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PREFACE

The number of manifestations from the general public with arguments against the use of horses in sports has increased. The most common argument is that using horses, or animals in general, for sports is an attack on the animal's dignity. However, most of these arguments are not evidence-based. As a rider, I have asked myself the questions "are we using or sharing activities with horses? Are horses enjoying this activity as I am?" for many years.

At the end of my veterinarian academic years I had the opportunity to perform an internship in IRSEA (Research Institute in Semiochemistry and Applied Ethology) for six months. In this internship, we have studied equine cognitive skills and abilities and some implications of chemical communication. At this point, I have realised that positive or negative interactions with horses could modify their perception of humans. I started asking the question about "how would horses perceive training". The only way to answer that question is by studying horses in the contexts of training or working.

The arguments of the lay population against the involvement of equines in sports needed to be investigated. Scientific information should demystify what is right or wrong, and most importantly help to improve equine living conditions, careers and social relationships with humans, thus improving equine welfare in general. This thesis provides some information on horses' perception of their training or working activities. The knowledge about the perception of horses is the beginning of an intellectual process that aims to improve equine welfare.

The perception of an activity depends on the methods employed in training, work or competition. In this study some exercises, that are common to many equestrian disciplines, were investigated and compared to see their pros and cons in relation to equine emotional responses. Finally, this study provides reliable information that can be used by riders, trainers and therapists to improve equine welfare.

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Merci à Céline, Estelle, Eva et Sana pour toutes les analyses statistiques, mais surtout de votre implication dans la préparation des protocoles de recherches et de vos visites sur le terrain pour mieux comprendre les objectifs de chaque essai. Votre équipe a été présente dès le début (préparation) à la fin (jour de la soutenance) de ma thèse. Cela est incroyable et je vous en remercie. **Merci beaucoup.**

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« The better part of one's life consists of his friendships » (Abraham Lincoln). Je voudrais remercier certains amis plus proches qui constituent ma nouvelle famille en France. Merci spécialement à Véronique et Patrick, Cécile et Sylvain, Míriam et Alessandro, Pietro et Elisa.

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TITRE: Influence of the activities imposed by the human being on equine emotional responses

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RESUME EN FRANÇAIS

Les activités des chevaux au bénéfice de l'être humain sont variées et ont évolué au cours du temps. Le cheval a exercé des fonctions de transport, il est devenu un avantage en temps de guerre et un athlète sur plusieurs disciplines sportives très variées. Actuellement, il participe aussi à des thérapies destinées à l'être humain. Les chevaux participent souvent à plusieurs disciplines équestres ou activités de travail, ce qui demande une grande capacité d'adaptation de la part de l'animal. En conséquence, l'implication des chevaux sur des activités très variées peut être à l'origine de réponses émotionnelles également variées (positives ou négatives).

Les réponses émotionnelles des chevaux aux différentes activités peuvent être étudiées à l'aide d'indicateurs comportementaux et physiologiques des émotions. L'objectif de cette thèse était d'étudier l'influence de certaines activités que l'être humain impose au cheval sur ses réponses émotionnelles. Un autre objectif de cette thèse était d'étudier des nouveaux indicateurs physiologiques (biologiques) qui peuvent avoir des implications au niveau des émotions (ex : ocytocine).

Les résultats ont démontré une variation des réponses émotionnelles des chevaux en fonction de l'activité à laquelle ils participaient. Les exercices latéraux ont été bénéfiques pour l'état émotionnel des chevaux impliqués dans différentes disciplines équestres (dressage, concours de sauts d'obstacles et concours complet d'équitation). L'équithérapie n'a pas

produit d'émotions négatives ni d'émotions positives aux chevaux qui y ont participé. Les réponses émotionnelles à un stimulus spécifique étaient différentes entre les chevaux de travail (travail de trait au Chili) e les chevaux de sport (rodéo). Les niveaux d'ocytocine plasmatique libre observés étaient différents entre les activités étudiées.

Ces résultats ouvrent plusieurs perspectives et voies de recherche. Les réponses émotionnelles des chevaux devraient être étudiées sur chaque discipline/activité séparément. L'étude de méthodes d'entraînement et de travail peut dévoiler des solutions pour améliorer le bien-être des chevaux. Des nouvelles études, sur des indicateurs biologiques (positifs ou négatifs) qui permettent d'étudier les réponses émotionnelles des chevaux sont nécessaires.

Mots-clés : bien-être, biomarqueurs, émotions, équidés, équitation, variabilité de la fréquence cardiaque

ABSTRACT

Humans have made profits from equine activities, which evolved through times. Horses worked as transport facilitator, become an advantage in times of war and are athletes in many sport disciplines. Nowadays, horses are involved in therapies to improve human medical conditions as well. Horses are often involved in different equestrian disciplines or work activities, which require a great adaptation capacity of the animal. Therefore, horses' implication in diverse activities may probably be the reason for the variability of equine emotional responses (either positive or negative).

Equine emotional responses to different activities can be investigated using behavioural and physiological indicators of emotions. The aim of this thesis was to study the influence of some activities imposed by humans in horses' emotional responses. The investigation of novel physiological indicators (biological ones) that may play a role in emotional responses (e.g. oxytocin) was also an aim of this thesis.

Results from this thesis demonstrated that equine emotional responses vary according to the activity in which horses are involved. Lateral exercises were beneficial for the emotional states of horses involved in different equestrian disciplines (dressage, jumping, eventing). Equine-assisted therapy did not produce negative nor positive emotions. Sport horses (Chilean rodeo) differed from working horses (urban draught work) in their emotional responses to a specific stimulus. Different plasma levels of free oxytocin were associated with different equine activities.

These results disclose many perspectives and research questions. Equine emotional responses should be investigated within each equestrian discipline or work activity separately. Training/working methods investigations may reveal innovative solutions to improve equine welfare. Further investigation on biological indicators (positive or negative) is still needed.

Keywords: biomarkers, emotions, equines, equitation, heart rate variability, welfare

LIST OF PUBLICATIONS:

1. **Articles:**

- Mendonça, T., Bienboire-Frosini, C., Kowalczyk, I., Leclercq, J., Arroub, S. and Pageat P., 2019. Equine activities influence horses' responses to different stimuli: could this have an impact on equine welfare? *Animals*, 9, 290, https://doi.org/10.3390/ani9060290

- Mendonça, T., Bienboire-Frosini, C., Sanchez, N., Kowalczyk, I., Teruel, E., Descout, E. and Pageat, P. de la Guérinière was right: shoulder-in is beneficial for horses physical and mental states. *Journal of Veterinary Behavior*, 38, 14-20, https://doi.org/10.1016/j.jveb.2020.05.003

- Mendonça, T., Bienboire-Frosini, C., Menuge, F., Leclercq, J., Lafont-Lecuelle, C., Arroub, S. and Pageat P., 2019. The impact of equine-assisted therapy on equine behavioral and physiological responses. *Animals*, 9, 409, https://doi.org/10.3390/ani9070409

- Rosselot, P., Mendonça, T., González, I. and Tadich, T., 2019. Behavioral and physiological differences between working horses and Chilean rodeo horses in a handling test. *Animals*, 9, 397, https://doi.org/10.3390/ani9070397

- Oliva, J., Mengoli, M., Mendonça, T., Cozzi, A., Pageat, P., Chabaud, C., Teruel, E., Lafont-Lecuelle, C. and Bienboire-Frosini, C., 2019. Working smarter not harder: oxytocin increases domestic dogs' (*Canis familiaris*) accuracy, but not attempts, on an object choice task. *Frontiers in psychology*, 10, 2141, https://doi.org/10.3389/fpsyg.2019.02141 (please see annex 1)

- Bienboire-Frosini, C., Chabaud, C., Mendonça, T., Mengoli, M., Arroub, S., Oliva, J., Cozzi, A. and Pageat, P., 2019. Investigating the oxytocinergic system: plasma measures of free vs. total oxytocin fractions in two animal models. Published as an abstract in *Psychoneuroendocrinology*, 107(S), 33, https://doi.org/10.1016/j.psyneuen.2019.07.094

2. International congresses proceedings, oral presentations and posters:

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- Mengoli, M., Mendonça, T., Oliva, J., Bienboire-Frosini, C., Chabaud, C., Codecasa, E., Jochem, M., Cozzi, A. and Pageat, P. Do assistance dogs show work overload? Canine prolactin as clinical parameter to detect chronic stress-related responses. IVBM, Samorin, Slovakia, 2017.

- Mendonça, T., Bienboire-Frosini, C., Kowalczyk, I., Leclercq, J., Lafont-Lecuelle, C. and Pageat P. A novelty test to assess temperament in equine athletes. EVCBMAW, Berlin, Germany, 2018.

- Mendonça, T., Bienboire-Frosini, C., Leclercq, J., Sanchez, N., Descout, E. and Pageat, P. Few stimuli versus several stimuli training sessions. IVBM, Washington DC, USA, 2019.

- Mendonça, T., Bienboire-Frosini, C., Menuge, F., Lafont-Lecuelle, C., Arroub, S. and Pageat P. How do horses perceive equine-assisted therapies: as a negative or a positive event? EVCBMAW, Eindhoven, Netherlands, 2019.

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- Mengoli, M., Mendonça, T. and Pageat, P. Monitoring dog's daily activity level with a remote device in separation related disorders: a clinical report. Berlin, Germany, 2018

- Bienboire-Frosini, C., Chabaud, C., Mendonça, T., Mengoli, M., Arroub, S., Oliva, J., Cozzi, A. and Pageat, P. Investigating the oxytocinergic system: plasma measures of free vs. total oxytocin fractions in two animal models. ISPNE, Milan, Italy, 2019

- Mendonça, T., Bienboire-Frosini, C., Arroub, S., Menuge, F., Lafont-Lecuelle, C. and Pageat P. Influence of patients' expectation on equine emotional responses during equine-assisted therapy sessions. EVCBMAW, Eindhoven, Netherlands, 2019. Prize for the best poster presentation by an ECAWBM-BM resident.

GENERAL INTRODUCTION

The horse:

Origin and evolution

Equids belong to the *Perissodactyla* order (Wilson and Reeder 2005). Animals from this order can be easily recognised as they are odd-toed ungulate (Wilson and Reeder 2005). The study of fossils allowed to identify animals from the *Hyracotherium* genus that lived fifty to thirty-eight million years ago in North America (McBane and Douglas-Cooper 1997). These animals were the size of lamb (10-50kg) and stand on three toes on the hindlimbs (McBane and Douglas-Cooper 1997). At the time North-America and Euro-Asia were united by the land, which facilitated the emigration of these ancient animals (McBane and Douglas-Cooper 1997). Fifteen to ten million years ago, through natural selection, these animals have seen anatomical modifications that included having only one toe on each foot in contact with the ground, longer limbs and heavier bodies (approximately 500kg) and noses (McBane and Douglas-Cooper 1997; MacFadden 2005). However, with the land rupture by the Bering strait, equids had different evolutions and during the ice age, almost all were extinguished, including all those that lived in North America (Peplow 1998). Equids that survived in Europe and Asia are at the origin of the modern equids (Peplow 1998).

Animals with only one limb were classified within the *Equus* genus and different species can be observed (McBane and Douglas-Cooper 1997; Pageat 2011a). The modern horse (*Equus caballus*) has genetic differences from all the other equids, e.g. Przewalski's horse, zebras or donkeys (Hatami-Monazah and Pandit 1979; McGreevy 2004a). The word "equine" refers to all the *Equus* species according to taxonomy. Please note that in this work the word "equine" will only be employed regarding the *Equus caballus*, thus the modern and domesticated horse, because only this species was investigated.

Equines are herbivores and nomads, which means that they would rather keep moving from one grazing field to another than stay at only one grazing field (McGreevy 2004a). Equines anatomy specificities (see anatomy and biomechanics section) are associated with an important time budget spent on feeding activities (Ferreira 2016). These animals are natural preys as they are not equipped with horns or antlers, so they rely on their perception, caution and agility to avoid predators (McGreevy 2004a). This is an important characteristic of these species because while they spend most of their time feeding, they should equally avoid predators. Equids have long noses that allows them to maintain surveillance while eating (McGreevy 2004a). Additionally, they rely on relationships with their conspecifics to increase the area of surveillance against predators (Heitor et al. 2006; Cozzi et al. 2010).

While predation is a real risk in nature, the modern horse is mostly protected from it thanks to the co-habitation with humans (Goodwin 1999; Cooper and Albentosa 2005). Horses intraspecies relationships may have been created at first to improve predator avoidance, but these relationships have social implications as well and were considered an indispensable need for horses (VanDierendonck and Spruijt 2012).

Equines have evolved sideways with humans through domestication and were used for many different purposes (Peplow 1998). Equine and human evolutions cannot be separated. Horses domestication allowed humans to travel across the world and meet populations with different genetic profiles, thus increasing human genetic variability (McGreevy 2004a). Domestication has produced many modifications to the equine natural environment; thus these animals needed to adapt to humans and to a whole new environment that has changed through times (Pageat 2011b).

Equine morphological evolution created this tall and large animal. While a horse seems very strong and resistant due to its dimensions, its anatomic structures are often challenged by the activities that humans perform with these animals (Baxter et al. 2011; Whitton 2016).

Anatomy, physiology and biomechanics

Equine anatomy and physiology are at some extent challenged by the environments and activities in which horses are involved. In this section only the structures that are most often challenged by the activities investigated in this PhD will be described.

Musculoskeletal system

Evolution caused modifications in equine musculoskeletal anatomy (Goff 2016). Limbs are lengthier, muscle tissues are located at the proximal end of the limb and the distal limb musculature was replaced by elastic tendons (see fig. 1; Kainer and Fails 2011; Goff 2016). These modifications occurred to decrease limb weight and to increase energy storage, thus reducing the energetic cost of locomotion (Goff 2016). This anatomical modification occurred in other species as well; e.g. Greyhound dog, a breed that was bred to sprint (Kemp et al. 2005).

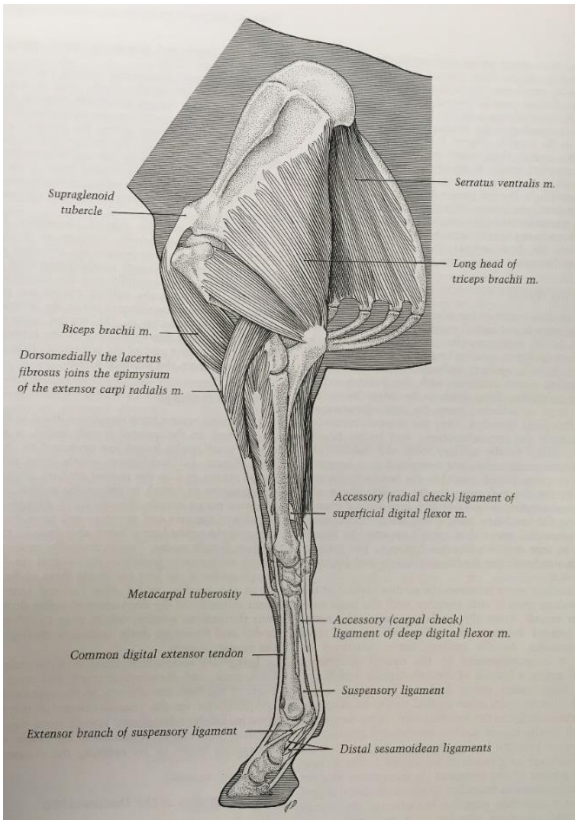


Figure 1: Equine limb anatomy with the proximal limb mainly composed by muscles and the distal limb composed by ligament and tendons. (Source: Kainer and Fails 2011)

Equine foot and pastern (see fig. 2) include the three phalanges, the distal sesamoid (as known as the navicular bone) and the associated structures, as the ligaments that are responsible for the stability of the joints between these bones (Gregory 2013). The distal articular surface of the middle phalanx, the articular surface of the distal phalanx and the articular surfaces of the navicular bone are limited in range of motion (Kainer and Fails 2011). Horses dispose of the digital cushion that has anticoncussive function, protecting the foot anatomic structures from impacts to some extent (Kainer and Fails 2011). Additionally, the hoof wall elasticity helps to sustain the impact of equine locomotion (Kainer and Fails 2011). The hoof wall regions are the toe, medial and lateral quarters and the heels (Kainer and Fails 2011). This structure is thick at the toe and becomes progressively thinner at the quarters, finally becoming thicker again in the heels (Douglas et al. 1996; Kainer and Fails 2011). Different elasticity levels are observed in the different regions of the hoof wall, which enables the wall to adapt to different sources of impact (Douglas et al. 1996; Kainer and Fails 2011). The superficial surface of the hoof wall has no sensitivity and protects the subjacent structures (bones, tendons, ligaments, digital cushion, blood vessels and nerves) from temperature challenges, dehydration and from infections (Martins da Silva 2009a). The ventral surface of the hoof (see fig. 3) is formed by the sole, frog, heels, bars and ground surface of the wall (Martins da Silva 2009a; Kainer and Fails 2011). The sole does not normally bear weight on its ground surface except near it junction with the white line (line between the hoof wall and the sole), however, it bears the internal weight from the solar surface of the third phalanx (Martins da Silva 2009a; Kainer and Fails 2011). The frog is a wedge-shaped elastic structure because of its water content (>40%), which should be in contact with the ground playing a role on decreasing the direct impact over the other hoof structures (Martins da Silva 2009a; Kainer and Fails 2011). When compressed, the frog induce pressure on the bars and the digital cushion, creating hoof expansion to protect its internal structures (Martins da Silva 2009a).

