) averaged for all horses across all trials compared between the phases of the experiment. The bold horizontal line indicates the median, the upper and lower hinge of the boxplot correspond to the 25 and 75 percentiles, comprising the interquartile range (IQR). The outwards pointing whiskers extend to the smallest and largest value at most $1.5 * \text{IQR}$ from the hinge. Data points smaller or greater are indicated as single dots. The mean heart rate was significantly higher following Drop 1 than Post experiment. Asterisks: *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

Based on these findings, the effect of the horses' social context on their heart rate in response to the events of the object tests was only assessed regarding the initial object presentation (Drop 1). Additionally, the effect of social context was assessed regarding the horses' heart rate during the phases of the experiment.

4.3.2.2 Cardiac activity and social context

The mean and maximum heart rate calculated for the 10-second period following Drop 1 did not differ between the test conditions (Friedman tests: $n = 8$, mean heart rate: $X^2 = 4$, $df = 2$, $p = 0.145$; maximum heart rate: $X^2 = 1.75$, $df = 2$, $p = 0.427$; Figure 4.8).

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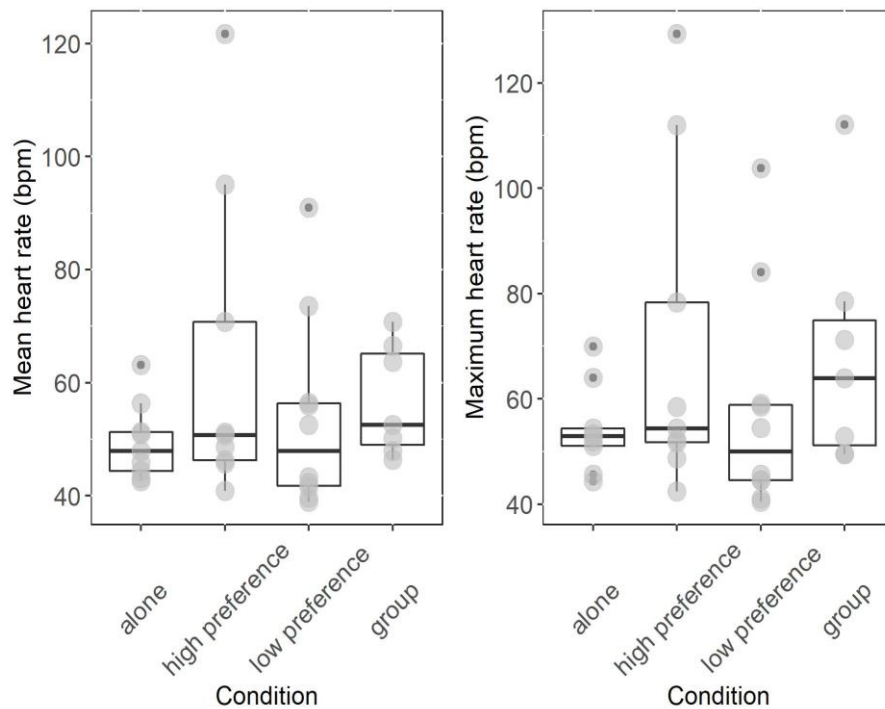


Figure 4.8 Cardiac response following Drop 1 per test condition. Tukey whiskers plots for the mean (left) and maximum (right) heart rate in beats per minute (bpm) calculated for the ten-second period after Drop 1. The bold horizontal line indicates the median, the upper and lower hinge of the boxplot correspond to the 25 and 75 percentiles, comprising the interquartile range (IQR). The outwards pointing whiskers extend to the smallest and largest value at most $1.5 * \text{IQR}$ from the hinge. Data points smaller or greater are indicated as single dots. There was no significant difference in heart rate between the four test conditions.

Moreover, mean heart rate during the experimental phases, that is, during the phases between the experimental events (Phase 1: Drop 1 – Move 1; Phase 2: Move 1 – Drop/Move2; Phase 3: Drop/Move 2 – Remove), did not differ between the social test conditions (Friedman tests, $n = 7$: Phase 1: $X^2 = 4.543$, $df = 3$, $p = 0.209$; Phase 2: $X^2 = 2.486$, $df = 3$, $p = 0.478$; Phase 3: $X^2 = 2.314$, $df = 3$, $p = 0.510$; Figure 4.9).

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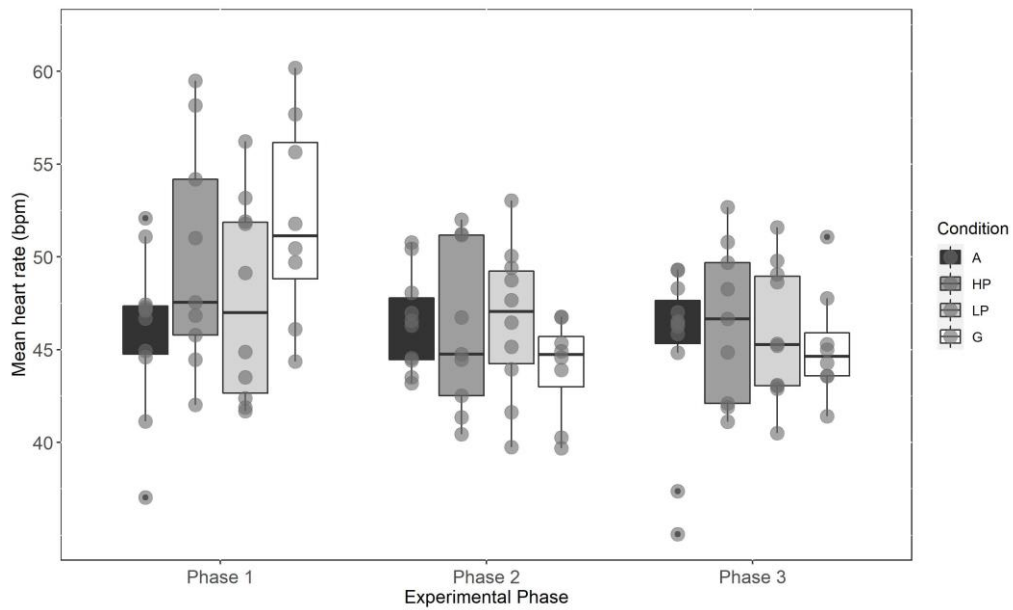


Figure 4.9 Mean heart rate for the experimental phases and test conditions. Tukey whiskers plots for mean rate in beats per minute (bpm) shown for the three phases of the object exposure and grouped per test condition alone (A, black), highly-preferred (HP, dark grey), less-preferred (LP, light grey), and group (G, white). The bold horizontal line indicates the median, the upper and lower hinge of the boxplot correspond to the 25 and 75 percentiles, comprising the interquartile range (IQR). The outwards pointing whiskers extend to the smallest and largest value at most $1.5 * \text{IQR}$ from the hinge. Data points smaller or greater are indicated as single dots. There was no difference in mean heart rate between the four test conditions during the experiment's phases.

4.3.3 Exploratory behaviour

In seven cases, horses approached the object immediately after Drop 1. The longest latency to approach was 856 seconds. In ten cases, horses did not approach. The focal horses' latency to approach did not depend on the social test condition (Friedman test: $n = 10$, $X^2 = 4.21$, $df = 3$, $p = 0.242$; Figure 4.10). There was no significant effect of trial number on the latency to approach, only a tendency (Friedman test: $n = 10$, $X^2 = 5.88$, $df = 2$, $p = 0.052$).

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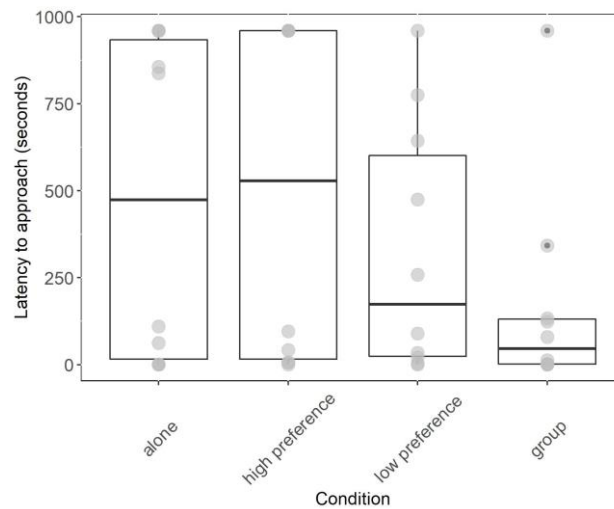


Figure 4.10 Latency to first approach. Tukey whiskers plot for the latency (in seconds) to the first approach of the object for each test condition. The bold horizontal line indicates the group median, the upper and lower hinge of the boxplot correspond to the 25 and 75 percentiles, comprising the interquartile range (IQR). The outwards pointing whiskers extend to the smallest and largest value at most $1.5 * \text{IQR}$ from the hinge. Data points smaller or greater are indicated as single dots. The maximum of 960 seconds represents the length of the trial, indicating that horses did not approach. A minimum of one second indicates that a horse immediately explored. The test condition did not affect the horses' latency to approach the novel object.