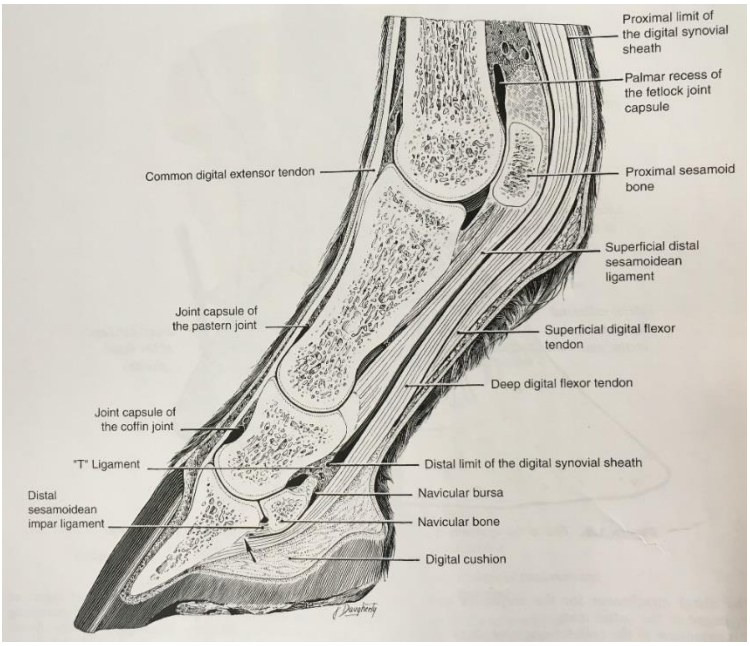


Figure 2: Equine anatomy of the distal limb with the fetlock and the digit. (Source: Kainer and Fails 2011)

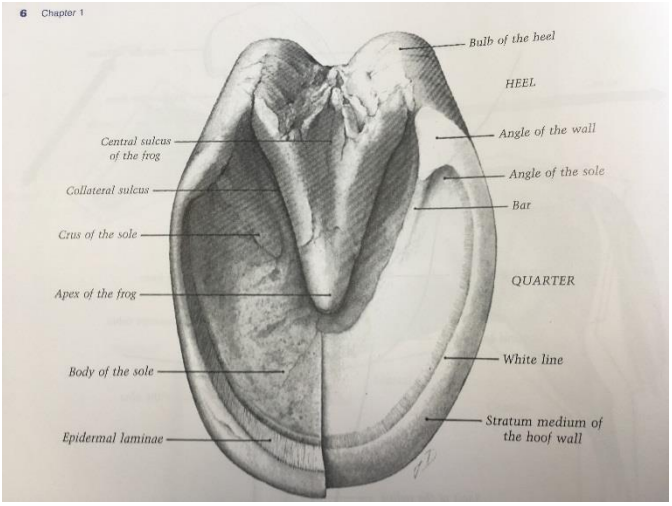


Figure 3: Equine anatomy ventral surface of the hoof. (Source: Kainer and Fails 2011)

Since the distal limb is not provided with muscles, weight bearing will solicit bones, tendons and ligaments, which play a role in avoiding joint instability or even tissue failure (Goff 2016). This means that horses are predisposed to musculoskeletal injuries, with higher prevalence and incidence of injury in the distal limb (Goff 2016). The study of anatomy and biomechanics provides evidence that explains how these animals who are very strong and fast can have their anatomical structure challenged, thus being predisposed to injuries.

Horse axial structures include 7 cervical, 18 thoracic, 6 lumbar, 5 sacral and 15-21 caudal vertebrae (Kainer and Fails 2011). The first two cervical vertebrae have a particular structure to allow vertical flexion and extension (first vertebra: atlas) and lateral flexion/rotation (second vertebra: axis) of the head (Martins da Silva 2009b). From the third to the seventh vertebrae, they are similar to one another and, as in other species, they are progressively shorter from cranial to caudal (Kainer and Fails 2011). Cervical structures might be challenged by hyperflexion or by increased contractibility of the dorsal muscles of the neck in training (Martins da Silva 2009c; Zebisch et al. 2014). Thoracic vertebrae are numerous when comparing to other species (dogs, cats and cows: 13; pigs: 14-15) and articulate with the ribs and the latter with the sternum, which along with dorsal muscles and upper forelimb muscles increase the stability of this region (Martins da Silva 2009b; Kainer and Fails 2011). Riders and the apparatus load this anatomical region in horseback riding activities and the communication provided by the riders with their legs will happen in this region as well (Netto de Almeida 1997b). Lumbar vertebrae are stabilised by the dorsal musculature, having limited flexion and extension ranges of motion due to intervertebral joints tightness (Martins da Silva 2009c; Kainer and Fails 2011). The joint between the 6th lumbar vertebra and the sacrum allows a huge range of motion, which allows the horse to move the hindlimb under the general body mass, creating impulsion or allowing the horse to move backward as well (Martins da Silva 2009b). The sacrum is a single bone that articulates with the hip in the sacroiliac joint (Kainer and Fails 2011). Thoracic, lumbar and sacroiliac lesions are often observed in horses, often due to improper training techniques (Kainer and Fails 2011).

The described anatomic structures are an adaption of horses' to travel over short distances at great speed or over long distances at a lesser speed, so they may promptly avoid predators, find new food sources and avoid unfavourable weather conditions (Gregory 2013). However, horses perform many activities that differ from grazing or avoiding predators, like racing or jumping, which challenge equine anatomy (Ely et al. 2009) and probably emotional responses as well.

Equine locomotion

The natural equine locomotion includes different gaits (Martins da Silva 2009d). Some gaits are specific to some breeds, as the pace or tolting in Icelandic horses (McGreevy 2004b). For this reason, in this work, only the general gaits (walk, trot and canter), which are the most observed, will be described (see tab. 1). The gaits can be defined according to some characteristics: rhythm, symmetry, and the duration of the suspension phases (Baxter et al. 2011). Rhythm corresponds to the cadence of the footfall within a gait, taking into account timing (number of beats) and impulsion (McGreevy et al. 2005; Baxter et al. 2011). Rhythm can be classified in 2, 3 or 4 beats (Martins da Silva 2009d; Back and Clayton 2013). Symmetry refers to the synchronisation of the members of one side of the horse (e.g. left) with the contralateral side (e.g. right), which means that the movements of one side should be very similar or equal to the movements of the other side (Martins da Silva 2009d; Baxter et al. 2011). The absence of suspension phases means that the horse should always have, at least, a member on the ground when moving (Martins da Silva 2009d). Suspension phases are moments in which, for a given time, the horse will not have any member in contact with the ground (Martins da Silva 2009d).

Gait	**Characteristics**	**Gait sequence**
Walk	4-beats Symmetric Without suspension phases	RH, RF, LH, LF
Trot	2-beats Symmetric With suspension phases	RH-LF, LH-RF
Canter	3-beats Asymmetric With suspension phases	RH, LH-RF, LF or LH, RH-LF, RF

Table 1: Gaits description according characteristics and sequences. RH: right hindlimb; LH: left hindlimb; RF: right forelimb; LF: left forelimb (Adapted from Martins da Silva 2009c)

Each of the described gaits is classified into other categories and exercises in competition, according to the Fédération Equestre International (FEI 2019). These categories include terms as “medium”, “collected”, “working”, “extended”, etc. (FEI 2019). Nevertheless, this information means that within equine locomotion many details and different exercises are researched by horseback riders. For this reason, some gait classifications in the literature separate natural and artificial gaits (Martins da Silva 2009d). The description of some of these categories, defined by the FEI, will be provided in the section “horses at sports and at work”.

Equine lateral preferences were previously described (McGreevy and Rogers 2005; Esch et al. 2019). Laterality means that horses would perform more easily to one side (right or left hand) than to the other (McGreevy and Rogers 2005; McGreevy and McLean 2010a; Marr et al. 2018). Even if equine laterality is not surprising for horseback riders, its consequences, a decrease in performance or an unusual response to a specific exercise at the right or the left hands, might be particularly surprising or unwelcomed for the rider (Mcgreevy 2004; Mcgreevy and McLean 2010). Characteristics of equine locomotion that influence the human-horse relationship will be described in the “horses at sport and at work” section.

Digestive system

Equine teeth allow horses to produce very efficient mastication (Martins da Silva 2009e). The particularity of equine teeth is their lifetime evolution. Herbivores, like horses, who eat fibrous diets, experience abrasion of their teeth (Dixon and Dacre 2005; Martins da Silva 2009e). Although dental abrasion is a natural process, equines dispose of continuous dental growth, which is a condition that helps horses on keeping healthy and efficient teeth (Dixon and Dacre 2005). Abrasion and growth were complementary in nature; however, humans influence on horses’ lives may have modified equine feeding conditions and both processes (abrasion and dental growth) may have been impacted (Martins da Silva 2009e). Human influences in equine dental health will be detailed in the following sections.

The equine stomach is a very small organ in proportion to the equine body structure, representing only 8-12% (8-15 litres) of the total volume of the digestive tract (Sisson and Grossman 1986; Ferreira 2016). Additionally, the equine stomach has a powerful cardia (entrance of the stomach) that does not allow any reflux or air coming back to the oesophagus in normal conditions (Sisson and Grossman 1986). This means that in normal health conditions horses cannot vomit (König and Liebich 2006; Cintra 2016). The digestion duration in the stomach is about one hour for the 2/3 of each meal and about five to six hours for the resting 1/3 (Martins da Silva 2009f). As a herbivore, the energy intake from food sources is very poor, which means that equines must eat great amounts of food to meet their daily energetic needs (Pageat 2011c). Therefore, having a small stomach and ingesting low energy food, equids need to repeatedly ingest small amounts of food (Ferreira 2016). These particularities of the equine stomach and digestion were introduced because they have implications on the management and husbandry of these animals. These implications will be further detailed in the following sections.

Behavioural neuroanatomy

Behavioural neuroanatomy include different components of the central nervous system (CNS): forebrain, brainstem, cerebellum and spinal cord (Hahn 2004). All the nerves outside CNS are collectively called the peripheral nervous system (de LaHunta 2009a).

The forebrain includes the cerebrum, hypothalamus and thalamus (Mills and Nankervis 1999). The cerebrum is composed by the two cerebral hemispheres, in which the cell bodies are in two different regions: superficial surface (gyri and sulci) and deep to the surface (basal nuclei). These neurons are connected by axons to the brainstem (Hahn 2004). The corpus callosum consists of axons connecting the two sides of the cerebral hemispheres (König et al. 2006). Only one cranial nerve is associated with the forebrain, which is the olfactory nerve, a very large nerve in horses that plays an important role in chemical detection (Cunningham 2004; Hahn 2004). The cerebrum is responsible by the perception, emotion, voluntary

movement and learning (Hahn 2004). The hypothalamus is the higher centre for regulation of the autonomic motor activity, operating without direct voluntary control (Greco and Stabenfeldt 2004). However, the cerebral cortex may influence the hypothalamic hormonal regulation, which is the case with stimuli that have been previously associated with emotionally relevant events (Hahn 2004). Therefore, hormonal consequences may influence behavioural responses (Hahn 2004). The thalamus is an integrating system with pathways relaying the brainstem and higher centres in the cerebrum (Cunningham 2004), All impulses heading to the cerebrum, with the exception of the ones produced by the olfactory nerve, must synapse in the thalamus (Cunningham 2004). In normal states, the thalamus should filter sensory inputs in response to different stimuli, being directly involved in the conscious perception of some sensations and playing a role in the maintenance of consciousness, the focusing of attention, and the initiation of sleep (Newman 1995).

The brainstem includes the midbrain, pons and medulla oblongata and includes the nuclei of most cranial nerves (Hahn 2004). The brainstem structures play important roles on controlling cardiac and lung functions, eye movement and many other critical functions, while the cerebellum is involved in the control of movement and balance (de LaHunta 2009a). The midbrain contains the pathways connecting the brainstem and the cerebrum, the nuclei of cranial nerves III (oculomotor) and IV (trochlear), which are involved in reflexive eye blinking, and reactions to visual or auditory stimuli, and the upper motor neurons that are involved in equine locomotion, especially in equine gait changes (de LaHunta 2009a). The pons includes the nuclei of the cranial nerves V (trigeminal), which is responsible for the mastication muscles and is also involved in facial sensation (de LaHunta 2009a). The medulla oblongata includes the nuclei of the last seven cranial nerves VI-XII, which are involved in movement of the eyes (VI: abducens) and face (VII: facial), balance and hearing (VIII: vestibulocochlear), taste and pharyngeal movements (IX: glossopharyngeal), control of larynx muscles and viscera (X: vagus), control of the neck (XI: spinal accessory) and tongue movement (XII: hypoglossal) (de LaHunta 2009a). In the medulla oblongata and cerebellum are located the sensory nuclei

involved in proprioception (de LaHunta 2009b). The cerebellum modulates the tone of contraction of opposite muscles, being involved in balance and responsible for smooth movements (de LaHunta 2009b).

The more relevant structures involved in emotional responses are located in different regions of the CNS (Hahn 2004). Cortex brain may be divided, according to their function, in three different regions: the paleocortex, neocortex and archicortex (Hahn 2004). The paleocortex is composed by the piriform lobes in both sides of the brain, which are concerned with smell (Hahn 2004). The neocortex includes somatosensory and motor, visual and auditory areas and the association cortex (Hahn 2004). The association cortex is involved in the collection of information, prioritisation and decisions about suitable responses (Hahn 2004). The archicortex is composed by medial structures included in the limbic system, such as the hippocampus, the amygdala and the hypothalamus, thus being involved in emotions and behaviours (Aggleton 1993; Beaver 2019a). The limbic system is involved in memory, learning, social and emotional responses and in processing olfactory input (Aggleton 1993). Horses have a large and well developed olfactory system, which plays a role in emotional changes related to different experiences (Hahn 2004). Additionally, the vomeronasal organ (VNO), which is involved in semiochemical detection, is well developed in horses and communicates directly with the limbic system, through the amygdala, and the frontal lobe of the cortex, thus having a direct effect on emotions and behaviour (Hahn 2004).

Equine perception and communication

Horses sensorial organs functions differ from humans (McGreevy 2004c); thus, their communication and perception of the environment differ from humans as well. Horses interact with their pairs by combining communication cues: tactile, visual (postural), acoustic and chemical cues (Pageat 2011d). Tactile intraspecific communication is involved in equine social relationships and plays a role in horses' emotional states (Pageat 2011d). Tactile information is very important for horses as they can identify and locate tactile stimuli very precisely (Pageat

2011c). Equine vision allows horses to perceive distant movement with little acuity, which is expected from a prey species, who need to perceive possible dangers to quickly escape, independently of the nature of the danger (Saslow 2002). Additionally, equine hearing abilities increase the perception of potential dangers (Saslow 2002). Horses respond to sounds at least at a distance of 4400 metres from their location coming from different directions as they can move their ears 180°, which make a total of 360° when both ears are combined (Pageat 2011c). However, they locate sounds with a possible bias of 25° (Pageat 2011c). In addition to these different paths of communication, horses use chemical communication as the main communication pathway for distant and long-term information (Saslow 2002). Chemical cues are involved in intraspecific communication (pheromones) and produce unconscious emotional and hormonal modifications on the receiver (Pageat 2011d).

Equine appeasing pheromone (EAP) is a semiochemical involved in equine chemical communication from birth with an appeasing effect (Cozzi et al. 2013). EAP reduces restless behaviours during foal separation from the mare with long-lasting effects, up to 6 weeks (Van Sommeren and Van Dierendonck 2010). Cozzi et al. (2013) demonstrated the appeasing effect of this pheromone by exposing adult horses to a cognitive test after a stressful situation (transport). Falewee et al. (2006) observed the same appeasing effect, either in behaviour or physiological responses after a fearful situation. Additionally, chemical communication is involved in reproduction (Bigiani et al. 2005). The different cited studies highlighted the importance of chemical communication for horses. Nevertheless, chemical messages are combined with visual and acoustic cues to ensure that the message is perceived by the receiver (Pageat 2011d).