Taken for all trials, the average rate at which horses investigated the object, i.e., looked towards the object but did not manipulate it, was 0.32 ± 0.64 (mean \pm SD) times per minute and the average duration of investigation was 0.60 ± 1.67 seconds per minute. The average rate of exploration, i.e., manipulation of the object, was 0.41 ± 0.66 seconds per minute, and the average duration of exploration was 0.77 ± 1.53 seconds per minute. In 18 trials, horses showed investigatory behaviour (eight horses), and in 19 trials, horses showed exploratory behaviour (seven horses). Only one horse never investigated or explored the novel objects.

Neither the frequency nor the duration of exploration and investigation differed between the test conditions. There was only a tendency that the frequency and duration of investigation was the highest in the group condition (Friedman tests: $n = 10$, frequency investigation: $X^2 = 6.72$, $df = 3$, $p = 0.08$, duration investigation: $X^2 = 6.88$, $df = 3$, $p = 0.076$; frequency exploration: $X^2 = 1.64$, $df = 3$, $p = 0.650$, duration exploration: $X^2 = 4.34$, $df = 3$, $p = 0.22$; investigation and exploration: $X^2 = 5.55$, $df = 3$, $p = 0.136$; Figure 4.11).

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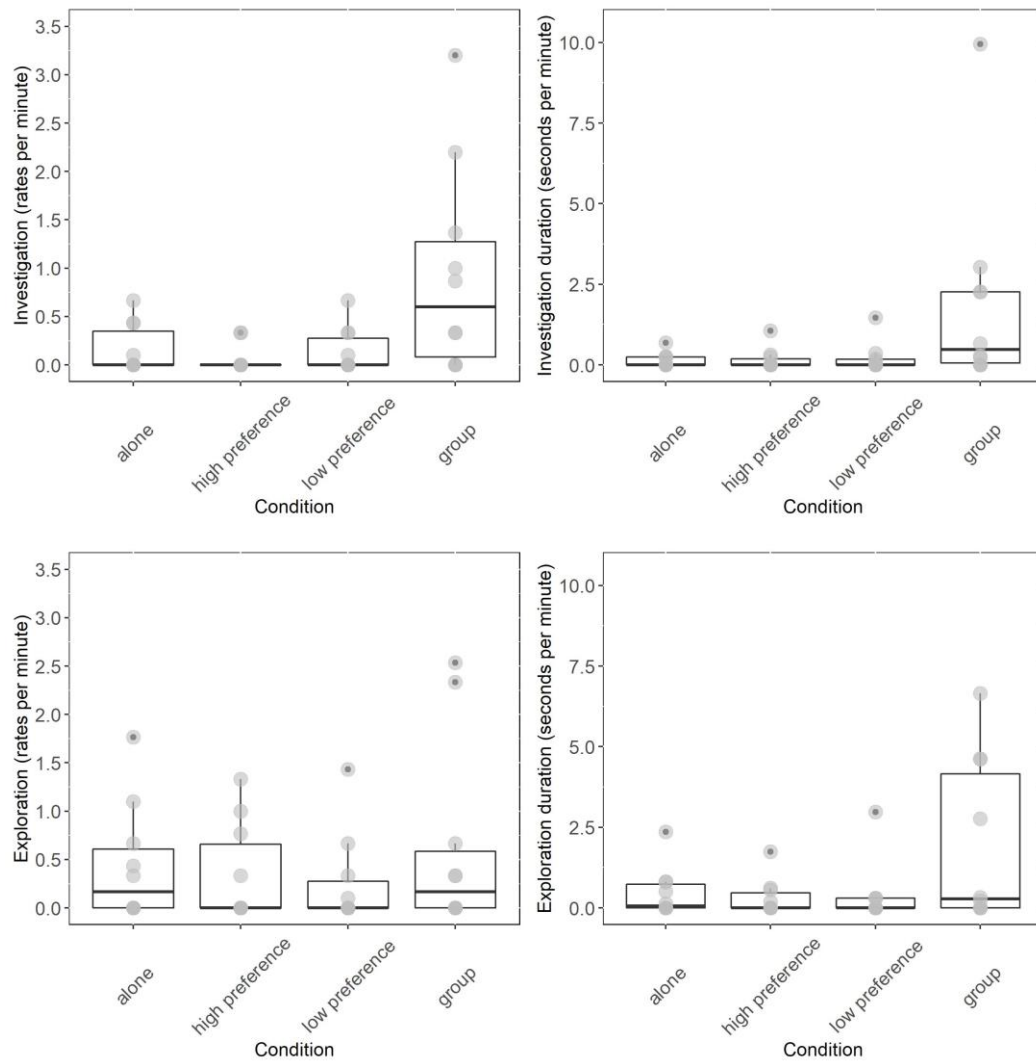


Figure 4.11 Investigation and exploration of the novel object. Tukey whisker plots for the occurrence of investigatory (top) and exploratory behaviour (bottom) in rates per minute (left) and their duration in seconds per minute (right) for the different test conditions. The bold horizontal line indicates the group median, the upper and lower hinge of the boxplot correspond to the 25 and 75 percentiles, comprising the interquartile range (IQR). The outwards pointing whiskers extend to the smallest and largest value at most $1.5 * \text{IQR}$ from the hinge. Data points smaller or greater are indicated as single dots. All variables did not significantly differ between test conditions.

The trial number did not affect the rate or the duration of investigation or exploration (Friedman tests: $n = 10$, frequency investigation: $X^2 = 1.68$, $df = 2$, $p = 0.431$; duration investigation: $X^2 = 1.08$, $df = 2$, $p = 0.582$; frequency exploration: $X^2 = 3.58$, $df = 2$, $p = 0.167$; duration exploration: $X^2 = 1.92$, $df = 2$, $p = 0.382$).

4.4 Discussion

In this chapter, it was assessed whether the social context affected the behavioural and physiological stress response, and exploratory behaviour of domestic horses (*Equus caballus*). The specific aim was to investigate whether the presence of closely bonded conspecifics of the same sex or of the whole group would induce buffering effects of the behavioural and physiological stress response and facilitate exploratory behaviour. Therefore, the heart rate and behaviour of ten horses were recorded when exposed to a novel object either alone, with a highly-preferred or less-preferred conspecific, or when with their whole group. The findings will be discussed first regarding social buffering and then regarding socially facilitated exploratory behaviour.

4.4.1 The effect of social context on the horses' behavioural and physiological stress response

Based on previous research, the prediction was that the horses' behavioural and physiological stress response to a novel object exposure would be buffered by the presence of a highly-preferred conspecific, and even more so by the horses' whole group. The findings presented in this chapter showed that the horses' responded with a significant but comparatively mild stress response to the novel object tests. Thereby, their behavioural and physiological stress response was independent of their social context. Neither the presence of a highly-preferred conspecific nor the presence of their group facilitated a buffering effect during the novel object experiment. Although this was a surprising finding, it goes in line with the findings of some previous studies. In non-human primate species, it was found that the individuals' physiological responses to a novel object exposure within their natural home range did not differ when they were alone or within their social unit (Vervet monkeys, *Chlorocebus pygerythrus*: Blaszczyk, 2017; Squirrel monkeys, *Saimiri sciureus*: Levine, 2000). Taken together, this suggests that animals do not always adapt their physiological and behavioural responses according to their social environment.

A possible explanation for the difference between the findings presented in this chapter and studies that found significant buffering effects (e.g., Hennessy et al., 2006; Hennessy, Kaiser and Sachser, 2009; Hodges et al., 2014; Kiyokawa

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et al., 2014b; Sachser, Dürschlag and Hirzel, 1998) could be the applied test paradigm. In many social buffering studies that have found significant buffering effects, the test subjects were exposed to stressors of high intensity that were inevitable and uncontrollable for the test subjects. Such test paradigms included, for example, pain-inducing fear conditioning (e.g., Kikusui, Winslow and Mori, 2006; Kiyokawa, et al., 2014a; Kiyokawa, Kawai and Takeuchi, 2018; Mikami et al., 2016), or a transfer into a completely novel environment (e.g., Apfelbeck and Raess, 2008; Hennessy, Zate and Maken, 2008; Kaiser et al., 2003; Sachser, Dürschlag and Hirzel, 1998).

In comparison to such test paradigms, a novel object is a punctual stressor in space and time. Moreover, the test arena was spacious. Therefore, horses in this study were able to ignore, avoid or to retreat from the stimulus. This potentially caused the horses to perceive the test situation as controllable, as indicated by their significant but overall mild stress response. Previous research has shown that the stress response decreased when the tested individuals could enhance the distance to the threatening stimulus or avoid it (Moberg, 2000). Moreover, perceived control over the stimulus can facilitate a reduction in the animals' stress response. For example, when test subjects have been provided with cues that predicted the onset of a stressor (Bassett, Cairncross and King, 1973), or when they perceived to be able to control or to stop a stressor (Hennessy, Kaiser and Sachser, 2009; McEwen and Wingfield, 2003; Swenson and Vogel, 1983; Uchino and Garvey, 1997), their stress response was alleviated. Levine (2000) concluded, based on his review, that social buffering effects can depend on the controllability of the presented stressor. When test subjects had the possibility to retreat from a stressor, the social context had no significant effect on their stress response.