Behavioural traits and fundamental needs

The description of the time spent by equines on grazing in relation to the anatomy and physiology of their stomach was previously provided, though behaviours related to feeding are very important for horses as well (McGreevy et al. 1995; Tadich and Araya 2012). Horses are

nomads that enjoy moving for long distances and to search for different sources and kinds of food (McGreevy 2004d). Therefore, tasting different foods is an activity with emotional value for horses (McGreevy 2004d; Nagy et al. 2009). As horses are social animals that live in groups, feeding is a very important social activity to this species (Pageat 2011a).

Horses spontaneously search for the company of their conspecifics (Pageat 2011d). Relationships created between horses will be involved in equine emotional balance (Pageat 2011d). Groups of horses can be divided into two categories: natal group (birth or family group) and bachelor group (McGreevy 2004e). The first group is composed by mares, their pre-dispersal offspring and one or more stallions (McGreevy 2004e). These groups are kept together until the foals are between 2-3 years old (Pageat 2011d). Then, the juvenile stallions and mares will disperse (Pageat 2011d). The juvenile mares may find a stallion and start a new natal group, while juvenile stallions will form new groups, the bachelor groups (McGreevy 2004e). The bachelor group is composed by juvenile stallions or mixed groups (mixed groups tend to be unstable and often changes of individuals in the group occur) until the age of 3 or more years old, and then the group will separate, so they can find mares to create new natal groups (Pageat 2011d).

In such complex groups, communication is fundamental to keep a balance between different individuals (Pageat 2011d). Communication between horses is possible through visual, acoustic, tactile and chemical information (Pageat 2011d). Communication through the different paths of communication is very important for horses as they are the means to avoid intraspecific aggression and to protect the group against predators (Pageat 2011d). This information is important because domestication and current husbandry conditions of horses created some modifications that may compromise or at least change the communication and the social behaviour of horses.

Horses feeding and social behaviour repertoire were impacted by the domestication process and understanding the consequences of domestication in equine behavioural and

physiological need is fundamental to understand equine behaviour and succeed in the human-horse relationship.

Human-horse relationship:

Domestication

Equine domestication was a long and gradual process (Beaver 2019b). This process is very likely to have started at first in an area that corresponds to the current Kazakhstan and Ukraine, the ancient Eurasia (Bruford et al. 2003). The timeline is yet to be exactly defined, but some evidence suggests that the process happened between 4000 and 6000 years ago, and perhaps up to 9500 years ago (Driscoll et al. 2009). In the Olympic games, 2500 years ago, four-horse chariot races were added to the event, then 60 years later mounted racing was added as well (Davis 2007).

Horses were a food source for humans at first, but humans quickly understood the value of horses for transport (Peplow 1998; Beaver 2019b). The movement of horses across borders is probably the main reason for the homogeneous pattern observed in horses nowadays (Bruford et al. 2003). Riding horses increased productivity and efficiency of shepherding because with horses, one person could shepherd more animals over larger grazing areas (Beaver 2019b). This means that horses, as dogs (Pageat 2019), played a role in human evolution, as they were involved at the beginning of animal production.

In addition, horses have become a representation of power, especially during wars 3000 years ago (Anthony and Brown 2011). Then, cavalry horses were used to pull chariots during the war when the ground was suitable for wheeled carts (Beaver 2019b). Until 600 years ago, an army using horses in battles was more likely to win the war (Beaver 2019b). This means that the horse was also involved in human evolution and history through wars, providing an advantage for the armies that had them at their disposal.

After the second world war, horses have been used by police forces as police mounts, and their purposes have been diversified from food source, police force, to many different types of work, pleasure, sports and competition (Beaver 2019b). This evolution of the equine industry was accompanied by modifications in equine environments and living conditions. Equines started to have an economic interest and different investments (e.g. selective breeding) were done in this industry (McGreevy 2004a).

Domestication along with evolution produced modifications in equine coats and body sizes (Macfadden 1986; Beaver 2019b), which suggests that selective breeding was part of the domestication process (Ludwig et al. 2009). Black horses appeared in the ancient territories that are now Portugal and Spain 6000 years ago and only 25% of the population had this coat colour (Beaver 2019b). Horses with chestnut coats have seen their number increase from 4000-5000 years ago and represented 25% of the total equine population (Ludwig et al. 2009). Other colour variations appeared through domestication as well (Beaver 2019b). Equine body sizes increased with evolution, respecting the Cope's Law, which defines a trend toward increased body sizes in different species (Macfadden 1986). This increase in body sizes was partially caused by human selection and breeding of horses for specific functions (Brooks et al. 2010). Breeding along with horse natural evolution including movements between different continents have created genetic diversity and originated different breeds (Kavar and Dovč 2008; Brooks et al. 2010; Petersen et al. 2013). The environment might have influenced horses body size, which would justify the differences observed on the body size of local breeds from different global regions (Kavar and Dovč 2008). Associated with the increasing economic value of horses thanks to breeding improvements, husbandry conditions were modified by humans with the aim of protecting horses from predators and to increase humans control over horses (McGreevy 2004a).

Humans created single stables for different reasons. Some of these reasons are the need to spare grazing areas from damage underfoot, the protection from extreme environmental

conditions related to the weather, to avoid intraspecific conflicts that can produce wounds or other injuries, to facilitate daily activities because horses are easier to catch if they are stabled, or to control food and water intakes as these are easy to monitor in stables and their modifications are clinical signs of disease (McGreevy 2004a). Additionally, trainers consider advantageous to limit physical activity by stabling horses to control the amount of daily exercise (McGreevy 2004a). This means that stable management was thought at first to improve equine health and protection from unnecessary injuries or diseases. However, some adverse consequences of these husbandry conditions are described (Bachmann et al. 2003; Hothersall and Casey 2012).

In pastures, horses time budget for feeding activities is 13-18h per day, however, stabled horses have this time reduced, because food is not available 24h/day (Cintra 2016). Stabled horses, who are fed with concentrates, spend approximately 1h on feeding activities and 15-17h with no specific tasks or activities (Cintra 2016). Equine gastrointestinal anatomy and physiology needs, as previously described, are to feed on small amounts multiple times per day. This means that husbandry conditions and feeding modified equine natural feeding activities, which was associated with gastrointestinal disorders (Cintra 2016). Some of these disorders, with direct impacts in the human-horse relationship, will be described in the following paragraphs.

Stabling horses in boxes do not allow horses to feed properly and continuously as they did in nature, which means that the natural dental abrasion does not occur (Martins da Silva 2009e). Nevertheless, dental growth continues to occur, which means that equines may now experience teeth overgrowth and mastication difficulties that did not occur in natural conditions (Martins da Silva 2009e). Dental growth in horses that are fed with concentrates undergo an irregular abrasion, which creates dental spikes, producing ulcers in the oral mucosa (Cintra 2016). Equine dental growth is very important to handlers and riders because the use of the bit produces direct consequences on the horse's mouth, including the teeth (Martins da Silva

2009e). For this reason, dental care has become a fundamental need for equines (Martins da Silva 2009e) and in this work, all horses were submitted to mouth examination to ensure that dental pain or discomfort would not influence the results.

The equine stomach is relatively small when compared to the equine full body dimensions (Cintra 2016). Excessive food intake was associated with gastric colic by overload, which is a very painful condition for horses (Cintra 2016). Additionally, equine secrete gastric acid continuously, which means that during the daily fasting periods horses gastric mucosa is exposed to a pH inferior to 2.0 (Sanchez 2018). This condition is the most probable aetiology of gastric ulceration in equines (Sanchez 2018). Gastric ulceration reduces equine performance, either in training, work and competition (Begg and O'Sullivan 2003), because this is a painful condition that creates nonspecific clinical signs and subclinical cases are described. The horse may associate this painful condition to training and to the rider, which may jeopardize the human-horse relationship (Mcgreevy 2004). Subclinical states are sometimes unperceived by owners and veterinarians, who might think that decreases in performance are due to behavioural problems (Marlin et al. 2019).

In conclusion, horses had to cope with many challenges through domestication and have adapted to human environments. Modifications to horse's natural lifestyle created by humans may have produced negative outcomes, however, thanks to many valuable researches these outcomes can be solved or prevented nowadays by creating husbandry and handling conditions that should meet horses' needs.

Horse at sports and at work

Horses are involved in many different activities. These activities differ from sport and work, however, there are some concepts that are common to almost all equestrian and working activities, which will be described in this section. Then, some details specific to sports or to working activities will be described as well.

Horse-rider or horse-handler communication was first theorised by Xenophon between 428 and 355 years before Christ and was the subject of the book of Blaineau (2011). This communication is based on the aids/cues, through the halter and the rope, the reins and the bit, the posture, the saddle, and the legs of the rider (Dyson 2017). This communication is very complex, because an aid may vary in intensity, anatomical region of application, and moment of application (Netto de Almeida 1997b; Mclean and McGreevy 2004). Therefore, modifications in the application of the aids may induce variable behavioural and physiological responses (Mclean and McGreevy 2004; ISES 2013; Zebisch et al. 2014). The precision of the cues is therefore essential, especially in dressage competition in which very similar exercises and gaits, that slightly differ in terms of the aids that should be provided are performed (Mclean and McGreevy 2004; Dyson 2017; FEI 2019). Dressage is included in training for many other disciplines, such as jumping, because dressage is the basis of those disciplines, like a gym for physical preparation of a human athlete (McGreevy and Mclean 2010e).

Dressage rules of the FEI (FEI 2019) define 4 types of walk and 5 types of trot and canter. The types of walk include “medium walk, collected walk, extended walk and free walk”. The five types of trot and canter include “working trot/canter, lengthening of steps/strides, collected trot/canter, medium trot/canter and extended trot/canter”. The canter has the specificity of an asymmetric gait, which means that in this gait there is one foreleg leading, usually the inside foreleg, though when the outside foreleg leads, this gait is called counter-canter and can be used to improve balance and strength. A description of each of these gaits details is provided in the Dressage rules document. Nevertheless, the details of each gait are very accurate and riders need to provide the horses with very precise aids to obtain the expected gait (Mclean and McGreevy 2004; Dyson 2017).

Horses gaits are usually described as symmetric (e.g. trot) in terms of the gait itself, however, most of the horses display individual variability and a natural asymmetry within the different gaits, which could be related to lateral preferences (McGreevy 2004b; Persson-Sjodin

et al. 2019). Training may improve horse's symmetry, which is an important goal for horseback riders, especially for those involved in dressage training and competition (Netto de Almeida 1997a; McGreevy 2004b; Clayton and Hobbs 2017).

The improvement of symmetry can be done by performing lateral exercises, which are asymmetrical movements (Denoix 2014), thus allowing the improvement of mobility and strength on one specific side of the horse (Netto de Almeida 1997a; Denoix 2014). However, lateral exercises differ from longitudinal exercises and should be correctly performed to achieve the results (de la Guérinière 1733; Oliveira 2006; de Coux 2007).

Lateral exercises benefits, as the shoulder-in (see fig. 4), include also an increase of horse's compliance with the horseback rider and the improvement of other biomechanical aspects as the impulsion (de la Guérinière 1733; Oliveira 2006; de Coux 2007). This means that the benefits of lateral exercises include also psychological aspects. However, the shoulder-in must be performed without coercion and with no resistance from the horse to produce the expected benefits (Netto de Almeida 1997b; Oliveira 2006; de Coux 2007). The horse should be relaxed, and the aids well harmonised (de Coux 2007).

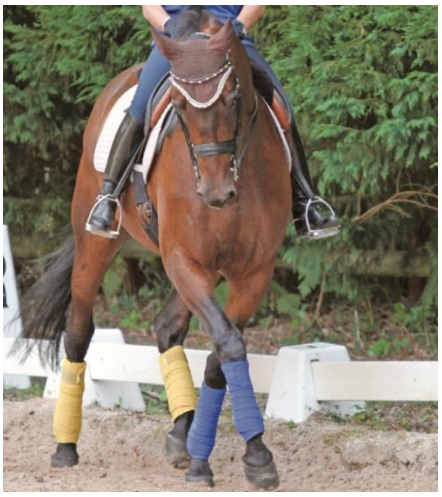


Figure 4: Shoulder-in illustration. (Source: Denoix 2014)

Horseback riders need to use adapted aids to communicate with the horse, which might be difficult once lateral exercises are more challenging to both the rider and the horse biomechanics and balance (De Cocq et al. 2010; de Cocq and Van Weeren 2013). This means that an influence from the rider on the results, either physical or psychological, of this exercise could be expected (Strunk et al. 2018), especially because riders are asymmetric as well (Hobbs et al. 2014). Nevertheless, scientific studies on the psychological consequences of these exercises have not been conducted to date. Lateral exercises may possibly be an interesting tool to improve equine welfare or manage behavioural problems of ridden horses, however, for working horses these exercises may be more difficult to perform.

Working activities are very diverse. They can be urban loading activities, therapeutic activities, mounted police, tourism, agriculture productions (e.g. wine), amongst others (Tadich et al. 2008; McGreevy and McLean 2010b; Freund et al. 2011). In this work, attention was placed on urban (see fig. 5) and therapeutic activities, so the following introduction of working activities will only consider these two activities.



Figure 5: Chilean urban draught working horse (Photograph courtesy of Tamara Tadich)

Working horses may play an important role in human life, as they may represent the main source of household incomes to some families (Tadich et al. 2008). Horses involved in draught work in the south of Chile are used for the transport of wood and products from agriculture for local markets (Tadich et al. 2008). Although this population of horses belongs to the poor community, their owners provide these animals with veterinary and hoof care, and generally, small amounts of health problems and negative behavioural responses are found (Tadich et al. 2008). Nevertheless, some welfare issues related to husbandry practices and hoof trimming can be observed and are usually related to a lack of understanding from the person involved, which could be improved by providing professional education on equine health and care to these individuals (Tadich et al. 2008). Welfare issues in working animals has become a societal concern. Therefore, a welfare assessment protocol was investigated in some populations of animals (Pritchard et al. 2005). This protocol aimed to identify or rule out any welfare problems or negative outcomes induced by these activities, which is the first step of any equine welfare investigation. However, the authors explained that in the absence of negative welfare outcomes the owners involved in this investigation tended to consider that their animals were experiencing positive welfare outcomes from their activities. Nevertheless, the definition of animal welfare also includes the experience of positive emotions or the production of positive outcomes (Marcet Rius et al. 2018a), which were not assessed in the previously cited study and requires further research.

Animal-assisted therapies have been used for different purposes (Edwards and Beck 2002; Cole et al. 2007; Kamioka et al. 2014). These therapies involve the use of animals to improve either physical and/or psychological states of human patients (Edwards and Beck 2002; Kamioka et al. 2014; Nurenberg et al. 2015). Equine-assisted therapy (EAT) is the branch of animal-assisted therapy that involves horses or donkeys (Borgi et al. 2015). EAT activities vary according to the patient's therapy goals between horseback adapted riding, interaction between the patient (on the ground) and the horse, which may include brushing, foot care, feeding or watering the horse, and in-hand work, which involve approaching the

horse, making the horse move forward or backward, leading the horse to pass parallel bars on the ground or lunging the horse, which could be done with a lead rope or with the horse free in an arena (Borgi et al. 2015; Malinowski et al. 2018). These activities have been used to improve social, emotional, and physical aspects in patients suffering from anxiety, depression, autism spectrum disorder, multiple sclerosis, Parkinson's disease and spinal cord injury or even to improve balance, muscle symmetry, coordination, and posture in patients suffering from genetic or post-traumatic conditions (Benda et al. 2003; Borgi et al. 2015; Nurenberg et al. 2015; Gonzalez-De Cara et al. 2016; Peppe et al. 2017; Kraft et al. 2019).

The influence of EAT on horses emotional responses was the subject of very few investigations, which suggested that no negative outcomes of these activities are produced (Kaiser et al. 2006, 2019; McKinney et al. 2015; Gonzalez-De Cara et al. 2016; Malinowski et al. 2018; Merkies et al. 2018). However, the absence of negative outcomes is not predictive of the presence of positive outcomes (Boissy et al. 2007; Marcet Rius et al. 2018a). Nevertheless, there was only one study published on positive outcomes of EAT (Malinowski et al. 2018), and the authors suggested that no positive outcomes were produced in their experiment. EAT from the equine perspective is a very recent research subject and needs further investigation to disclose the animals' perception of these activities.

Equine perception of the environment and activities imposed by humans

Temperament development at young ages may influence equine perception of the environment and may define individual responses in the animals for their whole life-time (Lansade et al. 2008; Fureix et al. 2009). Temperament is defined as a characteristic of an individual that appears early in life and tends to be stable over the course of time (Goldsmith et al. 1987; Lansade and Simon 2010). Some authors suggested that the development of temperamental traits might be influenced by environmental and genetics factors (Le Scolan et al. 1997; Wolff et al. 1997; Søndergaard and Ladewig 2004). The age at which young foals start to be handled (touched, brushed, accept to lift the foot, halter-leading) or gentled was

proved to influence the development of temperamental traits (Visser et al. 2002; Lansade et al. 2004). With age, equine temperament tends to be a determinant of the animal's behaviour (Henry et al. 2007). Therefore, environmental conditions created by humans might have an influence on the development of equine temperaments as well.

Equine evolution from natural conditions to human societies occurred with a protective approach created by humans, who unintentionally generated negative outcomes to equine health, and to equine psychological states (Davidson and Wilson 2002; Nagy et al. 2009; Malmkvist et al. 2012; Tadich and Araya 2012). Equine society awareness of these negative outcomes increased in the last decades and created a trend to progressively adapt equine husbandry to the equine physical and psychological need. The following paragraphs will provide information on how horses perceive living conditions that humans have created and their impact on psychological and behavioural aspects.

Social relationships between equines are, as previously described, very important to this species (Pageat 2011d). While humans have created stables with individual boxes to provide horses with the most protected environment, horses may perceive it differently (Cooper and Albentosa 2005; Falewee et al. 2006). The walls and the bars of a box partially or totally compromise equine communication, as some chemical cues need physical contact to be perceived, and most importantly tactile communication, which has been proven to be of considerable importance, is impaired (McGreevy 2004a). Additionally, as explained before, equine communication relies on combined cues (Pageat 2011d), which means that visual and acoustic cues might not be enough for a horse to emit a message. Some social behaviours as allogrooming or mutual fly-swatting cannot be performed by single-housed horses (McGreevy 2004a). The prevalence of some behavioural problems, as stereotypies, was associated with social isolation in stabled horses (Henry and Stephens 1977; Malmkvist et al. 2012), which reinforces the importance of social relationships in equine lives. Nevertheless, an important

individual variability in equine responses to housing conditions was observed (Blokhuis et al. 1998; Ijichi et al. 2013b; Yarnell et al. 2015).

The impact of stable management on equine gastrointestinal physiology was already described, however other welfare concerns need further attention, as the stables impact on behaviour. Horses feeding behaviour includes the choice of different food sources, which is limited in more restrictive environments, as stables (McGreevy 2004a). The movement between different pastures to select different food sources is a sensorial experience with emotional value for the animals; however, in stables horses cannot move or choose their food (McGreevy 2004d). Even if properly fed with a balanced diet, they continue being motivated to perform foraging/grazing, because this activity represents a sensorial experience and a physical activity, that is time consuming (Cooper and Albentosa 2005). Housing conditions influence equine behavioural responses, thus equine emotional states, so the observation of abnormal behaviours in stabled horses is not surprising (Visser et al. 2008; Budzyńska 2014). Equines are athletes and their performances are affected by their emotional states during training or competition (Bartolomé and Cockram 2016). For this reason, housing conditions are important to equine performance, because an animal that is kept under stressing conditions due to stable management may consequently have poor performances or present learning difficulties through work, training and competition (McGreevy and McLean 2010a; Tadich and Araya 2010).

Work and sport performances may also be influenced by other conditions. Social isolation due to stable managements and meeting unfamiliar conspecifics in familiar or unfamiliar environments may represent a source of excitement and/or stress for horses (Rivera et al. 2002; Hartmann et al. 2011; von Borstel et al. 2017). This excitement and stress may direct horse's attention to conspecifics or environmental stimuli and induce a lack of attention from the horses to the human's cues (Ioannou et al. 2004; Hall et al. 2018). The communication between the horse and the rider, either at work, therapy or sport, through the aids depends on

tactile stimuli, which makes this the most important path of communication in equitation (Saslow 2002). Additionally, as horses are able to identify with great precision the location of tactile information, miscommunication between humans and horses may easily occur (Mclean and McGreevy 2004; Pageat 2011c). Miscommunication between the handler/rider and the horse lead to decreases in performance and may impair a healthy horse-human relationship (Mclean and McGreevy 2004; Ijichi et al. 2013a). At the contrary, a healthy horse-human relationship may help horses on coping with disturbing environments (Le Scolan et al. 1997; McGreevy and Mclean 2010a). These relationships may differ according to the horse and handler/rider temperaments or personalities (Roberts et al. 2003; Hausberger et al. 2011).

Equines are involved in many activities that need different skills or abilities (McGreevy and McLean 2010b). Training methods differ significantly between equestrian disciplines and equine temperament may influence the discipline that humans will choose for a specific horse (König von Borstel et al. 2011; Graf et al. 2013, 2014; Suwała et al. 2016). Hausberger et al. (2011) and Baragli et al. (2017) described a relationship between equine personality and the type of work and suggested that the type of work may influence human personality. Therefore, temperaments may influence either positively or negatively the human-horse relationship (Fureix et al. 2009; Graf et al. 2013). Many studies have been developed to assess equine temperaments and their impact on performance, behaviour and equine welfare (Mills 1998; Hausberger et al. 2011; Graf et al. 2013, 2014). The influence of temperaments on learning performances is highly task-dependent (Lansade and Simon 2010). Regardless, many temperament tests were created to study equine reactivity when the horse was free in a controlled setting and their results were generalised to real conditions; however, to understand the influence of an individual's temperament in performance with a practical perspective, there is a need to observe the dyad horse-rider in real situations, because horses responses could differ when ridden or free in a paddock (Hausberger et al. 2011; König von Borstel et al. 2011).

Horse-human communication and relationships differ according to the performed disciplines or activities, as the specific skills and contexts are different as well (McGreevy and McLean 2010b). However, many horses are involved in different activities or equestrian disciplines, which means that these horses should acquire all the skills related to each of these activities (McGreevy and McLean 2010b). Additionally, horses are often handled/ridden by different handlers/riders, which means that they need to adapt and respond to cues/aids that vary with the handler/rider's skills (Strunk et al. 2018). The increasing knowledge and interest of the equestrian community about equine behaviour and training methods have unfolded welfare and ethical concerns related to the activities in which horses are involved (Jones and McGreevy 2010; McLean and McGreevy 2010).

Ethics and legislation on horse-related activities

Ethics could be defined as "the study of what is morally right and wrong, or a set of beliefs about what is morally right and wrong" (Cambridge 2019). To define what should be accepted or not, with ethics in perspective, there is a need to understand the reason why humans use horses from the past to the present, and to accept that, perhaps, what was previously accepted may become unaccepted in the present or in the future (Jones and McGreevy 2010). Additionally, one should have in mind the living conditions observed in different countries, which means that some practices may be accepted in some countries and not accepted in other countries (Jones and McGreevy 2010). This means that ethics is a subjective discipline, which relies on cultural and developmental conditions in different countries. Nevertheless, some ethical principles may be generalised to many countries, whose cultures, related to animal industries and sports, and development status are similar.

Ethical reasoning and questioning were the reason for the increasing number of research projects with the aim of assessing equine welfare and potentially originate legislation to ensure that welfare conditions are respected within the equine industry (McLean and McGreevy 2010). Some of these researches were produced by request of the European Union and the European

Parliament so they could become the basis of the legislation on animal protection, health, welfare and public health. Nevertheless, there is still a need for further research (European Parliament 2018).

The European Parliament ruled out some resolutions in 2017, which were published as a European Standard (EN 2018/C 263/06) in the Official Journal of the European Union in July 2018, concerning equine ownership and care (European Parliament 2018). In these recommendations, many issues requiring attention from the adequate authorities of the different Member States are pointed out and regulation/legislation are often demanded (European Parliament 2018). Some of the outlined subjects in this Standard are the transport, the end of life, husbandry conditions, working conditions and in general animal welfare of equines and the education of the equine industry to each of these subjects. Each of the Member States should then create their own legislation on each of these subjects, according to their social and cultural possibilities. However, no reference was made in this European Standard in terms of regulation of sports involving equines.

In France, some regulation and legislation for animal protection were created (Article L214-3 2010) and some penalisations are defined by law. However, legislation is often vague and different interpretations are possible. In the cited article is mentioned that animals should not be mistreated, though whether an animal is mistreated or not might be subjective and is most often open to interpretation (McGreevy and McLean 2009; Jones and McGreevy 2010; McLean and McGreevy 2010). An example of this is the Article 322-118 of the “Code du Sport”, which says that a horse ridden by a rider should be in good health, being capable and prepared to perform a specific exercise, and that this exercise should not involve any danger to the security of the rider and third persons (Article 322-118 2017). The problem with this legislation is that there is no means to assess whether a horse was capable and well prepared to perform a specific exercise so far (McLean and McGreevy 2010). Other countries created more specific and restrictive laws, like Sweden for instance. These laws are related to husbandry conditions.

For example, horses involved in any activity should have access to the paddock at least twice a week and two hours per day. This law is more specific and precise, which avoid different interpretations. However, most of the times, legislation should not be so restrictive, because laws have a direct impact on people's lives and on the industry. Having in mind the people who work in the equine industry (900,000 jobs from the equestrian sports industry) and the incomes this industry represents in the European Union (100 billion per annum), laws should define general principles and be adapted to each reality (European Parliament 2018). For these reasons, many of the acceptable or not acceptable conditions related to horses are ruled by the Féderation Equestre Internationale (FEI) and the local committees and depend on ethics.

The FEI has published a code of conduct for the welfare of the horse, though as in the law, the articles are vague and rely on the interpretation of the judges in competition (FEI 2013). Nevertheless, further research on the subject is demanded by the FEI in that code of conduct. In the French regulation of competition some articles are published for the protection of equines, however, they are also vague and include terms as "excessive use of the whips", or "excessive use of the spurs" or even "forbids the use of any dispositive that induces excessive pain" (FFE 2018), but there is no definition of what could be considered excessive and considering terms as "excessive pain", one could assume that pain within some amounts is acceptable in competition. Therefore, this prompts the question of how could one identify whether an action is excessive or not (Jones and McGreevy 2010). Finally, the definition of acceptable and not acceptable conditions in equine sports depend on professional ethics and ethical equitation have been encouraged in the last decade (McGreevy and McLean 2009; Jones and McGreevy 2010; McLean and McGreevy 2010).

The term ethical equitation is rather recent and was introduced by McGreevy and McLean (2009). This term implies that horses are sentient animals with the capacity to experience suffering, and the assumption that humans dispose of these animals for their own benefits, which should be associated with moral responsibility of ensuring equine welfare (McLean and

McGreevy 2010). Some ethical conditions related to the use of horses were highlighted and most often learning processes that horses experience are discussed, and some methods as the use of whips were discouraged (McGreevy and McLean 2009). Additionally, some activities may create negative mental or emotional states while no physical injury is perceived (Jones and McGreevy 2010). Research authors have highlighted the need to create objective measures of welfare to assess the consequences of different techniques in equitation (McLean and McGreevy 2010).

The public attention and the scientific scrutiny of ridden horses should lead governing bodies of horses sports to state their positions in different subjects (Jones and McGreevy 2010). Nevertheless, riders are already ethically responsible for minimizing the effect of their activities on horses' welfare (Jones and McGreevy 2010). The technology development and advances will most probably allow the assessment of different parameters, such as the rein tension, which will be key points to the assessment of equine welfare in the equestrian community (McLean and McGreevy 2010). Riders should be aware of equine learning capacities and learning processes to improve their use of horses, thus their welfare (McLean and McGreevy 2010).

Learning processes were the subject of many investigations and are mostly well-known in the scientific community (Mills 1998; Rowe 2005; Murphy and Arkins 2007; Hanggi and Ingersoll 2009; Gabor and Gerken 2010; Sankey et al. 2010). The need to educate riders, handlers, animals keepers and any person involved with horses in learning theory was stated by some authors (McLean and McGreevy 2010). Learning processes and their implications on equine welfare will be summarised in the following sections.

Equine Welfare (positive and negative):

Learning processes

Horses with the greatest capabilities to learn, understand and solve problems are more likely to succeed in environments created by humans (Murphy and Arkins 2007). This means that equine learning capabilities are fundamental to succeed in the horse-human relationships (Gabor and Gerken 2010). Learning is a process that modifies an individual's behavioural response, in a relatively permanent way, as a result of an experience (McGreevy and Mclean 2010f). The process of learning was described as an active or passive process (Murphy and Arkins 2007). Learning processes are often described in two categories: non-associative and associative learning (Nicol 2002).

The forms of non-associative learning directly involved in the human-horse relationship are the habituation and the sensitisation (McLean and Christensen 2017). Habituation is stimulus-specific and occurs when an animal is repeatedly exposed to an inconsequential stimulus at low-intensity levels (Hanggi 2005; Mengoli et al. 2014). The result of habituation is the decrease of an animal's response to a specific stimulus (McGreevy and Mclean 2010f). Habituation is important for any species because this process increases the predictability of well-known environments and avoids energy expenditure by determining the stimuli that do not need particular attention, thus preventing animals from being continuously attentive to the environment (Yin 2009). Sensitisation is the opposite of habituation. This means that sensitisation is the process by which the response of an individual increases after a repeated exposition to a specific stimulus at high-intensity levels (Hanggi 2005).

The non-associative learning processes described hereabove influence equines lives and are important in handling, training and competition contexts (Zentall 2005; McGreevy and Mclean 2010f). One example of habituation in horses daily lives is the presence of dogs in many equestrian facilities, which horses seem to find aversive at the first contact (McGreevy

and Mclean 2010f) and become habituated or sensitised depending on the dog's behaviour toward the horse. However, in some conditions, habituation is only desired to some extent. For example, the "contact" is considered as a balanced point in which rein tension (bit pressure), leg pressure and the seat should be neutral, meaning that the horse should become habituated to this specific point of pressure and any deviation from it must be associated with a specific response (McGreevy and Mclean 2010f). This process is difficult to achieve because riders want their horses to respond to the aids, though accepting the "contact" even if this represents a pressure that is still applied on the horse's body (McLean and Christensen 2017). Additionally, the "contact" is difficult to maintain and may easily become a punishment, which is an associative learning process.

The forms of associative learning involved in the human-horse relationship, and particularly involved in training, are classical and operant conditioning (McGreevy 2004f; McLean and Christensen 2017). In both cases, these learning processes rely on the association between different stimuli (McGreevy 2004f). Classical conditioning is the learning process by which an individual starts responding to a new stimulus by association with an old stimulus (McGreevy and Mclean 2010b). This process is usually described as the Pavlovian conditioning, thanks to study of Ivan Pavlov of gastric physiology, in which he observed that dogs started salivating when they heard a technician ringing a bell (new stimulus) before feeding them (old stimulus), creating an association between the sound of the bell and the food (McGreevy and Mclean 2010b). One example of this process in horses' lives is the association of an acoustic stimulus provided by the rider/handler with urination in horses involved in races for doping control (McGreevy and Mclean 2010b). At the beginning, the rider/handler may whistle when the horse urinates, but when the association between the acoustic stimulus and the behaviour is associated, horses may respond to the whistle by urinating (McGreevy and Mclean 2010b).

Operant conditioning is a form of associative learning that usually starts by trial-and-error learning until a voluntary response becomes associated with a consequence that could be a reinforcement or a punishment (McGreevy 2004f; McGreevy and Mclean 2010b; McLean and Christensen 2017). The use of reinforcement aims to increase the frequency of a behaviour while the use of punishment aims to reduce the frequency of a behaviour (Hockenhull and Creighton 2013; McLean and Christensen 2017). Additionally, both may be negative (subtraction of a stimulus) or positive (addition of a stimulus) and in equitation, the most commonly used technique for training is the negative reinforcement (Freymond et al. 2014; McLean and Christensen 2017). An example of negative reinforcement in the training of horses is the "acceleration" signal. The rider should increase leg pressure and once the horse increases the speed or perform an up gait transition the pressure is released (the stimulus is subtracted) (McLean and Christensen 2017).

Learning abilities are influenced by different factors. As explained before, temperament/personalities influence equine learning abilities (Visser et al. 2002; Lansade et al. 2004; Lansade and Simon 2010). Additionally, emotional states of horses influence learning abilities, thus playing a role for the success in training (McBride and Mills 2012). Therefore, given the importance of social relationships for horses, it is not surprising that they influence learning abilities as well (McVey et al. 2018). This is also applied to relationships with humans, especially in training or working situations (McGreevy and McLean 2009).