Moreover, social buffering could also be depending on the intensity of the stressor. Previous studies have shown that fish followed individual behaviour in a low-risk environment, however with increased risk, they suppressed individual behaviour and followed collective behaviour (Guppies, *Poecilia reticulata*: Ioannou, Ramnarine and Torney, 2017; Three-spined stickle backs, *Gasterosteus aculeatus*: McDonald et al. 2016). Furthermore, a study on social buffering in Barbary macaques (*Macaca sylvanus*) has shown that social buffering effects increased with the intensity of the social or environmental stressors. However, under mild stress conditions, no social buffering effects were detected (Young et

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al., 2014a). In low-risk environments, it is possibly a greater adaptive benefit if individuals exploit their environment independently of their social context and fulfil their individual needs (Jensen and Toates, 1993). Whereas adapting the behaviour to the social context can promote safety in high-risk environments (Webster and Ward, 2011).

Consequently, social buffering could be only one possible mechanism, among others, that can help individuals to cope with a stressor. Therefore, future research could assess which characteristics of a potentially stressful situation enable the individual to cope independently of their social environment and which characteristics promote a shift towards socially dependent coping strategies. This seems relevant as wild and domestic animals are exposed to different types and intensities of potential stressors in their daily lives (Burla, Siegwart and Nawroth, 2018; Emery Thompson et al., 2010; Esch et al., 2019; Lundblad et al., 2020). Studying social buffering effects in different test conditions and under different levels of stress intensities could further the understanding of the role of social buffering and under which conditions individuals gain benefits from their social integration when coping with stressors (Koolhaas et al., 2017; Snyder-Mackler et al., 2020).

Another prediction of this study was that horses would seek proximity to a highly-preferred conspecific during the novel object test. Based on the findings presented in this chapter, this prediction can be confirmed. After the initial object exposure, focal horses were in closer proximity to their highly-preferred than to their less-preferred test partner. This effect was small, but significant and indicates a differentiated and bond-dependent proximity seeking pattern in horses upon the exposure to a novel and sudden stimulus. Seeking proximity during or after threatening or stressful situations was first described as a functional trait of the attachment system and can promote the reduction of fear and distress (Bowlby, 1969). It has been argued that an attachment bond is necessary for proximity seeking to occur (Bowlby, 1969; Cassidy and Shaver, 1999; Hay, 1980; Mikulincer and Shaver, 2003). Thus, proximity seeking has been primarily described between offspring and their parents (Bowlby, 1969) or between pair partners (Dewitte et al., 2008) as these relationships are meant to be of attachment quality (Ainsworth et al., 1978; Dewitte et al., 2008; Rajecki, Lamb and Obmascher, 1978). However, proximity seeking has also been found between

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siblings after a novel object exposure (Stöwe et al., 2006b) or across species, for example, between dogs and their owners (Gácsi et al., 2001; Tuber et al., 1996). Taking these latter findings together with the findings presented in this chapter, they suggest that proximity seeking can also occur outside the classic attachment bond – or that other than the mother-offspring bonds also constitute attachment bonds. Interestingly, in contrast to the prediction, although the horses sought proximity to a highly-preferred conspecific in the dyadic test conditions, their heart rate did not differ between the test conditions; also see the additional analysis in **Appendix B**, page 227. This was surprising, as proximity seeking should serve the function of promoting safety and decrease the stress response (Bowlby, 1969; Dewitte et al., 2008). The presented finding could suggest that bond-dependent proximity seeking also serves other functions besides the reduction of the stress response, as proposed by Hay (1980). Consequently, future research is needed to study under which conditions horses show bond-dependent proximity seeking and what function it serves.

4.4.2 The effect of social context on the horses' exploratory behaviour

Based on previous research, it was predicted that the horses' exploratory behaviour would be facilitated by the presence of a closely bonded conspecific and even more so by the presence of the horses' whole group. The findings presented in this chapter did not support these predictions. Overall, the horses' latency to approach the novel object and their rates and durations of investigation and exploration were not significantly affected by their social context, or the social bonds with their test partners. Moreover, the findings of this study indicate that the horses exhibited individual behavioural strategies when exploring a novel object. As these findings contrast to results presented in previous studies (e.g., Mendonça et al., 2019; Miller et al., 2015; Moretti et al., 2015; Stöwe et al., 2006a;b; Stöwe and Kotrschal, 2007), aspects that might facilitate such socially independent behaviour and its potential adaptive benefits will be discussed.

Similar to the line of argumentation regarding the horses' stress response, the low stress intensity of the novel object tests could have promoted individual rather than socially dependent exploratory strategies (Ioannou, Ramnarine and Torney, 2017; McDonald et al., 2016). That the horses' exploratory behaviour was

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not significantly affected by their social context was, nevertheless, an interesting finding. The theory behind social buffering and socially facilitated exploration suggests that a reduction of the stress response allows the facilitation of exploratory behaviour (Ainsworth et al., 1978; Bowlby, 1969; Horn, Huber and Range, 2013; Palmer and Custance, 2008; Solomon et al., 2019). Consequently, the low-risk environment could have promoted socially facilitated exploratory behaviour. Nevertheless, the horses' social context did not significantly affect their exploratory behaviour. The high familiarity of the horses with the test environment could have contributed to this finding. Habituation to the test arenas did not only take place in preparation for this study, but they were often used as a housing area for the horses. This could have induced a 'home base' effect, first described by Eilam and Golani (1989) for rodents. The home base describes a location from which an animal starts exploration and returns to for comfort-seeking. Also, domestic horses were found to show a home base effect. A study demonstrated that horses, when exploring a novel environment, sought out a location from which they started exploring and returned to for comfort behaviour (Burke and Whishaw, 2020). Thus, the horses in this study might have been able to investigate or explore the novel object independently of their social environment due to the perceived safety of their environment. This seems in line with Blaszczyk's (2017) findings, who tested territorial vervet monkeys and presented them with novel objects within their territory. In her study, the monkeys also adopted an individual rather than socially-dependent exploratory behaviour.

Similarly, a recent study on how exploratory behaviour is connected with learning in young horses by Christensen et al. (2021) found that their exploratory behaviour was rather independent of their social environment. Young horses were exposed to a novel object at two time points. Their fear and exploratory behaviour were highly consistent, despite their dam being present at one test time point. These findings also indicate that the horses rather followed an individual behavioural strategy that was independent of their social environment. Adopting individual behavioural strategies in a low-risk environment can be adaptive as it allows the individual to fulfil their current needs (Jensen and Toates, 1993). Whether horses adopt the same exploration strategy in conditions that promote a more pronounced stress response needs to be assessed in future research. Exploring novel but potentially threatening features in the environment

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independently of the social context could increase the potential danger for the individual, especially in prey species. Consequently, under conditions of higher threat anti-predator strategies such as flocking and the selfish-herd principle (Foster and Treherne, 1981; Hamilton, 1971; Iranzo et al., 2018; King et al., 2012) could also influence exploration, and promote a synchronised co-exploration of potentially threatening novel features (Kim and Park, 2010; Ward, 2012). Investigating horses' exploratory behaviour under different stress-intensities could make it possible to assess whether horses deploy different stress coping and exploration strategies.

4.4.2.1 Linking social buffering and socially facilitated exploration

Another aim of this study was to assess bond-dependent buffering and socially facilitated exploration within the same experiment. Although many authors argue that social bonds promote social buffering and that social buffering promotes exploratory behaviour (Ainsworth et al., 1978; Bowlby, 1969; Horn, Huber and Range, 2013; Ijichi et al., 2018; Moretti et al., 2015; Payne et al., 2016; Solomon et al., 2019), comparatively few studies have investigated social relationships, social buffering, and socially facilitated exploration together. Studies that addressed the link between those aspects yielded differing results. One study found social buffering, but no socially facilitated exploration (Terranova, Cirulli and Laviola, 1999). Another study found no evidence for social buffering, but for socially facilitated exploration (Galhardo, Vitorino and Oliveira, 2012). The findings presented in this chapter showed again a different pattern: domestic horses showed neither (bond-dependent) social buffering nor socially facilitated exploration. This shows that the effect of an individual's social context and the regulation of its stress response and exploratory behaviour is not necessarily linked but possibly depends on multiple factors such as (perceived) controllability, the intensity of the stress response, or type of stressor.

Future studies could further investigate factors that determine whether an animals' social context affects its stress response or exploratory behaviour. Such knowledge could further the understanding about the potential benefits of group-living (Krause and Ruxton, 2002; Snyder-Mackler et al., 2020), and about adaptive mechanisms that facilitate a balance between individual and socially dependent behaviour (Ioannou, Ramnarine and Torney, 2017; McDonald et al., 2016; Young et al., 2014a).