Training horses to respond to humans cues/aids is fundamental to succeed in the human-horse relationship, however, some techniques, as punishments, require very precise timing and consistency to be effective (McGreevy and McLean 2009). Regardless, handlers, trainers and riders receive few, if any, education on the learning theory, and most important on its application to handling, riding or just on how to interact with horses. Some authors have proposed the inclusion of applied learning theory in education programmes for people involved with horses (McLean and McGreevy 2010). Nevertheless, the application of the learning theory

in training has some implications in equine welfare. These implications will be described in the following sections.

Implications of learning processes for equine welfare

Horses learn in predictable ways and this is probably the reason why humans can train and interact so effectively with members of this species (McLean and Christensen 2017). Understanding learning theory can help horse trainers work by accelerating the learning processes of their horses, however, respecting equine welfare (McLean and Christensen 2017). The application of learning theory does not necessarily mean that equine welfare is respected, because some principles of learning theory, were associated with negative outcomes and negative emotions. Punishment, for example, was associated with negative emotions and negative outcomes (Mills 1998; McGreevy and McLean 2007).:

- Increased hyper-reactivity, fear and stress;
- Fearful associations with the punisher or the surrounding context;
- Animals displaying fear of trying new responses.

This means, that riders should be aware of the principles of learning theory, but of its consequences as well. Many studies were performed on equine memory and suggest that horses memories may last for at least seven years (Hanggi and Ingersoll 2009). This means that positive or negative experiences that induce positive and negative outcomes, respectively, may last for long periods. Additionally, misuses or inadequate applications of learning theory may result in discomfort, pain and stress as well (McGreevy and Mclean 2010c).

Some undesired behavioural events may result from pain, stress, fear or any other sources of distress, which are very difficult to objectively assess in training and competing contexts (Hall et al. 2013). Many horses are described as “lazy” or “stubborn” when they are actually experiencing pain (Hall et al. 2013). Dental pain may be at the origin of “contact” issues (Hall et al. 2013). For example, pulling the reins on a horse experiencing subclinical dental pain, will

induce acute pain, and will probably result in abnormal behaviours, hence to recognise normal and abnormal behaviours is fundamental for riders and trainers (McGreevy and Mclean 2010d). This example highlights the importance of being aware of learning theory and of its results because a rider who understands the principles and the results of learning theory will be able to discriminate a normal behavioural response (e.g. trial-and-error) from a pain-related response.

Small amounts of stress may be helpful for learning, while high levels of stress (referred to as distress) inhibit learning (Bartolomé and Cockram 2016). The threshold that defines the line between small or high amounts of stress is still to be defined (McLean and Christensen 2017). Nevertheless, stress induces modifications of the equine physiological and behavioural responses (Koolhaas et al. 1999; González et al. 2019; MacDougall-Shackleton et al. 2019). These modifications could be positive or negative for the animal welfare state or its future emotional responses (Broom 1991; Bartolomé and Cockram 2016; Esch et al. 2019).

Equine behavioural responses are modified by the environment (Visser et al. 2008). Horses stabled individually display more stress-related behaviours than horses stabled in groups (Wechsler 1995; Visser et al. 2008). Habituation is the learning process involved in the capacity of horses to adapt to confinement (Groothuis and Carere 2005; McGreevy and Mclean 2010f). Some horses display more difficulties in adapting to confined environments than others, which may be related to their early life experiences and temperament (Lansade and Simon 2010; Lesimple et al. 2011).

Evolution through training requires the development of different abilities according to the aimed work activity or equestrian discipline (McGreevy and Mclean 2010a). The development of these very specific abilities depends on learning processes, which may create stress or distress as previously described (McLean and Christensen 2017). Additionally, as horses dispose of a great memory and recall abilities, their early experiences are very important for their future (Hanggi and Ingersoll 2009; Mengoli et al. 2014; Pereira-figueiredo et al. 2017).

This means that the specific learning processes and learning conditions associated with one specific activity may modify equine emotional responses, either in physiological or behavioural terms, for the entire life of the animal (Hausberger et al. 2011; Kiley-Worthingthon 2011).

The study of equine emotional responses and welfare requires the use of objective physiological and behavioural indicators (Broom 1991; Désiré et al. 2002). Additionally, the definition of animal welfare includes not only the absence of negative experiences or feelings but also the presence of positive experiences and feelings (Boissy et al. 2007). Therefore, animal welfare assessments must consider both negative and positive indicators (Marcet-Rius et al. 2019). Many studies aimed to investigate negative emotional responses and negative welfare (Górecka et al. 2007; Fureix et al. 2009; Peeters et al. 2013; Johnson et al. 2017; Usui and Nishida 2017; DuBois et al. 2018; Arhant et al. 2019; Clark et al. 2019). Some studies in different species were done to identify positive behavioural and physiological indicators of animal welfare (Marcet Rius et al. 2018a; Marcet-Rius et al. 2019).

There are different indicators used for the study of animal welfare and most of them are based on principles related to physiology and behaviour. These concepts will be introduced in the following sections.

Physiological indicators of positive and negative emotions

In general, physiological indicators of animal welfare are related to the functions of the hypothalamus-pituitary-adrenal (HPA) axis (Hahn 2004; Costantini et al. 2008; Tadich et al. 2013; von Borstel et al. 2017) and the autonomic nervous system (Kuwahara et al. 1996; Bowen and Marr 1998; Ille et al. 2014; von Borstel et al. 2017). Abnormal levels of neurotransmitters, which production and/or function is related to the HPA axis have been associated with mental diseases and abnormal behaviours in humans and animals (Hahn 2004). These neurotransmitters are included in three different groups: biogenic amines, amino acids and neuropeptides (Hahn 2004).

Biogenic Amines

The biogenic amines group includes different neurotransmitters, which were associated with emotional responses or states: dopamine, epinephrine, norepinephrine and serotonin (Hahn 2004). Dopamine receptors in the basal nuclei of the brain play a role in the initiation of motor responses and the regulation of movement (Hahn 2004). High density of D_2 receptors of dopamine was associated with high levels of detachment and aggressiveness (Breier et al. 1998; Hector 1998). Moreover, there is evidence of the dopaminergic system implications in the brain reward system in the pre-frontal cortex (Hahn 2004; Arias-Carrión and Pöppel 2007). Therefore, the dopaminergic system activity plays a role in learning activities, especially through operant conditioning, which is involved in any training session. High dopaminergic activity was also observed in stereotipic horses, which suggests that stereotypies in horses might be long-lasting thanks to their rewarding effect (Dodman 1998; Taber et al. 2012). Even if the activity of the dopaminergic system has an evident association with emotional responses, studies on the implications of this system activity on equine emotional states are still needed.

Epinephrine and norepinephrine levels in serum increase in painful and stressful situations (Greco and Stabenfeldt 2004; Ayala et al. 2012). Epinephrine is in higher concentration in the serum and is responsible from sweating and increased heart rate when horses experience stress or excitation, while norepinephrine is more abundant in the brain and plays a role of modulator of the autonomic reflexes and pain sensations (Hahn 2004). In humans, noradrenergic activity was associated with aggressive behaviour, and a low level of noradrenergic activity was associated with narcolepsy in horses (Elliott 1977; Peck et al. 2001). These neurotransmitters are difficult to study because their half-life in the plasma is inferior to 30 seconds (Snow et al. 1992). Additionally, the process of blood sampling may be stressful, influencing the results (von Borstel et al. 2017). Therefore, they are not reliable behavioural indicators.

Serotonin plays a role of moderator of behavioural responses, thus having a major adaptive role in social relationships (Hahn 2004). Low concentrations of serotonin in the rostral pons and midbrain (synaptic clefts) were associated with the inability of adopting passive or waiting attitudes, which might be associated with stereotypic behaviours in horses (Overall 1998). Serotonin is a neurotransmitter involved in the regulation of mood and emotional states, as fear and aggression, impulse control, arousal, sleep-wake cycle, food intake and even pain (David et al. 2004; Landsberg et al. 2013). This neurotransmitter has been studied in relation to age, circadian cycle, physical exercise and medical conditions in horses (Alberghina et al. 2010b, a; Ayala et al. 2012; Ferlazzo et al. 2012; Bruschetta et al. 2013, 2014, 2017). However, to the best of my knowledge, there are no studies on serotonin influence on equine emotional states, probably because all the previously cited factors influencing serotonin levels in equine serum or plasma needed to be rulled out. Additionally, the ELISA kit to analyse this neurotransmitter was only recently validated (Chabaud et al. 2018). This ELISA kit is a recent tool that will probably allow the development of new study designs, because ELISA kits do not have the contraints of radioimunoassay techniques (Bienboire-Frosini et al. 2017). Therefore, further research is still needed to investigate the serotoninergic mechanisms involved in emotional responses.

Amino acids

There are many amino acids in the brain (Fatjó et al. 2013). In this section, only two major amino acids that function as neurotransmitters in the brain will be introduced: gamma-aminobutyric acid (GABA) and the glutamate (Hahn 2004). GABA is the most wide-spread neurotransmitter in the brain and has inhibitory effects (Fatjó et al. 2013). GABA decreases were associated with seizure activity and Parkinson's disease (Fatjó et al. 2013). Aggression behaviours were associated with low levels of GABA in the neural tissue on post-mortem analysis, which is not surprising given the inhibitory role of GABA (Almeida et al. 2005). Other studies have associated higher plasma levels of GABA with aggressiveness, however, the

authors stated that it is still unclear how plasma levels are related to those in the neural tissue (Bjork et al. 2004). Therefore GABA is not a reliable indicator of equine emotional states.

Glutamate is the major excitatory neurotransmitter in the brain (Hahn 2004). This amino acid plays a crucial role in learning and memory (Hahn 2004). Glutamate is involved in aggressive, impulsive and defensive behaviour (Hahn 2004; Fatjó et al. 2013). Some authors suggested that behaviour modifications, in human patients experiencing dementia, might be associated with dysfunctional glutamate neurotransmission via N-methyl-D-aspartate (NMDA) receptors in the cortex and hippocampus regions of the brain (Francis 2010). Glutamate concentrations are often assessed through imaging or indirectly through the observation of patient's response to a specific medication (Francis 2010; van Veenendaal et al. 2016), which might be expensive and time-consuming. Therefore, these emotional indicators are not easy to measure, especially in healthy animals, which make them impractical.

Neuropeptides

Neuropeptides are short-chained amino acids, of which the main function is to modulate other neurotransmitters (Fatjó et al. 2013). Mammal neuropeptides with demonstrated relevance in emotional responses include endorphins, oxytocin and vasopressin (Heinrichs and Domes 2008; Dore et al. 2013).

Endorphins are endogenous opiate-like peptides released from the pituitary gland in response to sources of stress, either physical or mental (Mehl et al. 1999; Ferlazzo et al. 2017). Endorphins play a role in pain perception modulation as well (Mehl et al. 1999). Considering endorphins, only beta-endorphins were investigated (Mehl et al. 1999; Pell and McGreevy 1999; Micera et al. 2012; Ferlazzo et al. 2017). Some authors hypothesised that horses displaying stereotypic behaviours would have higher beta-endorphin concentration in plasma, based on the positive response of these animals to opioid antagonists (Pell and McGreevy 1999), however other authors investigated the plasma concentration of beta-endorphins in

horses displaying stereotypic behaviour and observed no significant difference when compared to a control group (Hemmann et al. 2012). They assessed beta-endorphins using radioimmunoassay (RIA) techniques, though, this method is being gradually replaced by enzyme immunoassay (EIA) techniques (Bienboire-Frosini et al. 2017). There is in the market an EIA kit available to study beta-endorphins in horses for research purposes, but few studies were done with this kit (Hemmann et al. 2012; Micera et al. 2012). Authors often highlight the need for further research on endorphins implication in equine behaviour and emotional responses (Hemmann et al. 2012; Fazio et al. 2013), therefore this is not yet a reliable indicator of equine emotional responses.

Oxytocin and vasopressin are neuropeptides composed by nine amino acids, both primarly synthesised in the hypothalamus, projected through the axons of hypothalamic neurons into various parts of the brain and released from the pituitary gland into the blood stream (Dhakar et al. 2013). Oxytocin and vasopressin are involved in different emotional responses (Lim and Young 2006; Neumann 2008; Lee et al. 2009; Koolhaas et al. 2010). These neuropeptides were associated with the regulation of social behaviours (social memory, affiliative behaviours or aggression) and appears to modulate stress- and anxiety-related behaviours (Heinrichs and Domes 2008; Neumann 2008; Lee et al. 2009; Ziegler and Crockford 2017). Additionally, oxytocin was associated with positive social interactions, both intra- and inter-specific interactions in different species (Numan and Young 2016; Rault 2016; Marcet Rius et al. 2018b). Therefore, some authors suggested that oxytocin might play a role in enhancing the well-being of animals (Ishak et al. 2011), while other authors suggested that oxytocin stability may indicate an animal's ability to cope with different situations that occur in their routines, which means that oxytocin could be a potential interesting indicator of animal welfare (Marcet Rius et al. 2018b). Nevertheless, the analysis of these neuropeptide concentrations in plasma through EIA techniques requires the validation of the available kits. The application of the EIA oxytocin kit originally designed to be used with human plasma was validated to assess free oxytocin concentrations in equine plasma (Bienboire-Frosini et al. 2017). However, some

authors suggested that free oxytocin concentrations might be a confounding factor because free oxytocin concentrations can be modified by different factors, as e.g. age or drugs, which produce samples with non-detectable levels of oxytocin (Doneanu and Rainville 2013). Therefore, some authors developed a method to assess the total fraction of oxytocin (bound to plasma proteins and free) in plasma samples of dogs (Brandtzaeg et al. 2016). Nevertheless, the correlation between free and total oxytocin concentrations was not established for equines, thus the meaning of total oxytocin concentrations as a behavioural indicator of animal welfare requires further research.

Glucocorticoids

Cortisol is a natural glucocorticoid hormone produced in response to adrenocorticotropic hormone (ACTH) stimulation of receptors in the adrenal cortex and was associated with physical and psychological stress (Covalesky et al. 1992). Some studies have investigated the HPA axis through the assessment of serum or salivary cortisol (Peeters et al. 2011, 2013; McKinney et al. 2015; Sauer et al. 2019). Some of these authors observed no significant differences on salivary cortisol reactivity between elite sport horses and amateur horses in multiple samples after ACTH stimulation, while breed and management conditions influenced the amounts of cortisol release (Sauer et al. 2019). This is interesting, because this probably means that cortisol is not a good welfare indicator for work/training activities (Johnson et al. 2017; Malinowski et al. 2018; Sauer et al. 2019), but would be more related to husbandry conditions (Ayala et al. 2012; Sauer et al. 2019). Peeters et al. (2013) suggested that cortisol levels in both riders and horses increase during competition, however they compared cortisol baseline (at rest) levels with competing levels and cortisol also sees a physiological increase with physical activity, which means that one could not discern whether an increase of cortisol is related to the physical activity or stress (Tadich et al. 2013; MacDougall-Shackleton et al. 2019). Additionally, cortisol release varies with the circadian cycle (Evans et al. 1977). This means that samplings should always be taken at the same day time, which might be

unpractical either in research or real conditions. Therefore, cortisol is not a reliable indicator for the study of emotional responses during physical activities.

Autonomic nervous system (ANS)

Stress-related responses have been associated with an increase in the activity of the autonomic nervous system (ANS) in horses (von Lewinski et al. 2013), especially with increases in sympathetic activity, heart rate and facial temperature (Hamlin et al. 1972; Pagani et al. 1986; Friedman and Thayer 1998; Janczarek et al. 2013; Hall et al. 2018).

Heart rate variability (HRV) analysis has often been used to study equine emotional responses to cognitive (Mengoli et al. 2014; Baragli et al. 2017) and temperament tests (König von Borstel et al. 2011), and as an indicator for equine welfare assessments (von Borell et al. 2007; Baragli et al. 2009; Stewart et al. 2011; Vitale et al. 2013). Fear and avoidance responses along with anxious states were associated with increases of the HR and decreases of some parameters of HRV (Rietmann et al. 2004; Villas-Boas et al. 2016). This means that HR and HRV provide information related to equine emotional responses, notably in the context of human-horse relationships (Friedman and Thayer 1998; Munsters et al. 2012; Baragli et al. 2014; von Rosenberg et al. 2017; Hall et al. 2018). Additionally, HRV provides information related to anticipation of a specific situation or recovery capacities (Smiet et al. 2014; Usui and Nishida 2017).