4.4.3 Implications for equine welfare

Although the horses in this study displayed bond-dependent proximity seeking behaviour, their stress-related behaviours and heart rate were independent of their social context. These findings showed that horses predominantly adopted individual coping strategies in a condition of mild stress. For equestrian practice, these findings suggest that horses do not necessarily benefit from the presence of a closely bonded horse or conspecifics in general when exposed to novel objects in their familiar environment which induce a mild stress response.

Whether these findings apply to more excitable horses or conditions that are less controllable and/or provoke a higher stress response needs to be investigated in future research. Previous studies have shown that horses' stress reactivity can be breed-dependent, with ponies being less reactive than sports horses such as thoroughbreds or warmbloods (Lloyd et al., 2008; Roberts et al., 2016; Sackman and Houpt, 2019). Therefore, future research could assess whether more temperamental horses benefit more from their social context when exposed to novelty, as suggested by previous studies in warmbloods (Christensen et al., 2008; Rørvang and Christensen, 2018). Domestic horses are often exposed to novel stimuli during handling or training routines which they can perceive as stressors (Burla, Siegwart and Nawroth, 2018; Esch et al., 2019; Lundblad et al., 2020; Schmidt et al., 2010a;b;c; Yarnell, Hall and Billett, 2013). Consequently, further research that helps determine sources of stress and sources of (social) support for domestic horses is important from an equine welfare perspective as it can help to facilitate an environment that reduces stress for horses under human care (Dalla Costa et al., 2014; Fraser, 2008).

4.5 Conclusion

This study aimed at investigating whether domestic horses benefitted from the presence of a closely bonded conspecific or their whole group when exposed to a novel object. The horses under study showed a significant but mild stress response to the object exposure. The horses spent the period after the initial object presentation in closer proximity to their highly-preferred than to a less-preferred field companion. However, closer proximity was not accompanied by a lower heart rate. Furthermore, the social context did not significantly affect the

Chapter 4 – Novel object test

horses' behavioural and physiological stress response, or exploratory behaviour during the novel object test. Taken together, these findings suggest that in the context of a novel object test, which facilitated a mild stress response, horses adopted individual behavioural and physiological strategies that did not depend on their social context. This behavioural and physiological response pattern could constitute an adaptive strategy under mild stress conditions.

The presented study provided novel insights into the effects of horses' social context on their behavioural and physiological response to novelty. Moreover, it prompted further questions about what factors determine whether animals individually adapt to potentially stressful stimuli in their environment, or whether their adaptation depends on their social context.

CHAPTER 5

Benefits of social bonds in domestic horses
(*Equus caballus*) – General discussion



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5.1 General discussion

5.1.1 Summary of the research

The aim of this project was to assess the benefits of close social bonds among unrelated domestic horses (*Equus caballus*) from a mechanistic perspective. Horses are one of the few species that form long and durable social bonds between unrelated individuals and between individuals of the same sex (Cameron, Setsaas and Linklater, 2009; Seyfarth and Cheney, 2012). Therefore, they are an ideal species to study the advantages of such bond formations. One benefit of social bonds is social buffering. Social buffering describes the alleviation of the behavioural and physiological stress response in or after stressful situations facilitated by the presence of or interactions with a closely bonded conspecific (Cohen and Wills, 1985; Hennessy et al., 2006; Hennessy, Zate and Maken, 2008; Kaiser et al., 2003; Sachser, Dürschlag and Hirzel, 1998). The central question of this thesis was whether horses benefit from social buffering effects facilitated by the presence of or interaction with their closely bonded conspecifics.

To study social buffering-effects, three contexts were selected that have been found to provoke a stress response and are of daily relevance to domestic horses: (1) social interactions (Sgoifo et al., 1998; Viblanc et al., 2012; Wascher, Arnold and Kotrschal, 2008), (2) the separation of individuals from their social unit (Boissy and Le Neindre, 1997; Bolt et al., 2017; Erber et al., 2012; Hartmann, Christensen and Keeling, 2011; Pérez Manrique et al., 2019; Pollard and Littlejohn, 1995), and (3) the exposure to a novel object in the animals' familiar environment (Błaszczuk, 2017; Bonnot et al., 2018; Coe et al., 1982; Emery Thompson et al., 2010; Levine, 2000). The aim was to assess social buffering effects on behavioural and sympathetic-adrenal-medullary (SAM) axis activity levels. Consequently, a combination of behavioural observations and heart rate measurements was applied to answer the research questions.

In the subsequent sections, the main findings (Table 5.1) will be discussed in the context of behavioural ecology and social buffering research. This is followed by a discussion of the main findings and their implications for equine science and equine welfare. Limitations and suggestions for future research will be discussed within the respective sections of both subchapters.

Chapter 5 – General discussion

Table 5.1 Summary of key findings. Research questions, hypotheses and key findings are listed by thesis chapter.

Chapter	Research questions, hypotheses, and key findings
Chapter 2	1. Do social interactions affect heart rate in domestic horses?
	Hypothesis A: Agonistic interactions constitute a potent stressor and facilitate a physiological stress response on the SAM-axis activity level.
	<ul style="list-style-type: none"> The majority of agonistic interactions were of low intensity, which did not facilitate a measurable stress response on the SAM-axis activity level.
	Hypothesis B: Short affiliative interactions and mutual grooming facilitate relaxation and correspond to lower cardiac activity.
	<ul style="list-style-type: none"> Overall, short affiliative interactions did not significantly affect the horses' heart rate. Mutual grooming corresponded to a significantly lower heart rate than when horses were standing, indicating a relaxation effect.
	2. Does heart rate differ between initiators and receivers of social interactions?
	Hypothesis C: The role of the interacting horses has an effect on their heart rate during social interactions.
	<ul style="list-style-type: none"> Receiving an agonistic interaction corresponded to an increase in heart rate. Thereby, the horses' heart rate was similar to walking, reflecting their retreat. The horses' heart rate did not differ when either initiating or receiving short affiliative interactions.
	3. Does heart rate during social interactions depend on the relationship quality (bonds, rank) between the interacting horses?
	Hypothesis D: Social relationships between the interacting horses have an effect on their SAM-axis activity. <ul style="list-style-type: none"> Rank but not bond relationships affected horses' heart rate during agonistic interactions. The heart rate of receivers was higher, the higher the initiator in rank compared to the receiver. Bond relationships had an effect on horses' heart rate during short affiliative interactions but not during grooming. Receiving short affiliative interactions from closely bonded group members facilitated a slight increase in heart rate, potentially reflecting a positive emotional state, e.g., excitement.

Chapter 5 – General discussion

Table 5.1 continued.

Chapter 3	1. Does the presence of a closely bonded conspecific of the same sex buffer the behavioural and physiological stress response during separation from the social group?
	Hypothesis A: The presence of a closely bonded horse of the same sex facilitates social buffering effects on behavioural and SAM-axis activity levels.
	<ul style="list-style-type: none"> The horses behavioural and physiological responses to separation did not differ between separation with a closely bonded or a less closely bonded horse. Alert and startle behaviour was lower when with another horse compared to when isolated alone.
	2. Does the occurrence of short affiliative interactions depend on the social bond between the horses?
	Hypothesis B: The rate of short affiliative interactions and the duration of mutual grooming depend on the social bond between the horses.
	<ul style="list-style-type: none"> Horses exhibited the same number of affiliative interactions towards highly-preferred and less-preferred horses when separated from their group.
	3. Does the occurrence of short affiliative interactions between horses facilitate a bond-related buffering effect on cardiac activity level?
	Hypothesis C: Higher rates of short affiliative interactions and longer mutual grooming durations between bonded conspecifics will facilitate social buffering.
	<ul style="list-style-type: none"> A longer grooming duration corresponded to slightly higher RMSSD values. The frequency of short affiliative interactions did not affect SAM-axis activity during separation.
	4. Does spatial proximity between horses depend on their social bond?
	Hypothesis D: The spatial proximity between the horses depends on their social bond.
	<ul style="list-style-type: none"> When separated, horses showed similar proximity patterns when with a closely bonded horse or a less closely bonded horse.
	5. Does close spatial proximity between horses facilitate a bond-related buffering effect on cardiac activity level?
	Hypothesis E: Close spatial proximity between bonded horses facilitates social buffering.
	<ul style="list-style-type: none"> Close spatial proximity to a highly-preferred or less-preferred horse did not affect the horses' heart rate during separation.

Table 5.1 continued.

Chapter 4	1. Does the horses' behavioural and physiological stress response to a novel object exposure depend on their social context?
	Hypothesis A: The presence of a closely bonded conspecific of the same sex buffers the focal horse's behavioural and physiological stress response.
	Hypothesis B: The presence of the whole group facilitates a stronger buffering effect than the presence of a closely bonded conspecific.
	<ul style="list-style-type: none"> • In the context of the novel object experiments, which constituted a mild stressor, horses behavioural and physiological stress response did not depend on the test condition (alone, with a highly-preferred or less-preferred horse, group). • Horses showed proximity seeking to a highly-preferred horse directly after the initial exposure to the novel object. This proximity pattern did not affect their SAM-axis activity.
	2. Does the exploratory behaviour that horses exhibit towards a novel object depend on their social context?
	Hypothesis C: The presence of a closely bonded conspecific of the same sex facilitates exploratory behaviour.
	Hypothesis D: The group's presence has a stronger facilitatory effect on exploratory behaviour than the presence of a single conspecific.
	<ul style="list-style-type: none"> • The horses' exploratory behaviour (latency to approach, investigation, exploration) did not differ between the four test conditions (alone, with a highly-preferred or less-preferred horse, group).

5.1.2 Benefits of social bonds in domestic horses: a discussion of main findings and future directions

5.1.2.1 Rank but not bond relationships affected heart rate during agonistic interactions

The findings presented in **Chapter 2** indicated that horses' heart rate during low-intensity agonistic interactions was not affected by the bond relationship between the interactors but by their rank relationship. The receivers' heart rate was slightly higher, the higher the initiator was in rank relative to themselves. As rank relationships are based on the proportion of won and lost conflicts with a conspecific (Van Dierendonck, Schilder and De Vries, 1995), the chances are higher, that an agonistic interaction with a higher-ranked conspecific results in defeat or harm. Consequently, the receiver is more likely to prepare for or carry out a necessary behavioural action, such as retreating and avoiding conflict (Sigurjónsdóttir et al., 2003). A close social bond between horses had no significant effect on heart rate during agonistic interactions. Therefore, no bond-dependent buffering was found in the context of low-intensity agonistic interactions among horses in established groups.

Agonistic interactions are central components of group-living animals' daily lives (Hinde, 1976) which can, if causing chronic stress, have a detrimental impact on the animals' long-term health (McEwen, 2008; Sapolsky, Romero and Munck, 2000; Sgoifo et al., 2001; Sgoifo, Carnevali and Grippio, 2014). Therefore, it seems necessary to further investigate under which circumstances agonistic social interactions provoke a stress response, and under which circumstances they do not. Several factors that contribute to social stress have already been determined. For example, receiving a high amount of aggression (Abbott et al., 2003; Aureli, Preston and de Waal, 1999; Yamanashi et al., 2018), group instability (Crockford et al., 2008; Emery Thompson et al., 2010; Noller et al., 2013; Wittig et al., 2008), or actively defending a rank position (Creel et al., 2013) can facilitate social stress. Rank stability, however, was found to contribute to the predictability of agonistic interactions and their outcomes (Barrett, Henzi and Lusseau, 2012; Kaufmann, 1983) which can reduce social stress (Koolhaas et al.,

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2017). Compared to existing knowledge about the effect of rank relationships and agonistic interactions on social stress, little is still known to what extent bond relationships affect agonistic interactions and their underlying physiological mechanisms. However, animals live in multi-dimensional social structures that comprise agonistic and affiliative interactions as well as rank and bond relationships (Barrett, Henzi and Lusseau, 2012). As these dimensions are not independent of each other (Barrett, Henzi and Lusseau, 2012; Flack et al., 2006), future research could further investigate whether and how bond relationships affect agonistic interactions and their underlying physiological mechanisms. A meaningful next step could be to assess whether the findings presented in this study also apply to high-intensity agonistic interactions, for example, during competitive feeding situations (Beery and Kaufer, 2015; Kotrschal, Hemetsberger and Dittami, 1993). Close bonds have been found to facilitate tolerance in competitive contexts and reduce aggression (Asakawa-Haas et al., 2016; Dale et al., 2017; Lehmann and Boesch, 2009; Ostner and Schülke, 2014). Consequently, agonistic interactions among closely bonded horses could be of lower intensity even under competitive situations. This could provide bonded individuals with an indirect physiological benefit, as low-intensity aggression corresponds to a reduced physiological cost (Romero, Dickens and Cyr, 2009; Viblanc et al., 2012; Wascher et al., 2009; Wascher, Arnold and Kotrschal, 2008). Such research could extend the existing knowledge about the potential cost and benefits of group-living and bond formations (Snyder-Mackler et al., 2020; Viblanc et al., 2012; Wascher et al., 2009; Wascher, Arnold and Kotrschal, 2008).

5.1.2.2 Social bonds had different effects on heart rate during grooming and other affiliative interactions

The analysis in **Chapter 2** showed that mutual grooming did correspond to a lower heart rate than when standing, a behaviour of similar physiological activity. This goes in line with findings from mutual grooming in other species such as primates (Aureli and Yates, 2010; Wittig et al., 2016), which indicates a low arousal level and possibly a positive affective state (Boissy et al., 2007; Reefmann, Wechsler and Gyga, 2009). Moreover, the study presented in **Chapter 3** revealed that a longer grooming duration between horses during separation from their group corresponded to a higher heart rate variability

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(RMSSD). However, these effects were independent of the bond between the horses. In contrast to these findings, a previous study by Wittig et al. (2016) demonstrated that the decrease of HPA-axis activity levels was bond-dependent. Thereby, glucocorticoid metabolite levels after grooming were lower, the stronger the bond between the grooming partners. The difference between the findings presented by Wittig et al. (2016) and those in this thesis could be explained by intercorrelation patterns among different types of affiliative interactions. In the domestic horses under study the grooming behaviour was not strongly correlated with other behaviours that can indicate a close social bond, such as approaches, following, body contact, and spatial proximity (Cameron, Setsaas and Linklater, 2009; Silk, Cheney and Seyfarth, 2013); see details about intercorrelations between affiliative behaviours in **Appendix A**, page 225. In reference to the biological market theory, this can suggest that horses might not primarily choose their grooming partners according to the strength of their bond but by the value of the provided grooming commodity (Noë and Hammerstein, 1994). This would reflect some observations in domestic and feral horses, where horses seek out specific group members for grooming, thereby leaving their nearest neighbours behind (D.V.H., personal observation).

Other affiliative interactions, such as sniffs, touches, and rubbing heads, did not correspond to a decrease in heart rate. On the contrary, the findings in **Chapter 2** showed a small but significant effect that the receivers' heart rate was slightly higher, the higher the bond strength to the initiator. This finding contrasts with the hypothesis that affiliative interactions, especially among closely bonded conspecifics, facilitate relaxation as indicated by a decrease in the activity of the physiological stress axes (Aureli, Preston and de Waal, 1999; Briefer et al., 2015; Wittig et al., 2008). However, interpreting this finding from the perspective of animal emotion research, the slight increase in heart rate could suggest a physiological arousal due to positive emotional valence (Mendl, Burman and Paul, 2010; Reefmann et al., 2009). However, the findings presented in **Chapter 3** showed that the rate of initiated or received short affiliative interactions during the separation trials had no effect on the horses' heart rate or heart rate variability.

To further investigate the effect of social bonds on heart rate during affiliative interactions and their possible link to a positive emotional state (Hall et al., 2018; Kremer et al., 2020; Mendl, Burman and Paul, 2010), more research is

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needed. Future studies could employ different data collection techniques to gather more information about the animals' physiological and emotional responses to different social experiences. Hall et al. (2018) suggested that such techniques could include measures of surface and core temperature, electrodermal response, or the assessment of hormone levels such as oxytocin or prolactin. Moreover, they could include behavioural measures, such as spontaneous blink rate shown to be related to dopamine secretion (Jongkees and Colzato, 2016), or EquiFACS (Wathan et al., 2015), a systematic catalogue of equine facial movements that can be linked to the emotional state of horses (Lundblad et al., 2020).

The findings regarding heart rate during mutual grooming and other affiliative interactions presented in this thesis suggest that different types of affiliative interactions correspond to different underlying physiological mechanisms that can link social integrations with long-term health (Cohen and Janicki-Deverts, 2009; Lakey and Orehek, 2011; Snyder-Mackler et al. 2020; Thoits, 2011). Previous research has shown that socially well-integrated individuals, who engage in social bonds and affiliative interactions, benefitted from increased health and a higher life expectancy (Holt-Lunstad, Smith and Layton, 2010; House et al., 1988; Nuñez, Adelman and Rubenstein, 2015; Snyder-Mackler et al., 2020). On the one hand, it has been proposed that this benefit of social integration arises from an accumulative effect of affiliative interactions and social support in stressful situations which buffers and alleviates the physiological stress response, i.e., social buffering (Cohen and Wills, 1985; Romero, Dickens and Cyr, 2009; Snyder-Mackler et al., 2020). This mechanism could be primarily triggered by grooming behaviour, as grooming corresponded to lower heart rate (Aureli, Preston and de Waal, 1999; **Chapter 2**), and a higher heart rate variability (**Chapter 3**). Similarly, previous studies have shown that individuals who were embedded in grooming networks showed an alleviated or less pronounced HPA-axis activity (Fürtbauer et al., 2014; Puehringer-Sturmayr et al., 2018; Wittig et al., 2016; Yamanashi et al., 2018; Young et al., 2014a;b).

On the other hand, social integration can be beneficial and promote long-term health through regular positive experiences and overall well-being. This is described as the 'main effect of social support'; a complementary model to the model of social buffering (Cohen and Wills, 1985). A mediator of such main effects

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can be the instalment of positively valenced emotion (Van Dierendonck and Spruijt, 2012; Mendl, Burman and Paul, 2010) that is generated and updated during repeated affiliative interactions with bonded conspecifics (Cohen and Wills, 1985). The findings that short affiliative interactions between closely bonded horses corresponded to a slightly increased heart rate (**Chapter 2**) could support this idea.