Sympathetic activity may be modified by psychological states without any perceived alteration in HR or respiratory rate (Sleigh and Henderson 1995; Tiller et al. 1996). However, modifications in sympathetic activity should be reflected in HRV (von Borell et al. 2007). HRV outputs include time domain analysis and frequency domain analysis. Time domain analysis has different parameters (von Borell et al. 2007; Stucke et al. 2015). The most common time domain parameters used to study HRV in horses are the HR, the standard deviation of the R-R wave intervals (SDNN) and the square root of the mean of the sum of the squares of

differences between successive interbeat-intervals (RMSSD) (Visser et al. 2002; Stucke et al. 2015). Frequency domain analysis includes parameters that are influenced by the respiratory rate, which varies according to species; therefore, the frequency bandwidths should be adapted in this type of analysis (Després et al. 2002; Bowen 2010). These bandwidths concern the following parameters: LF, HF and VLF. However, there is no established agreement concerning these frequency bandwidths. Some authors suggest that, for LF, HF, and VLF the bandwidth thresholds should be 0.01–0.07 Hz, 0.07–0.6 Hz and 0.0033–0.04 Hz respectively (Kuwahara et al. 1996; Gehrke et al. 2011; Zebisch et al. 2014), while other authors suggest thresholds of 0.005–0.07 Hz for LH, 0.07–0.5 Hz for HF and 0.001–0.005 Hz for VLF (Bowen and Marr 1998; Bowen 2010).

Each of the described parameters reflects different aspects of HRV (Stucke et al. 2015). The HR represents the net interactions between vagal and sympathetic regulation (von Borell et al. 2007). The SDNN reflects the long-term variability of cardiac activity and could be influenced by both the sympathetic and parasympathetic systems of the ANS (von Borell et al. 2007; Stucke et al. 2015). The RMSSD represents high-frequency beat-to-beat variations reflecting the vagal activity (von Borell et al. 2007; Stucke et al. 2015). Notably, no agreement has been established regarding the meaning of LF. Some authors have suggested that this parameter may represent sympathetic activity (Rietmann et al. 2004; von Borell et al. 2007), while others have suggested that LF may represent both sympathetic and parasympathetic activities (Bowen and Marr 1998; Bowen 2010). Additionally, some have suggested that the sympathetic tone should not be directly derived from HRV parameters (Kuwahara et al. 1996; von Borell et al. 2007). In contrast, HF is commonly considered a marker of the cardiac vagal tone and, consequently, the parasympathetic system (Kuwahara et al. 1996; von Borell et al. 2007; Evans and Young 2010; Stucke et al. 2015; Ohmura and Jones 2017). The VLF component of HRV has been associated with a slow recovery of the ANS balance after a mental or physical effort (Usui and Nishida 2017). Finally, the most widely accepted aspect of frequency domain analysis is that the LF/HF ratio parameter reflects the sympatho-vagal

balance or the sympathetic-parasympathetic balance (Kuwahara and Hiraga 1999; von Borell et al. 2007; Stucke et al. 2015). The analysis of the ANS activity provides an objective tool to study both positive and negative emotions (Désiré et al. 2002; Boissy et al. 2007).

The noninvasive character of and the information provided by HRV make this assessment an interesting tool for studying ANS activity in horses during physical or psychological challenges (Malinowski et al. 2018). However, the assessment of HRV or any other physiological indicators should be combined with behavioural indicators to be correctly interpreted (Paul et al. 2005; Hall et al. 2018).

Behavioural indicators of positive and negative emotions

Emotions are often assessed through behavioural responses, as changes in locomotion patterns, approach and avoidance, or more subtle signs as body posture and facial expressions (Porsolt et al. 1977; Hall et al. 2018). Vocalisations are also studied as an emotional indicator, however, they are variable and probably similar sounds may represent different valences (positive or negative), which made vocalisations difficult to assess and sometimes their interpretation might differ between authors (Yeon 2012; Stomp et al. 2018b). Further research on vocalisation, specifically, in acoustic signals and their emotional valences are still needed (Hall et al. 2018).

High head and tail position, snorting and locomotory responses were previously used as indicators of anxiety and fear responses (Forkman et al. 2007). Facial expression and ear movements were equally used in some researches to study signs of discomfort or pain and emotional responses as well (Dalla Costa et al. 2014; Hintze et al. 2016; van Dierendonck and van Loon 2016; Waran and Randle 2017). These behaviours were assessed in horses at resting conditions and were associated with negative emotions, but their assessment in ridden horses might be difficult (Waran and Randle 2017). In terms of positive indicators of emotions, few studies have investigated them, and they are mostly related to social play, affiliative

behaviours and sometimes vocalisations (Waran and Randle 2017). These parameters, except for vocalisations, can not be observed in ridden horses.

Some studies were done to investigate behavioural indicators of emotions in ridden horses and to assess whether they can be identified by professionals or not (Fleming et al. 2013; Hall et al. 2014; Smiet et al. 2014). Head position, head/neck carriage, head movement, looking around, ear position, palying/chewing the bit, mouth and tongue positions, tail movements, salivation and auditory signals were investigated. Equestrian professionals did not respond consistently, suggesting that these behaviours are difficult to assess, so the authors concluded that further research on behavioural indicators of emotions in ridden horses is needed (Hall et al. 2014). Additionally, the training of horses to practice a specific activity has an influence in the way horses manifest fear and stress, which means that some animals may be more tolerant and others more reactive to the exact same stimulus depending on the activity in which they are involved (von Borstel et al. 2010). Therefore, the activity factor needs to be considered for the assessment of behavioural indicators in horses (Yarnell et al. 2013).

Squibb et al. (2018) suggested that the influence of training and the extent to which a horse is under stimulus-control may over-shadow inherent emotional responses. This reinforces the need of behavioural indicators to be combined with other measures, as physiological indicators to allow a global understanding of equine emotions (Hall et al. 2018).

OBJECTIVES

The main objective of this thesis was to investigate how different activities, imposed by humans, impact equine emotional responses and welfare.

More precisely, the aims were defined as follows:

- Investigate whether equine emotional responses would vary or not with the activity in which horses were involved in their usual routines.

- Demystify some of the beliefs, held by the equestrian community, by investigating equine emotional responses, through behavioural and physiological indicators, to standardised training sessions.

- Explore the rider's influence on the horse's biomechanics and on the horse's emotional responses to training.

- Investigate equine emotional responses to different working conditions.

RESULTS

Chapter 1: Equine activities influence horses' responses to different stimuli: could this have an impact on equine welfare?

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1.1. Introduction

The involvement of horses in activities imposed by humans may modify the horse's emotional responses. Different training methods, specific to each activity, are used to let horses learn how to respond or react to the diverse situations they will face. Since horses involved in different activities will experience different training methods, they may learn to respond differently to certain stimuli. The disclosure of this hypothesis was a fundamental condition for the general investigation because if horses who were involved in a specific equestrian or equine activity responded differently to some stimuli, this condition should be considered in the following studies. Controlled conditions were then created to study the influences of an equestrian or equine activity on the horses behavioural and physiological responses to different stimuli.

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Equine Activities Influence Horses' Responses to Different Stimuli: Could This Have an Impact on Equine Welfare?

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Simple Summary: Horses are required to perform a wide variety of activities. Training a horse for these activities may influence the horse's perception of and reactions to different stimuli. This study investigated the reactivity and emotional responses of horses involved by humans in different equine activities (dressage, jumping, eventing and equine-assisted activity/therapy) by studying their physiological and behavioral responses to different stimuli. A test setting with five phases was created to test equine responses to five different stimuli and compare these responses among horses from the different disciplines. It was demonstrated that the horses involved in the different activities had different responses, both physiologically and behaviorally, to the studied stimuli. These findings suggest that training a horse for a specific activity modifies the perception of stimuli and the strategies that the horse uses to balance its emotional state. Thus, horses involved in different activities probably behave differently according to their training. Such information is of great importance in improving training methods, with the aim of increasing equine welfare.

Abstract: The learning and cognitive challenges that horses may face differ according to the activities in which they are involved. The aim of this investigation was to study the influence of equine activities on the behavioral responses and autonomic nervous system (ANS) activity of adult horses. Forty-one horses were divided into four groups: dressage (9), jumping (10), eventing (13) and equine-assisted activity/therapy (9). A test was created to compare the horses' behavioral and physiological responses to different stimuli. The goal was always to obtain a treat. To study the ANS activity, heart rate variability was assessed using the standard deviation of the R-R intervals (SDNN), square root of the mean of the sum of the squares of differences between successive interbeat-intervals (RMSSD) and low frequency/high frequency (LF/HF). To assess behavioral responses, video analysis was performed considering the following behaviors: exploration, interactions with another horse, and latency to approach. Significant differences in SDNN (DF = 3; F = 3.36; $p = 0.0202$), RMSSD (DF = 3; F = 4.09; $p = 0.0078$), LF/HF (DF = 3; F = 4.79; $p = 0.0031$), exploration (DF = 3; F = 5.79; $p = 0.0013$) and latency to approach (DF = 3; F = 8.97; $p < 0.0001$) were found among horses from different equine activities. The activity that adult horses practice appears to influence behavioral and physiological responses to different stimuli, thus impacting equine welfare.

Keywords: behavior; horse; equitation; heart rate variability; reactivity; welfare

1. Introduction

The concept of animal welfare includes both physical and mental elements, suggesting that emotions are an important component of welfare [1,2]. Animal emotions have been widely studied and many studies have investigated the influence of emotions on animal welfare [3–5]. Stress has often been associated with negative emotions [6], and the latter has been associated with poor animal welfare [1].

Some authors have suggested that horses from different activities might express their emotions through different behaviors [7,8]. For example, fear reactions differ between horses from different activities [9]. Jumping horses appear to be less reactive to frightening stimuli than dressage horses [9]. The cited study suggested that jumping horses may have a genetic component involved in the development of such behavior, while no correlation was found between genetics and the development of behavior in dressage horses. Learning not to respond to frightening stimuli may be associated with a habituation or desensitization to these stimuli through training, but horses might not generalize this reduced fear response to all potentially fear-inducing situations related to competition and training [10].

Learning capacities are influenced by emotions [3], which means that horses trained to perform different equine activities may use different behavioral and physiological strategies to adapt to the training conditions in which they are involved. These differences could have consequences in the development of the animals' behavioral traits and emotional management [11].

The learning and cognitive challenges that horses may face differ with the sports discipline they practice [12]. Dressage horses must discriminate between similar cues provided by riders, while jumping horses must learn to jump obstacles, while ignoring the obstacle color or structure [12]. Eventing horses must combine the needs of dressage and jumping horses [12]. Equine-assisted activity/therapy (EAA/T) involves activities that include both recreational and therapeutic interventions for people with mental and/or physical conditions [13–15]. Horses involved in these activities may experience stress induced by the inconsistency of the patients' contact or cues, as these people are usually experiencing physical, mental or combined medical conditions [16,17].

As a social species, horses devote a significant amount of their time to intra- and interspecific interactions, and even to reconciliation after conflict [18,19]. The husbandry conditions and management of these animals should encourage socialization because equine behavioral and emotional responses might be influenced by the animals' sociability [20]. Housing conditions that allow horses to interact with conspecifics have been associated with an improvement in learning abilities and training performance [21]. Additionally, housing conditions that involve social isolation have been associated to increases in psychological stress expressed by behavioral and physiological changes [22,23]. These findings suggest that improving the welfare (e.g., improving housing conditions) of horses may improve training performance as well. Nevertheless, training performance could also be influenced by other factors [8].

Stress-related responses have been associated with an increase in the activity of the hypothalamic-pituitary-adrenocortical (HPA) axis, adrenomedullary system and autonomic nervous system (ANS) in horses [24]. Some studies have investigated the HPA axis through the assessment of serum or salivary cortisol [15,25,26]. Other studies have investigated the ANS through the assessment of heart rate variability (HRV), which is a noninvasive technique for studying the ANS responses of horses [27]. Additionally, HRV analysis in horses has often been used to study emotional responses to cognitive [3] and temperament tests [11], and as an indicator for equine welfare assessments [27].

Sympathetic activity may be modified by psychological states without any perceived alteration in heart rate (HR) or in respiratory rate [28]. However, modifications in sympathetic activity should be reflected in HRV [27]. HRV might be useful to study sympathetic and parasympathetic activities during psychological or physical challenges via parameters such as the low frequency/high frequency (LF/HF) ratio [27].

HRV outputs include time domain analysis and frequency domain analysis. Time domain analysis has different parameters [27,29]. The most commonly used parameters to study HRV in horses are

the HR, the standard deviation of all the R-R wave intervals (SDNN) and the square root of the mean of the sum of the squares of differences between successive interbeat-intervals (RMSSD) [29,30]. Frequency domain analysis includes parameters that are influenced by the respiratory rate, which varies according to species; therefore, the frequency band widths should be adapted in this type of analysis [31,32]. These band widths concern the following parameters: LF and HF. However, there is no established agreement concerning these frequency band widths. Some authors suggest that, for LH and HF, the band width thresholds should be 0.01–0.07 Hz and 0.07–0.6 Hz, respectively [33,34], while other authors suggest thresholds of 0.005–0.07 Hz for LH and 0.07–0.5 Hz for HF [32,35].

Each of the described parameters reflects different aspects of HRV [29]. The HR represents the net interactions between vagal and sympathetic regulation [27]. The SDNN reflects the long-term variability of cardiac activity and could be influenced by both the sympathetic and parasympathetic systems of the ANS [27,29]. The RMSSD represents high-frequency beat-to-beat variations reflecting the vagal activity [27,29]. Notably, no agreement has been established regarding the meaning of LF. Some authors have suggested that this parameter may represent sympathetic activity [27,36], while others have suggested that LF may represent both sympathetic and parasympathetic activities [32,35]. Additionally, some have suggested that the sympathetic tone should not be directly derived from HRV parameters [27,33]. In contrast, HF is commonly considered a marker of the cardiac vagal tone and, consequently, the parasympathetic system [27,29,33]. Finally, the most widely accepted aspect of frequency domain analysis is that the LF/HF ratio parameter reflects the sympatho-vagal balance or the sympathetic-parasympathetic balance [27,29,37].

The noninvasive character of and the information provided by HRV make this assessment an interesting tool for studying ANS activity in horses during physical or psychological challenges [14].

Therefore, the aim of the present investigation was to study the influence of the activity in which animals were involved on the behavioral responses and ANS activity of adult horses. We hypothesized that the behavioral responses and the ANS activity of horses were influenced by the activity in which horses were involved.

2. Materials and Methods

This investigation included two experiments. The first experiment investigated equine behavior and physiological responses under different types of stimuli. The second experiment investigated the influence of several equine activities on horses' behavior and physiological responses.

This project was approved by IRSEA's (Research Institute in Semiochemistry and Applied Ethology) Ethics Committee (C2EA125) and the French Ministry of Research (APAFIS Process number 11949).

2.1. Population

The horse populations differed between the two experiments. Common inclusion criteria were as follows: horses should be more than 4 years old, be performing any equestrian or therapeutic activity at the time of the experiment, and according to a physical consultation performed by a veterinary practitioner, should be healthy. All horses included in the experiments had access to paddocks during the daytime and to a single box during the night. On rainy days, the horses were kept indoors.

In the first experiment, the population of horses comprised forty-one horses (1 stallion, 23 geldings and 17 females) with a mean age of 10.41 ± 4.28 years old. These horses were involved in one of the following activities in their usual daily lives: jumping, eventing, dressage, endurance, reining or leisure. Leisure horses comprised horses that were involved in various riding activities (dressage, jumping, reining, equestrian rides) with no specific purpose, as a hobby of the rider.

For the second experiment, as part of the inclusion criteria, horses had to be involved in one of the following equine activities as part of their usual routine: jumping, eventing, dressage or EAA/T. These horses had been involved in one of these activities since the age of 3–4 years. To reduce the number of animals used for research proposals and to respect legislation concerning ethics and equine welfare, particularly the 3R's rule, data obtained in the first experiment concerning horses involved in jumping,

eventing and dressage were used for the second experiment. Nine new horses involved in EAA/T were also included. Ultimately, a population of forty-one horses (25 geldings and 16 females) with a mean age of 10.76 ± 3.99 years old was considered for this experiment. The population was divided into the following groups: dressage (9), jumping (10), eventing (13) and equine assisted activity/therapy (9).

2.2. Study Design and Data Collection

Each experiment was divided into different phases (described below). The first experiment investigated the phase effects to understand the behavioral and physiological responses of the tested horses in the different phases. The second experiment studied two factors: activity and phase. The phase factor was studied to ensure that any possible observed significant difference in activity was not influenced by a cross effect between phase and activity.

The setting and testing conditions were the same for both experiments.