Based on the assumption that different types of affiliative interactions trigger different underlying physiological mechanisms, future studies could continue this line of research and specifically address single types of affiliative interactions instead of pooling them together for the analysis. This could further our understanding regarding the link between the underlying mechanisms of different types of social interactions, bond-formations, and potentially long-term health (Cohen and Wills, 1985; Dunbar and Shultz, 2010; Snyder-Mackler et al., 2020).

5.1.2.3 Social bonds and proximity seeking

After the initial exposure to the novel object (**Chapter 4**) focal horses spent significantly more time in close proximity to their preferred group member than to their less-preferred group member. Proximity seeking has been primarily described in the context of attachment bonds, such as between offspring and their parents (Bowlby, 1969) or pair partners (Dewitte et al., 2008), and should promote safety during potentially threatening situations (Bowlby, 1969). However, proximity seeking was also found outside of these attachment bond formations, for example, among siblings after novelty exposure (Stöwe et al., 2006b). Furthermore, proximity seeking was found across species, for example, between dogs and their owners after the exposure to a threatening stimulus, or during social separation (Gácsi et al., 2001; Solomon et al., 2019; Tuber et al., 1996). The finding from the novel object experiment (**Chapter 4**) indicated that proximity seeking can also occur between closely bonded horses of the same sex. This provides further evidence that proximity seeking can occur outside the classic attachment bond, or that other types of social bonds could be of attachment quality (Solomon et al., 2019).

There was no bond-related difference in spatial proximity between the horses in the other phases of the novel object exposure (**Chapter 4**) or during the

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dyadic separation trials (**Chapter 3**). Comparing the different test situations of the separation and novel object tests, the horses showed the greatest behavioural and physiological stress response upon the initial object presentation; and this was the test-event where bond-dependent proximity seeking occurred. Taken together, this suggests that proximity seeking between closely bonded horses of the same sex depends on the intensity or the type of the stimulus.

On a physiological level, the heart rate of focal horses in the phase following the initial object exposure did not differ between the four test conditions (alone, with a highly-preferred horse, with a less-preferred horse, or with their group) (**Chapter 4**), or more specifically, between the two dyadic test conditions (**Appendix B**, page 227). These findings showed that the horses' proximity seeking in this phase of the experiment, and thus spatial proximity, did not facilitate a calming effect indicated by lower physiological activity levels, as suggested by previous studies (Dujardin et al., 2019; Kiyokawa et al., 2009, 2013; Palestini et al., 2005). This is an interesting finding because it is thought that the primary function of proximity seeking during potentially stressful situations is to promote safety and to alleviate stress (Ainsworth et al., 1978; Bowlby, 1969; Dewitte et al., 2008). There may be a chance that the horses' stress response was not high enough for social buffering to occur, as previous research demonstrated that mild stress conditions did not necessarily facilitate social buffering (Young et al., 2014a). However, Hay (1980) proposed that proximity seeking could also have other functions than the alleviation of the stress response, such as promoting social learning. However, the analyses in **Chapter 4** revealed that the presence of a closely bonded conspecific did not facilitate the horses' exploratory behaviour. Consequently, more research is needed to determine whether bond-dependent proximity seeking depends on the intensity of a stressor and what specific function it serves if it does not promote an alleviation of the animal's stress response.

5.1.2.4 Generalised and independent behavioural and physiological responses

Apart from the findings discussed in the previous subsections, the findings revealed no other bond-dependent effects on the horses' behaviour or cardiac activity in the three investigated contexts. Rather, horses showed behavioural and physiological responses that were generalised towards all test partners,

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regardless of their social preference, or they responded independently of their social context.

The rate of affiliative interactions and the spatial proximity did not differ between highly-preferred and less-preferred dyads during the dyadic experiments presented in **Chapters 3** and **4**. This provided an interesting result, as nearest neighbour assessments and directed affiliative interactions (**Chapter 3**) indicated a significant preference of specific other horses when within their whole group. Moreover, the findings from **Chapter 3** showed that spatial proximity to group-members of different preference did not affect the horses' heart rate or heart rate variability. Firstly, horses' SAM-axis activity levels were not different when they were in close proximity or apart from their highly-preferred or less-preferred group member. Secondly, when comparing the dyads, there was no difference in SAM-axis activity levels when being close to a highly-preferred or less-preferred field companion.

Overall, these findings suggest that horses, as prey animals, maybe generalise their behaviour towards conspecifics when separated from the social group independently of their actual social preference. The findings on vigilance behaviour, presented in **Chapter 3**, point in a similar direction. The vigilance of horses significantly decreased when they were with a group member, independently of their bond. These generalised behavioural patterns could also be contextualised based on the biological market theory (Noë and Hammerstein, 1994). Being selective in a situation where fewer social partners are available can be costly (Noë and Hammerstein, 1994). For horses, as prey animals, grouping was found to be an anti-predator strategy (Feh, Boldsukh and Tourenq, 1994; Rubenstein, 1978; Van Der Post, Verbrugge and Hemelrijk, 2015). Therefore, selectiveness could be costly when being separated from their social group as it could increase the potential risk of predation. Thus, affiliating and spending time in proximity could be a generalised (i.e., not bond-dependent) and possibly a more beneficial strategy when there is a limited number of conspecifics available.

The other findings presented in **Chapters 3** and **4** showed that the horses' behavioural and physiological responses were independent of their social context during the two experiments. Their cardiac activity during the separation or the novel object test and their exploratory behaviour did not depend on the presence of a specific group member or the whole group. This suggests that domestic

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horses coped independently of their social environment when exposed to a stressor of mild intensity. The relationship between threat-level and behavioural strategies has been demonstrated in previous studies. In guppies (*Poecilia reticulata*) and three-spined sticklebacks (*Gasterosteus aculeatus*), individuals resumed personal behavioural strategies in a low-risk environment. However, with increasing risk, the fish suppressed individual strategies and followed collective behaviour (Ioannou, Ramnarine and Torney, 2017; McDonald et al., 2016). In male Barbary macaques (*Macaca sylvanus*), social buffering effects were identified under high stress intensity. However, no buffering effects were detected under conditions of mild stress (Young et al., 2014a). These studies have shown that the individuals' behavioural and physiological response, and social buffering, can depend on the intensity of the stressor. Both strategies are evolutionary beneficial. In contexts of low risk, individuals possibly gain the most benefits by exploiting their environment through socially independent behaviour and fulfilling their individual needs (Jensen and Toates, 1993). Whereas, adapting the behaviour to the social context can promote safety in high-risk environments (Webster and Ward, 2011).

Future research could further investigate this interplay between individual behaviour, social buffering, and stress intensity. Conducting such research with domestic horses seems to be a great opportunity. Previous studies have shown, that the stress reactivity in horses can be breed-dependent (Lloyd et al., 2008; Roberts et al., 2016; Sackman and Houpt, 2019; Wolff, Hausberger and Le Scolan, 1997). Exposing horses of specific different breeds, and thus possibly reactivity types, in different social conditions to stressors of varying intensity could provide novel insights in two areas: (1) whether specific individuals benefit more from social buffering than others, and (2) which factors determine whether individuals exhibit an independent or a socially-dependent coping strategy (Koolhaas et al., 2010, 1999). Such research could highlight under which conditions social buffering plays a role in enhancing an individual's stress coping capacity and under which conditions socially independent coping strategies are more beneficial to the individual. This can help to gain a better understanding of the benefits and the role of social bond formations and social integration that can, on the long-run, promote health and longevity (Cohen and Wills, 1985; Snyder-Mackler et al., 2020).

5.1.2.5 Social buffering among closely bonded but unrelated domestic horses – current evidence and outlook

Overall, the three studies did not yield strong evidence that domestic horses benefitted from social buffering provided by closely bonded but unrelated conspecifics under mild stress conditions. Upon a sudden exposure to a novel object, closely bonded horses were in closer proximity than less closely bonded horses. However, this proximity seeking was not accompanied by a lower heart rate. The bond between horses had no alleviating effect on their heart rate during agonistic interactions or their behavioural and physiological response to experimental separation from their group, or exposure to the novel objects. During the separation trials, horses showed generalised responses towards any conspecific, regardless of their bond. During the novel object exposure, the horses' behavioural and physiological responses were independent of their social context. It was discussed that such strategies may be adaptive for horses, as a prey species, or might be beneficial in conditions of low stress intensity.

These findings highlight the importance of studying social buffering in different species and under different test conditions; especially as they deviate from previous studies that have found significant (bond-dependent) buffering effects (Kiyokawa et al., 2014b; Rørvang, Ahrendt and Christensen, 2015; Rørvang and Christensen, 2018; Sachser, Dürschlag and Hirzel, 1998). Generating more data on social buffering outside the traditional (experimental) contexts can help to understand what role social buffering can play besides individual or generalised behavioural and physiological responses.

Nevertheless, the findings, that were presented in this thesis apply to the described test conditions which were of low stress intensity; and they need to be generalised with caution due to the restricted sample size. Furthermore, this thesis primarily investigated social buffering effects in male-male dyads. To gain a comprehensive understanding of social buffering in horses and whether social bonds affect social buffering, further research is needed. Besides testing horses under different stress intensities, as discussed in the previous subchapter (5.1.2.4), the presented research can be extended to studying other bond types that are typical for horse sociality, such as female-female and female-male bonds. Horses also form strong bonds between mares (Cameron, Setsaas and Linklater, 2009; Stanley et al., 2018). However, mares were not included in the experimental

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studies as they were either related or had to be excluded based on ethical considerations. Besides bonds among group members of the same sex, horses form long-lasting bonds between stallions and mares (Kaseda and Khalil, 1996; Linklater et al., 1999), and as indicated by the initial observations of this study, between mares and geldings. Studying social buffering regarding different bond types that are typical for horse sociality makes it possible to determine whether different types of social bonds play a role in social buffering or whether they primarily serve other functions.

It could be the case that close bonds among unrelated horses (of the same sex) primarily serve functions other than social buffering, such as facilitating group cohesion (Khalil and Kaseda, 1998; Stanley et al., 2018) which can provide horses with a defence against predation (Feh, Boldsukh and Tourenq, 1994; Rubenstein, 1978). Moreover, mature stallions were found to form coalitions to jointly defend a group of mares (Linklater et al., 1999), which can increase their reproductive success (Feh, 1999). Furthermore, feral mares can benefit from close social bonds as they reduce male harassment, forced copulation and abortion, thus increasing their reproductive success (Cameron, Setsaas and Linklater, 2009; Stanley et al., 2018). Consequently, more research is needed to gain a comprehensive understanding of whether and under what conditions social bonds provide horses with benefits such as social buffering and thereby contribute to the benefits of social integration regarding long-term health (Snyder-Mackler et al., 2020).

5.1.3 Implications for equine welfare and a discussion of future directions in equine welfare research

The presented research touches upon two pillars of animal welfare: (1) 'natural living'; this concept aims at promoting housing systems that allow animals to live as naturally as possible and express their species-specific, including social, behaviour. And (2) 'affective states', which aims at facilitating positive states of the animal and reducing or preventing negative states, including stress responses (Boissy et al., 2007; Fraser, 2008). There are two areas where the findings regarding socio-physiology and social buffering have implications for equine welfare: firstly, housing and secondly, handling and training. In the subsequent sections, the implications of the presented findings for horse housing and handling

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will be discussed, and possible future directions for equine welfare research will be outlined.

5.1.3.1 Implications for horse housing

In **Chapter 2**, data were presented on how social interactions in free-ranging domestic horses impact their heart rate. The findings showed that the majority of agonistic interactions were of low intensity and that these agonistic interactions did not provoke a significant stress response on the SAM-axis activity level. The findings showing that agonistic interactions did not facilitate a significant stress response are of importance, as concerns about social interactions, stress and the risk of injuries are used in arguments against group housing of horses (Hartmann, Søndergaard and Keeling, 2012).

Furthermore, mutual grooming was accompanied by a lower heart rate than when standing, an activity with comparable physical activity. This indicates relaxation and possibly a positive emotional state (Lansade et al., 2018). Grooming has been found to elicit the release of opioids and dopamine in the brain (reviewed in: Boissy et al., 2007; VanDierendonck and Spruijt, 2012), which both mediate motivation and gratification (Alcaro, Huber and Panksepp, 2007; Burgdorf and Panksepp, 2006; Ikemoto and Panksepp, 1999). These findings provide further evidence about the positive effect of mutual grooming. Allowing horses to perform this behaviour is therefore another step towards positive welfare, promoting positive experiences rather than just preventing the occurrence of negative ones (Fraser et al., 1997; Hall et al., 2018; Yeates and Main, 2008).

These first findings about socio-physiology in horses are promising evidence for promoting group housing in domestic horses. Nevertheless, these findings should be generalised with caution due to the context in which the data were collected. The presented studies included horses from established groups that experienced regular turnouts in spacious environments. However, different behavioural and physiological responses in social interactions might be observed in horses from other housing conditions. For example, aggression levels were found to be higher in unestablished groups (Christensen et al., 2002, 2011; Crockford et al., 2008; Granquist, Thorhallsdottir and Sigurjonsdottir, 2012; Jørgensen et al., 2009; Noller et al., 2013; Nuñez et al., 2014; Vandeleest et al.,

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2016), in horses with limited turnout experience (Jørgensen et al., 2009a), or under limited space allowance (Flauger and Krueger, 2013; Jørgensen et al., 2009; Pierard, McGreevy and Geers, 2019). Furthermore, heart rate measurements were taken under regular field situations, i.e., not during feeding times or when horses had to access limited resources, situations which have been found to act as a stressor (Aschwanden et al., 2008; Kotrschal, Hemetsberger and Dittami, 1993).

Therefore, future research could systematically assess whether different group housing environments impact horses' physiological response during social interactions, and how different breeds and personality-types cope with different conditions. Further understanding of how group housing impacts domestic horses' behaviour and physiology can inform the design and conceptualisation of group housing systems that promote equine welfare by reducing stress and promoting positive affect.

5.1.3.2 Implications for horse handling and husbandry routines

In **Chapters 3 and 4**, it was asked whether domestic horses' behaviour and cardiac activity did depend on the presence of a closely bonded conspecific during stressful situations, an aspect that has not previously been explored. During the novel object exposures, horses reacted mostly independently of their social context. Only following the object exposure, they spent more time in closer proximity, i.e., within two horse-lengths of their highly-preferred group member as compared to their least preferred one. During the separation from their social group, the horses showed reduced vigilance when they were in the company of a member of their group, independent of the bond strength with their separation partner. This could indicate a general response to being in the company of any familiar conspecific as opposed to a bond-specific effect. Thus, in the context of mild stress, there was little evidence that horses gained a social buffering effect from the presence of a highly-preferred other horse. Several possible reasons for these findings were discussed previously, see section 5.1.2.4, page 170ff. Overall, the presented findings suggest that horses predominantly self-regulate their behavioural and physiological stress response in the context of mild stress in a familiar environment. Furthermore, the presence of any familiar conspecific can promote safety, as shown by decreased vigilance in the presence of a highly and a less-preferred conspecific. Therefore, during handling procedures that

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require a separation of horses from their group, the presence of any group member possibly enhances their perceived safety.

Domestic horses are exposed to many different stimuli in their environment, during handling, or equestrian activities that can potentially be stressful, such as clipping (Yarnell, Hall and Billett, 2013), road transport (Schmidt et al., 2010b;c), training (Fowler, Kennedy and Marlin, 2012; Munsters et al., 2013; Schmidt et al., 2010a), weaning (Rogers et al., 2012), or separation (Collyer and Wilson, 2016; Hartmann, Christensen and Keeling, 2011). Therefore, future studies on social buffering could expand to other types of stressors that horses encounter during human handling and assess whether horses benefit from the presence of closely bonded conspecifics. Such findings could be translated into evidence-based suggestions for handling strategies that reduce horses' experiences of stress.

5.1.3.3 Discrepancy between behavioural and physiological indicators of stress

Another finding that is relevant for equine welfare is that the behavioural and physiological stress response during separation did not correspond (**Chapter 4**). Despite a higher rate of vigilance during isolation, the horse's heart rate and heart rate variability did not differ across the three test conditions (alone, with a highly-preferred, and a with less-preferred other horse). This finding seems unexpected; however, similar patterns have been described in other studies. Squibb et al. (2018) have found that horses that showed greater behavioural resistance in a novel handling task did not show a higher heart rate than compliant horses. Similarly, it has been found that horses that showed a higher behavioural reactivity during clipping, did not show a significantly higher heart rate than less reactive horses (Yarnell, Hall and Billett, 2013). In contrast, other studies have shown the opposite effect. Munsters et al. (2013) have found that horses that stood still and did not exhibit a behavioural stress response to an external stressor showed a significant increase in heart rate. Furthermore, horses that showed less behavioural arousal when awaiting high caloric food showed a significantly higher heart rate (Safryghin, Hebesberger and Wascher, 2019).

Taking these findings together, it seems relevant to make equestrians aware that, on the one hand, the occurrence of behaviours that can indicate stress

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does not necessarily correspond with a physiological stress response. Rather, it could indicate learned behaviour in specific contexts (Cooper and McGreevy, 2002); and that, on the other hand, the absence of stress behaviours does not always indicate that the horse does not experience stress. Future research could address this phenomenon and assess whether there are specific contexts where these discrepancies between the behavioural and physiological stress response occur. In addition, more indicators seem necessary to (1) reliably distinguish between stress-related and learned behaviour. And (2) that can help detect a stressed horse that does not display a behavioural stress response. Detecting horses' stress responses and adjusting handling, training, or husbandry accordingly can help to reduce stress and to promote positive welfare (Fraser, 2008) and, furthermore, reduce handling related risks (Christensen, Zharkikh and Chovaux, 2011).