A testing area was created (Figure 1) to investigate equine physiological and behavioral responses to varying stimuli. The testing area was made of a 10 m^2 paddock with an open field structure. A smaller paddock attached to the testing area hosted a familiar horse during the trials, respecting the horse's need for interaction with other horses [21]. The familiar horses did not participate in the experiments and were provided with hay and water. A chair and a bucket containing treats (carrots, commonly used as treats in this equine population) were positioned at the side of the testing area opposite to the testing area's entrance. The horse approaching and obtaining the treats from the bucket was always the goal.

A habituation period was used to let the horses spontaneously explore and get used to the setting and to create the motivation to approach the bucket and acquire the treats. This period lasted no more than 5 min. A horse was considered as habituated to the setting when it explored the setting, walked to the bucket and fed.

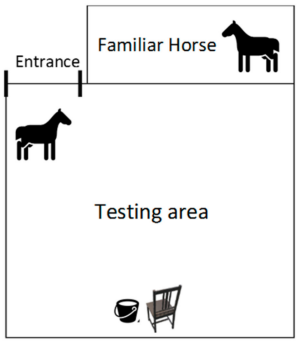


Figure 1. Scheme of the testing area. This setting was designed to test horse reactivity to different stimuli, while avoiding social isolation.

Each experiment included five independent phases (Table 1): baseline; unknown person; umbrella opening; ground obstacle; social isolation. These phases lasted no more than 5 min each, except for the baseline phase. Horses were led and removed from the testing area between each phase by an operator. During each testing phase, the horses were free in the testing area.

HRV data were collected using the Polar V800 Equine® system (Kempele, Finland). The data collected with the Polar system were transformed with Kubios® software (Kuopio, Finland) and only normal R-R intervals were considered for the HRV analysis. The final data included time domain and frequency domain analysis results (Table 2). Frequency band thresholds were established within each frequency interval using the following parameters: LF power = 0.005–0.07 Hz; HF power = 0.07–0.5 Hz; and LF/HF ratio [32].

During the last four phases, behavioral data were collected via video recording using a Sony Handycam HDR-CX450® camera (Weybridge, UK). Behaviors were assessed through video analysis, and with the help of an Excel® matrix (Redmond, WA, USA), behavior durations were determined

by one operator according to an ethogram (Table 3). This ethogram was developed based on the importance of these behaviors for equines [18,19,38–40]. For experiment 1, raw data were used for statistical analysis, and the test was considered to last 5 min for each phase for each horse. However, most of the horses did not need 5 min to achieve the goal, so in the second experiment, each phase was considered as finished once the horse achieved the goal. As the time to achieve the goal could vary between individuals, the proportion of the duration of each specific behavior over the latency to obtain the treat was estimated.

Table 1. Description of the aims of each phase of the experiment.

Phase	Aim	Description
Baseline (1)	Collection of HRV data at rest	HRV data was collected over a period of twenty minutes under normal life conditions of the horse (paddock or box) without any human influence.
Unknown person (2)	To test interspecific socialization	An unknown person sat on a chair close to the bucket (1 m of distance), so the horse needed to approach the person to access the treat.
Umbrella opening (3)	To test responses to a sudden stimulus	This stimulus has been used in many studies to investigate equine responses to sudden stimuli [37,38]. In this phase, the person sitting on the chair opened an umbrella when the horse starts eating from the bucket.
Ground obstacle (4)	To test recovery capacities and strategies	Obstacle bars were placed in the middle of the testing area, dividing the setting into two parts. To approach the treat and the person on the chair, the horse had to cross the bars.
Social Isolation (5)	To test equine responses to social isolation	The familiar horse and the person were removed from the testing area and from the horse's visual field.

Table 2. Adapted description of HRV variables from Stucke et al., (2015) [29].

Variable	Definition	Interpretation
HR (bpm)	Heart rate mean	Overall variability of cardiac activity
SDNN (ms)	Standard deviation of RR intervals	Long-term variability of cardiac activity influenced by the autonomous nervous system
RMSSD (ms)	Square root of the mean of the sum of the squares of differences between successive interbeat-intervals (IBI)	High frequency of IBI variations correlated to parasympathetic activity
LF/HF ratio	Low frequency/high frequency ratio	Representation of the balance between sympathetic and parasympathetic nervous systems

Table 3. Ethogram used for video analysis.

Behavior	Definition
Exploration	The animal searches for information by walking around the testing area or by sniffing or touching the person or object.
Interactions with another horse (Interaction)	Interactions with the untested horse through physical or visual contact.
Latency to obtain the treat (Latency 1)	Time the horse takes to approach the bucket and obtain the treat in all the phases.
Latency to obtain the treat after the umbrella opens (Latency 2; only in the 2nd experiment)	Time the horse takes to approach the bucket again after the umbrella opens in phase 3.

2.3. Statistical Analysis

Statistical analysis was carried out using SAS 9.4 software (SAS Institute Inc., Cary, NC, USA). The significance threshold was classically fixed at 5%.

The behavioral parameter "interaction with another horse" was not considered in phase 5 because the untested horse was removed from the testing area.

2.3.1. Experiment 1

For HR, SDNN, RMSSD, LF/HF ratio and Latency 1, the assumption of normality of the data was tested using the residual diagnostics plots and the UNIVARIATE procedure. Comparisons between phases were performed with the general linear mixed model using the MIXED procedure. If significant differences were found, multiple comparisons were analyzed with the Tukey test using the LSMEANS statement.

For Exploration and Interaction, the assumption of normality of the data was tested with the same procedure, although it was not verified. Then, comparisons between phases were performed using the nonparametric Friedman test using the FREQ procedure. If significant differences were found, multiple comparisons were analyzed with the signed-rank test for each pair of times with the UNIVARIATE procedure, and then Bonferroni correction was applied with the MULTTEST procedure to control the error risk due to the multiplicity of the tests.

2.3.2. Experiment 2

For SDNN, RMSSD, LF/HF ratio and Latency 1, the assumption of normality of the data was tested using residual diagnostics plots and the UNIVARIATE procedure. Homoscedasticity was tested with Levene's test using the GLM procedure. Comparisons between phases and activity were performed with the general linear mixed model using the MIXED procedure. When significant differences were found, multiple comparisons were analyzed with the Tukey test using the LSMEANS statement.

For HR and Exploration, the assumption of normality was verified, but the homogeneity of the variances was not. The GROUP = option was added in the REPEATED statement of the MIXED procedure. Then, comparisons between phases and activity were performed with the general linear mixed model using the MIXED procedure. If significant differences were found, multiple comparisons were analyzed with the Tukey test using the LSMEANS statement.

For Latency 2 (after the opening of the umbrella), the assumption of normality was not verified. Consequently, the nonparametric Kruskal-Wallis test was performed using the NPAR1WAY procedure. If significant differences were found, multiple comparisons were analyzed with the Wilcoxon test using the NPAR1WAY procedure, and then Bonferroni correction was applied with the MULTTEST procedure to control the error risk due to the multiplicity of the tests.

For Interaction, data were transformed into binary variables because the animals did not perform this behavior very often. In this case, the following code was considered: 1—there was an interaction with the untested horse; 0—there was no interaction with the untested horse. Comparisons between phases and activity were performed with the generalized linear mixed model using the GLIMMIX procedure. If significant differences were found, multiple comparisons were analyzed with the Tukey test using the LSMEANS statement.

3. Results

3.1. Experiment 1

Due to technical issues concerning the HR monitor, the data of 3 horses were not recorded. Additionally, artifacts concerning SDNN, RMSSD and the LF/HF ratio parameter of 2 horses were found. Aberrant data were excluded from the statistical analysis.

Additionally, for technical reasons, the video camera did not record some time frames, and some data were absent for 1 horse and could not be considered in the statistical analysis.

Some horses in the original population (one horse in phase 2; one horse in phase 3; three horses in phase 4; and two horses in phase 5) did not achieve the goal within 5 min, so their data were removed from the statistical analysis considering Latency 1.

All the included horses succeeded in the habituation phase.

3.1.1. Physiological Data (Table 4)

Significant differences in HR were observed between phases (DF = 4; F = 20.55; $p < 0.0001$). More specifically, HR was significantly lower in phase 1 than in all the other phases, and HR was significantly higher in phase 3 than in phases 2 and 4 (Table 4).

There was no significant difference in SDNN between phases (DF = 4; F = 1.74; $p = 0.1446$) (Table 4).

Significant differences in RMSSD were observed between phases (DF = 4; F = 2.74; $p = 0.0309$). However, after performing the Tukey adjustment for multiple comparisons, significant differences between individual phases could not be identified (Table 4).

There was no significant difference in the LF/HF ratio between phases (DF = 4; F = 0.45; $p = 0.7723$) (Table 4).

Table 4. Physiological data results of experiment 1.

Parameter	Phase	N	Mean	Std. Dev.	Median	Minimum	Maximum
Heart Rate	Baseline	41	42 [a]	7	43	30	55
	Unknown person	39	52 [b]	12	49	35	80
	Umbrella opening	41	58 [bc]	13	56	34	88
	Ground obstacle	40	52 [c]	10	50	34	78
	Social isolation	40	54	17	52	31	129
SDNN	Baseline	41	218	167	163	70	1026
	Unknown person	39	244	184	216	70	1192
	Umbrella opening	41	207	100	176	59	408
	Ground obstacle	39	177	111	145	44	482
	Social isolation	38	227	191	167	29	1024
RMSSD	Baseline	41	191	150	127	48	568
	Unknown person	39	172	103	183	29	441
	Umbrella opening	41	133	92	116	28	404
	Ground obstacle	39	124	86	90	27	409
	Social isolation	38	156	116	136	23	453
LF/HF Ratio	Baseline	41	3	3	3	0	13
	Unknown person	39	7	11	3	0	59
	Umbrella opening	41	8	13	5	0	78
	Ground obstacle	40	5	7	3	0	35
	Social isolation	40	8	15	3	0	67

[a] Significant difference between the marked phase and all the other phases (Tukey test). [b,c] Significant difference between the marked phases (Tukey test).

3.1.2. Behavioral Data (Table 5)

For Exploration, significant differences were observed between phases (DF = 3; Chi-square = 27.35; $p < 0.0001$). More specifically, Exploration was significantly lower in phase 5 than all the other phases (Table 5).

There was no significant difference in interactions with another horse between phases (DF = 2; Chi-square = 2.71; $p = 0.2576$) (Table 5).

Significant differences were observed between phases considering Latency 1 (DF = 3; F = 14.53; $p < 0.0001$). More precisely, Latency 1 was significantly higher in phase 2 than in phase 3. Additionally, Latency 1 was significantly lower in phase 5 than all the other phases (Table 5).

Table 5. Behavioral data results of experiment 1.

Parameter	Phase	N	Mean	Std. Dev.	Median	Minimum	Maximum
Exploration time (s)	Unknown person	41	42	48	22	0	199
	Umbrella opening	41	41	40	31	0	183
	Ground obstacle	40	35	53	14	0	233
	Social isolation	40	12	16	7 [b]	0	86
Interaction time (s)	Unknown person	41	7	19	0	0	98
	Umbrella opening	41	5	12	0	0	55
	Ground obstacle	40	3	8	0	0	40
Latency 1 time (s)	Unknown person	40	71 [c]	76	38	10	300
	Umbrella opening	40	37 [c]	27	29	9	123
	Ground obstacle	38	48	62	24	10	300
	Social isolation	39	21 [a]	18	14	5	89

[a] Significant difference between the marked phase and all the other phases (Tukey test). [b] Significant difference between the marked phase and all the other phases (Signed-rank test). [c] Significant difference between the marked phases (Tukey test).

3.2. Experiment 2

Due to technical issues concerning the HR monitor, the data of 5 horses were not recorded. Additionally, artifacts concerning HR, SDNN, RMSSD, and the LF/HF ratio of a few horses were found. Aberrant data were excluded from the statistical analysis.

Furthermore, because of technical reasons, the video camera did not record some time frames of 1 horse, so these data were absent and could not be considered in the statistical analysis.

Some horses in the original population did not achieve the goal within the 5 min, so their data were removed from the statistical analysis for Latency 1 (one horse in phase 2, one horse in phase 3, three horses in phase 4, and two horses in phase 5) and Latency 2 (three horses only in phase 3).

All the included horses succeeded in the habituation phase.

3.2.1. Physiological Data (Table 6)

There was no significant difference in HR between activities (DF = 3; F = 1.02; $p = 0.3886$) (Table 6).

Table 6. Physiological data results of experiment 2.

Parameter	Activity	N	Mean	Std. Dev.	Median	Minimum	Maximum
Heart Rate	Jumping	49	50	10	49	32	81
	Eventing	65	51	13	49	30	82
	Dressage	37	50	15	49	30	85
	EAA/T	43	47	8	46	35	76
SDNN	Jumping	48	189	93	170	67	443
	Eventing	64	188	112	153	29	515
	Dressage	38	216 [a]	114	188	57	500
	EAA/T	43	152 [a]	94	110	32	406
RMSSD	Jumping	48	138	84	117	45	351
	Eventing	65	136	105	106	25	441
	Dressage	36	171 [a]	106	154	23	436
	EAA/T	43	108 [a]	89	81	32	466
LF/HF Ratio	Jumping	49	5	6	3	0	38
	Eventing	64	6 [a]	8	4	0	54
	Dressage	38	2 [ab]	2	2	0	12
	EAA/T	43	7 [b]	9	4	0	57

[a,b] significant differences between the marked phases (Tukey test).

Significant differences in SDNN were observed between activities (DF = 3; F = 3.36; $p = 0.0202$). More specifically, SDNN was significantly higher in the dressage group than in the EAA/T group. A trend was also observed between the jumping and the EAA/T groups, with a higher SDNN value

in the jumping group than in the EAA/T group. No significant difference was observed in the phase effect or in the interaction between the activity and phase factors (Table 6).

Significant differences in RMSSD were observed between activities (DF = 3; F = 4.09; $p = 0.0078$). More specifically, RMSSD was significantly higher in the dressage group than in the EAA/T group. A trend was also observed between the jumping and the EAA/T groups, with a higher RMSSD value in the jumping group than in the EAA/T group. No significant difference was observed in the phase effect or in the interaction between the activity and phase factors (Table 6).

Significant differences in LF/HF were observed between activities (DF = 3; F = 4.79; $p = 0.0031$). More specifically, LF/HF was significantly higher in the EAA/T and eventing groups than in the dressage group. No significant difference was observed in the phase effect or in the interaction between the activity and phase factors (Table 6).

3.2.2. Behavioral Data (Table 7)

Significant differences in Exploration were observed between activities (DF = 3; F = 5.79; $p = 0.0013$). More specifically, Exploration was significantly higher in the jumping and EAA/T groups than in the eventing and dressage groups. No significant difference was observed in the phase effect or in the interaction between the activity and phase factors (Table 7).

Table 7. Behavioral data results of experiment 2.

Parameter	Activity	N	Mean	Std. Dev.	Median	Minimum	Maximum
Exploration	Jumping	40	68 ^{ab}	20	72	13	97
	Eventing	51	51 ^{ac}	25	55	0	100
	Dressage	35	57 ^{bd}	21	60	8	86
	EAA/T	36	69 ^{cd}	29	68	14	100
Latency 1	Jumping	38	66 ^{a}	69	40	10	300
	Eventing	49	29 ^{ab}	31	19	8	179
	Dressage	36	44 ^{c}	61	25	5	300
	EAA/T	35	82 ^{bc}	77	62	7	294
Latency 2	Jumping	10	41	32	38	2	80
	Eventing	12	36	82	9	0	295
	Dressage	9	44	80	6	2	247
	EAA/T	7	7	7	6	0	18

^{a,b,c,d} significant differences between the marked phases (Tukey test).

There was no significant difference in Interaction between activities (DF = 3; F = 1.11; $p = 0.3486$). Interaction was only performed by 47% of the horses in the jumping group, 24% in the eventing group, 15% in the dressage group and 7% in the EAA/T group (Descriptive statistics: 0 = absence of interaction; 1 = presence of interaction; jumping: 0 = 16, 1 = 14; eventing: 0 = 29, 1 = 9; dressage: 0 = 23, 1 = 4; EAA/T: 0 = 25, 1 = 2) (Table 7).

Significant differences in Latency 1 were observed between activities (DF = 3; F = 8.97; $p < 0.0001$). More specifically, Latency 1 was significantly higher in the jumping and EAA/T groups than in the eventing group. Additionally, Latency 1 was significantly higher in the EAA/T group than in the dressage group. A significant difference was also observed in the phase effect (DF = 3; F = 5.87; $p < 0.001$). More precisely, Latency 1 was significantly higher in phase 2 than in phase 5. No significant difference was observed in interaction between activity and phase factors (Table 7).

There was no significant difference in Latency 2 between activities (DF = 3; Chi-Square = 4.58; $p = 0.2052$) (Table 7).