5.1.3.4 Future perspectives: social buffering between humans and horses

As horses are domestic animals, their human handlers may be an important source of social buffering during handling, training, or husbandry routines. Previous research has demonstrated that horses reference their behaviour to a human's positive and negative facial expressions during a novel object test (Schrimpf, Single and Nawroth, 2020). Whether this behavioural adaptation depends on the relationship quality between the human and the horses is not yet known. Some studies have addressed the effect of the relationship quality on human-facilitated buffering effects during novel handling tasks. These studies have provided different findings: one study operationalised the relationship quality as 'owner' vs 'unfamiliar' handler and found that the horses' behavioural and physiological response did not differ between the two handler categories (Ijichi et al., 2018). Another study found that horses' heart rate was lower when handled by a familiar person than a stranger (Hockenhull et al., 2015). These findings suggest a variation in the strength of bond or relationship quality between horses and their owners, possibly depending on their tenure and training practices (Fureix et al., 2009; Trösch et al., 2020). Thus, horses may differ in their response to stressful situations when they are with owners or handlers of different relationship qualities. Studies in dogs, another domestic species, have shown that they were calmer and more explorative when exposed to a novel environment

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with their owner than when with a stranger or alone (Horn, Huber and Range, 2013; Palmer and Custance, 2008; Payne et al., 2016; Schöberl et al., 2016). Furthermore, it has been found that the buffering efficacy of the dog owner can depend on the relationship quality of the dog-human pair (Schöberl et al., 2009, 2016). Despite horses not being domesticated as long as dogs (horses: min. 5000 years (Levine, 2005); dogs: min. 60.000 years (Kotrschal, 2013)), there could be a similar underlying mechanism between horses and their human handlers.

Another aspect that could be addressed in future research is a comparative assessment of buffering provided by conspecifics versus human handlers. Previous studies have either addressed the impact of conspecifics on horses' fear responses (Christensen et al., 2008; Rørvang, Ahrendt and Christensen, 2015; Rørvang and Christensen, 2018) or have compared calming effects of familiar and unfamiliar human handlers (Hockenhull et al., 2015; Ijichi et al., 2018). However, it is unknown whether and how these effects relate to each other, and whether there is a difference in how horses benefit from the presence of a conspecific compared to the presence of a human. Experiments in dogs showed that humans were a more effective buffer of their physiological stress response when exposed to a novel environment than with familiar conspecifics (Tuber et al., 1996). Studies comparing effects of social support provided by conspecifics or humans could provide more insight into how domestication processes altered horses' behaviour and physiological responses, and what role human handlers take in our domestic animals' lives.

5.2 Conclusion

This study investigated whether domestic horses (*Equus caballus*) benefit from social buffering provided by closely bonded, but unrelated, members of their group. In a combination of behavioural observations and experiments bond-related buffering effects on behavioural and cardiac activity levels were assessed in three contexts: during social interactions, during separation from their social group, and when exposed to a novel object.

In conclusion, there was no strong evidence that closely bonded but unrelated domestic horses provided each other with social buffering in the specified study contexts. During social interactions, the bond relationship did not affect the horses' heart rate. Besides bond-dependent proximity seeking behaviour in response to the novel object exposure, the horses either exhibited generalised behavioural and physiological responses towards closely bonded and less closely conspecifics throughout the experiments, or individual responses that were independent of their social context. It was discussed that these generalised strategies could have an adaptive benefit for horses, as a prey species, and that individual behavioural strategies could be adaptive in contexts of low stress intensity.

The results of this thesis are also relevant to equine science and equine welfare. They demonstrated that horses, which are kept in established groups, predominantly engaged in low-intensity agonistic interactions. These did not elicit a stress response. Furthermore, allowing horses to interact socially can facilitate positive welfare. Mutual grooming was accompanied by a low heart rate, indicating relaxation and potentially a positive emotional state. Nevertheless, the presented findings need to be generalised with caution as they apply to mild stress conditions. The restricted sample size must also be taken into account.

Additionally, possible future directions in social buffering research were identified. For example, studying social buffering specifically under different stress intensities could yield further insight into which factors determine whether horses display individual or socially-dependent responses and whether the social context affects horses' behavioural and physiological (stress) responses. Moreover, extending the research to other types of social bonds that are typical for horses' social organisation can provide a more comprehensive understanding of whether and how different bonds play a role in the context of social buffering. End of word count.

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APPENDIX A – Social interactions

A.I Intercorrelations of affiliative behaviours

Bond strengths between all horses of the groups were quantified by calculating the Dyadic Composite Sociality Index (DSI), following Silk et al. (2013). The DSI-calculations were based on recorded behaviours during the initial scan and focal sampling procedures (**Chapter 2**, sections 2.2.2 and 2.2.2.1, page 40f). The candidate behaviours for the DSI were grooming, body contact (sniffs, touches and rubbing head), approach and following, and the rate of horses being nearest neighbours (Cameron, Setsaas and Linklater, 2009). To assess which of those behaviours were intercorrelated a Mantel test was carried out using the R package ‘vegan’ (Oksanen et al., 2019). Correlated behaviours with a correlation coefficient $r \geq 0.5$ (Field, Miles and Field, 2012) were included in the DSI. These were nearest neighbour rates, approach and following rates, and rates of body contact (Table A.1).

Table A.1 Results of the Mantel test to assess the candidate behaviours' intercorrelation for calculating the Dyadic Composite Sociality Index. Given are behaviours and their correlation coefficient (r) and the significance level (p). Significant results and results with a correlation coefficient of $r \geq 0.5$ are marked in bold.

Variable 1	Variable 2	Stable A		Stable B	
		r	p	r	p
Groom	Body contact	0.23	0.057	-0.04	0.051
Groom	Proximity	0.23	0.058	0.44	0.042
Groom	Approach/Follow	0.24	0.044	0.47	0.037
Body contact	Nearest neighbour	0.79	0.001	0.68	0.003
Body contact	Approach/Follow	0.85	0.001	0.29	0.075
Nearest neighbour	Approach/Follow	0.89	0.001	0.57	0.006

Appendices

APPENDIX B – Novel object test

B.I Heart rate during test phases in the highly-preferred and less-preferred conditions

As shown in **Chapter 4**, section 4.3.1.2, page 142, highly-preferred dyads were in closer proximity after the initial object exposure than less-preferred dyads. An additional comparison was conducted to assess whether this difference in proximity corresponded to a difference in the horses' heart rate. It was assessed whether focal horses' mean heart rate during the single test phases (Pre, Phase 1, Phase 2, Phase 3, Post) differed between the two dyadic test conditions (highly-preferred, less-preferred). Wilcoxon signed-rank tests did not reveal any differences ($n = 9$: Pre: $V = 12$, $p = 0.441$; Phase 1: $V = 9$, $p = 0.234$; Phase 2: $V = 20$, $p = 0.834$; Phase 3: $V = p = 0.944$; Post: $V = 13$, $p = 0.529$; Figure B.1).

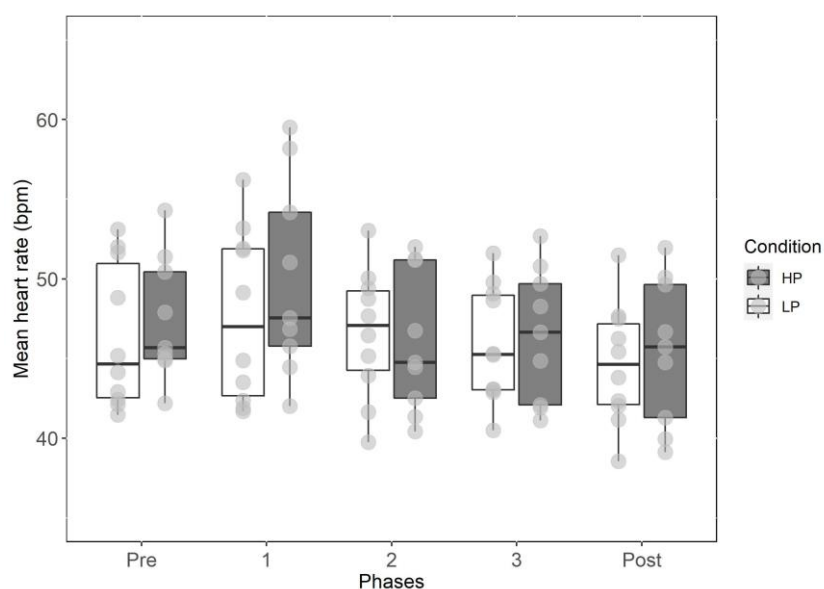


Figure B.1 Mean heart rate per phase for the dyadic test conditions. Tukey whiskers plot for mean heart rate in beats per minute (bpm) for the different phases of the experiment and test conditions: HP, highly-preferred (grey), LP less-preferred (white). The outwards pointing whiskers extend to the smallest and largest value at most $1.5 \times \text{IQR}$ from the hinge. There was no difference in mean heart rate between the test conditions taken for the five phases of the novel object test.