4. Discussion

In general, the results obtained in this study have shown that equine behavioral and physiological responses can be influenced by the activities in which horses are involved.

The first experiment confirmed that the stimuli in the different phases were neither too frightening nor challenging for the animals. Significant differences in RMSSD meant that the parasympathetic nervous system response differed in the different phases, but an overall balance of the ANS was maintained, as demonstrated by the absence of significant differences in SDNN and the LF/HF ratio.

In terms of the HR, an increase between the baseline HR and the HR of all the other phases was expected because the baseline represents an inactive phase from both physical and psychological perspectives. In the other phases, horses were exposed to new environmental conditions that they had to explore and were thus active. Significant differences concerning HR in phase 3 were most likely associated with the physical reaction of moving away from the sudden stimulus (the umbrella opening) rather than with emotional unbalance, as no other modifications in physiological parameters were found.

The decrease in the time spent exploring the testing area, as well as the decrease in Latency 1 between phases 2 and 3, could be explained by the learning capacities of the horses. The decrease in Exploration and Latency 1 in phase 5 might be interpreted as a strategy to cope with social isolation. This coping strategy was successful in balancing the emotional state of the horses, as demonstrated in the HR, SDNN and LF/HF ratio results.

The general lack of interactions between horses was an interesting finding, especially since horses are a social species. Nevertheless, even though interactions between horses were not observed as often as expected, the removal of the familiar horse modified the behavior of the tested horse. This behavioral modification was expressed by a reduction in exploratory behavior and Latency 1. These findings mean that even if the horses did not interact, the presence of a familiar horse appeared to have an emotional value for the tested horse.

To investigate the influence of equines' activities on the emotional responses of horses, the selected stimuli should not create excessive fear. This condition was important because stimuli that are too frightening may induce maladaptive responses [41,42], which was not the subject of the present investigation. The first experiment demonstrated that the stimuli were suitable for the investigation of the influence of equines' activities on horses' emotional responses.

The results of the second experiment showed that the activities in which horses are involved influence horses' physiological and behavioral responses to different stimuli.

Jumping horses balanced the activity of the ANS during the test, as demonstrated by the SDNN and LF/HF ratio results. This group required more time than the dressage and eventing groups to explore the testing area before approaching the person and obtaining the reward, as expressed by the Exploration and Latency 1 results. Nevertheless, the time spent exploring did not induce any modification of ANS activity.

Dressage horses had a higher value of SDNN than EAA/T horses, meaning that the former experienced a higher overall variability of the ANS than the latter during the test. However, the differences in the RMSSD and LF/HF ratio results demonstrated a higher influence of the parasympathetic nervous system in the overall variability of horses in the dressage group than in the EAA/T group. The EAA/T group had a lower overall variability and higher sympathetic nervous system activity than the dressage group during the test, as described by the RMSSD and LF/HF ratio results. This means that horses in the dressage group were more successful than horses in the EAA/T group in balancing the ANS during the test. Dressage horses took less time than EAA/T horses to explore the testing area and approach the person and the bucket to obtain the treat. Considering the physiological data, EAA/T horses showed an unbalance in the ANS and needed more time than dressage horses to approach the unknown person, which suggests that some social apprehension in horses may be induced by equine-assisted activities/therapy.

Eventing horses also had a high LF/HF ratio. Even if no other differences were found considering the physiological data, these results mean that an unbalance in the ANS was induced by the test, and this unbalance was most likely related to the sympathetic nervous system, as no differences were found in RMSSD. Eventing horses approached the person and achieved the goal more quickly than

jumping horses, but eventing horses experienced an emotional unbalance, while jumping horses did not.

All horses needed a similar length of time to approach the person after the opening of the umbrella. This is an interesting finding because it means that, independent of the activity they have been trained to practice, horses will demonstrate the same response to a sudden stimulus: running away and then returning and exploring the area.

The influence of the equine's activity on equine behavioral and physiological responses has been studied by different authors [8–10,17,43].

Some authors have suggested that jumping horses react significantly less than other horses to novelty [9]. Indeed, in our test, the jumping horses had a lower reactivity than the other horses. This low reactivity is usually desired in jumping horses by riders to reduce the risk of accidents [9]. However, some undesirable side effects can be expected, such as undesirable behaviors related to low fearfulness [9].

Another study [8] described differences in the reactional state of horses from different activities, suggesting that dressage horses could be more emotionally reactive than other horses. In agreement with the cited studies, the dressage horses reacted quickly to the different stimuli in our study. However, they rapidly recovered emotional homeostasis and were more successful in coping with the different stimuli than eventing and EAA/T horses. These findings suggest that dressage horses react to different stimuli faster than the other horses, but they also recover faster than other horses from an emotional challenge. A factor that could have a role in the observed difference between the reactivity of dressage horses in different studies is the housing conditions of the animals and their temperament [43–45]. Horses living in single boxes are deprived of many stimuli and may become more reactive to novelty, while horses with access to paddocks, likes the ones involved in this study, may be less reactive [22,23]. Additionally, equitation science has encouraged the application of training techniques that improve equine welfare in recent years [46], which may result in horses that are less inhibited and learn to manage their emotions in different situations [47].

A difference in the endocrinal responses (salivary cortisol) between eventing and dressage horses was previously described [10]. In the present study, eventing horses showed a higher increase in sympathetic system activity than jumping and dressage horses. These differences suggest that either the endocrinal or autonomic responses of eventing horses differ from those of other horses.

Studies on EAA/T horses have suggested that therapeutic riding may be as stressful as recreational riding [15,17]. In our study, even free horses demonstrated some emotional responses (HRV and behavior results) and probably some apprehension in approaching the person. These findings suggest that EAA/T horses may cope well with therapeutic activities but have some apprehension towards human contact; thus, producing poorer interspecific socialization.

The training exercises and conditions for performing different activities are very variable as horses need to acquire different competences to succeed in each of the activities, as described previously in [12,16,17]. The different learning situations associated to specific training for the different activities may influence the behavioral and physiological responses of horses in adulthood.

Equine welfare can be influenced by handling, training and housing conditions [44,48–50]. This investigation highlighted the variability of equine behavioral and physiological responses to different stimuli, and the influence of some equine activities on the reactional state and emotional management of adult horses. This means that the specific training for different activities may influence equine welfare in different ways. Understandings of equine perception of different stimuli and the corresponding emotional responses are of great importance to the equestrian community and for improving equine welfare.

5. Conclusions

The activity in which adult horses are involved appears to have an influence on their behavioral and physiological responses to different stimuli, and thus affects the welfare of horses. Understanding

this influence is of great interest for improving equine welfare, with the aim of habituating and/or desensitizing horses to disturbing stimuli.

It might be interesting to study the same responses in foals before starting the training process for any of the described activities. This investigation would allow us to better understand how the genetic component, and the learning process through training and environmental stimulation are related in the development of behavioral and physiological responses of horses.

Further studies should be performed to acquire more knowledge about equine behavior and physiological responses related to emotions. Equine housing and general management conditions have been improved thanks to increased knowledge based on scientific evidence. Therefore, strategies to balance the emotional state of equines will probably change in the future.

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1.3. Discussion

Results concerning behavioural and physiological responses to the different stimuli differed between the four groups (dressage, jumping, eventing and equine-assisted therapy) in this study. This means that equine emotional responses vary with the activity in which horses are involved. Consequently, the activity should be considered a source of variability for studies on equine emotional responses. These findings have been taken into consideration for the study design and the management of the following experiments. Accordingly, a decision was made to continue this investigation focusing on the emotional responses of equine athletes and working equines separately.

Chapter 2: de la Guérinière was right: shoulder-in is beneficial for horses physical and mental states

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2.1. Introduction

Equine athletes face many challenges through training and competition. Riders may play a role in their horse's emotional balance in training contexts. They communicate by different means (leg pressure, reins, body posture and balance) with their horses. This communication may help or confuse the horses. This study aimed to investigate equine emotional responses, through behavioural and physiological parameters, to a training with two riders with different levels of expertise in equitation. Equine behavioural and physiological responses to the two riders, one amateur and one professional, and to two training sessions were compared. One training session in which the horses were in control of their locomotion by moving around a training arena (longitudinal exercise) and another session in which the rider controlled the horse's locomotion performing standardised lateral exercises.

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Research

de la Guérinière was right: Shoulder-in is beneficial for the physical and mental states of horses

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ABSTRACT

Lateral exercises are commonly practiced in equitation. Studies on the influence of these exercises on horses' emotional responses are scarce. This study investigated equine behavioral and physiological responses to lateral exercises. Forty horses (11 ± 4 years old) and two riders (one professional and one amateur) were involved in the research. Two sessions of approximately 10 minutes each were performed. In session 1, longitudinal exercises were performed at three gaits: walk, trot, and canter. In session 2, horses performed a lateral exercise (shoulder-in) at the three gaits. To study autonomic nervous system activity, heart rate variability was assessed using heart rate (HR), low-frequency/high-frequency ratio (LF/HF), and very-low-frequency (VLF) data. Behaviors previously associated with a horse's lack of attention (looking around frequency [LAF]) and relaxation (playing with/chewing the bit duration [PCB]) were collected. LAF was significantly highest in session 1 ($P < 0.001$). PCB was significantly highest in session 2 ($P < 0.01$). In session 2, HR was significantly higher with the professional rider ($P < 0.05$). A trend was observed for LF/HF ($P = 0.066$), which was 2.3 times higher in session 1 than in session 2 with the amateur rider and higher in session 1 than in session 2 independent of the rider. A trend was observed for VLF ($P = 0.053$), which was lowest in session 2. These results showed longitudinal exercise was associated with increased sympathetic activity and distraction, while lateral exercise was associated with increased parasympathetic activity and relaxation. Thus, lateral exercises may be used to manage stress-related responses during training sessions, especially with lower-level riders.

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Introduction

Horses involved in different equestrian activities experience different training techniques and learning processes, which may modify equine responses to different stimuli over time (Mendonça et al., 2019a; McGreevy and McLean, 2010). However, independent of activities, horses must learn how to focus on a given task, select the stimuli they should respond to, and respond to these stimuli (Zentall, 2005). This is important because horses must continuously adapt to their rider's instructions and to the environment (McLean and Christensen, 2017).

The communication between the rider and the horse is based on aids/cues, through the reins, the bit, the saddle, and the posture and legs of the rider (Dyson, 2017). The intensity of these cues induces modifications in horses' behavioral and physiological responses (Zebisch et al., 2014; ISES, 2013; Mclean and McGreevy, 2004). In addition, these aids/cues differ in not only intensity but also the anatomical region of application (Mclean and McGreevy, 2004; Netto de Almeida, 1997). Thus, aids/cues differ between longitudinal and lateral exercises (De Cocq et al., 2010; Symes and Ellis, 2009). Compared to longitudinal exercises, lateral exercises are difficult to perform, as they challenge the biomechanics and

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balance of both the rider and the horse (de Cocq and Van Weeren, 2013; De Cocq et al., 2010). However, in the equestrian literature, some authors have reported that the lateral exercise known as "shoulder-in" (FEI, 2019) provides physical and psychological benefits to horses involved in equestrian activities (de Coux, 2007; Oliveira, 2006; de la Guérinière, 1733).

The described benefits of this exercise are an increase in the horse's compliance and the improvement of biomechanical aspects such as impulsion (de Coux, 2007; de la Guérinière, 1733). However, these authors have also explained that this exercise must be performed correctly to obtain its benefits and has limitations when performed incorrectly. Shoulder-in should be performed without coercion and with no resistance from the horse to produce the expected benefits (de Coux, 2007; Oliveira, 2006; Netto de Almeida, 1997). The horse should be relaxed, and the aids should be well harmonized to achieve the exercise aims (de Coux, 2007). This suggests that the rider's experience may influence the success of this exercise and that his/her training is a prerequisite for achieving its benefits.

The aim of this study was to investigate equine behavioral and physiological responses to longitudinal and lateral training sessions with two riders of different skill levels in equitation. Heart rate variability (HRV) analysis, along with behavioral assessment, has proven to be a valuable noninvasive tool to study equine emotional responses through the activity of the autonomic nervous system (Mendonça et al., 2019b; Rosselot et al., 2019; Mengoli et al., 2014). We hypothesized that horses would experience higher behavioral and physiological (HRV) changes in the longitudinal session than in the lateral session because, as previously mentioned in the equestrian literature, lateral exercises appear to reduce undesired behavioral responses and increase a horse's compliance with the rider. In addition, we hypothesized that the influence of the rider on the horse's behavioral and physiological responses would be higher with an amateur rider than with a professional rider because the lower precision, balance, and coordination of the amateur might create ambivalent information that could be misinterpreted by the horse.

Methods

Subjects

This study included 40 horses (11 ± 4 years old): 16 females, 23 geldings, and 1 stallion. Twelve horses were housed in paddocks 24 h/day, while the other 28 horses had access to the paddock during the daytime and were kept in boxes at night. Horses were generally fed four times daily: industrial pellets (7 am and 4 pm) and with hay (8 am and 7 pm).

All the studied horses had received previous training in longitudinal and lateral exercises and should have learned and frequently practiced the "shoulder-in" exercise (FEI, 2019). These horses' routines included four training sessions of 50 minutes per week. All horses were engaged in competition at the time this study was conducted. Horses were ridden with their usual apparatus, including bit and bridle, to avoid any response to novelty related to the equipment. Nosebands were used respecting the International Society for Equitation Science (ISES) and the Fédération Equestre Internationale (FEI) code of conduct criteria, being applied at least 1.5 cm of space between the nose and the noseband. Horses were recruited from eight equestrian structures in the southeast of France.

Two dressage horseback riders who were involved in competition, one at the professional level and the other at the amateur level of the "Fédération Equestre International", were involved in this study. The riders were unknown to the horses.

Procedure

The experiment included longitudinal exercises (session 1) and lateral exercises (session 2). A five-minute break between the two sessions was needed to stop the data collection in session 1 and to start the data collection in session 2. The interactions between the rider and the horse differed between the two sessions. For the longitudinal exercises, the horses could organize their locomotion freely, needing relatively few aids from the rider, while for the lateral exercises, the rider provided the horses with many aids and organized the locomotion of the horse to perform the exercises (Strunk et al., 2018; de Cocq and Van Weeren, 2013; De Cocq et al., 2010). Each session lasted for 10 minutes. Before each session, the rider rode the horse around the arena as a warm-up. No lateral exercises were performed during the warm-up period, which lasted between 5 and 10 minutes depending on the compliance of the horse with the rider.

In session 1, horses were required to perform longitudinal movements around a training arena (Figure 1A) at three gaits: walk, trot, and canter. The rider did not provide the horses with any cues other than the cues involved in gait changes. This session was organized as follows: five minutes per direction (right and left), with one and a half minutes at walk, two and a half minutes at trot, and one minute at canter in each direction. Gait durations represented the proportion of time per gait that was practice in routine training sessions.

In session 2, the shoulder-in was performed at the three gaits for the two sides. To change direction, horses passed road cones that were in the middle of the arena, and at the end of the arena, they turned to the opposite direction, as illustrated in Figure 1B–D. Once the activity was finished at walk, the exercise was repeated at trot and then at canter. Gait transitions were made on the wide side of the arena, and the exercises were performed on the long side of the arena. This schema aimed to replicate the timeline of a normal training session and was designed to allow the collection of the biomechanical, behavioral, and physiological data.

The analysis of biomechanics parameters was conducted to validate the execution and quality of the lateral exercises in session 2. Biomechanics data were collected using an Equisense Motion Sensor® (Lille, France). This material was used following the manufacturer's guidelines, and different parameters were obtained (Table 1). All data were provided and calculated by the equipment directly.

Behavioral data were collected via video recording using a Sony Handycam HDR-CX450® camera (Weybridge, UK) and a Move 'N see Pixio Robot Cameraman® (Brest, France). Behaviors were assessed through video analysis in continuous sampling at normal speed and with the help of an Excel® matrix (Redmond, WA), behavioral durations or frequencies were determined by one operator according to an ethogram (Table 2). Slight differences in the duration of session 2 (few seconds) were observed between horses. Then, the proportion of the duration of "playing with/chewing the bit" (PCB) over the total time of each session was calculated.

HRV data were collected in the two phases using the Polar V800 Equine® system (Kempele, Finland). The collected data were transformed with Kubios® software (Kuopio, Finland), and only normal R-R intervals were considered for the HRV analysis. The final data included heart rate (HR) and frequency domain analysis results (Table 3). Frequency band thresholds were established within each frequency interval using the following parameters: low-frequency (LF) power = 0.005–0.07 Hz; high-frequency (HF) power = 0.07–0.5 Hz; very low-frequency (VLF) power = 0.001–0.005 Hz (Bowen, 2010).

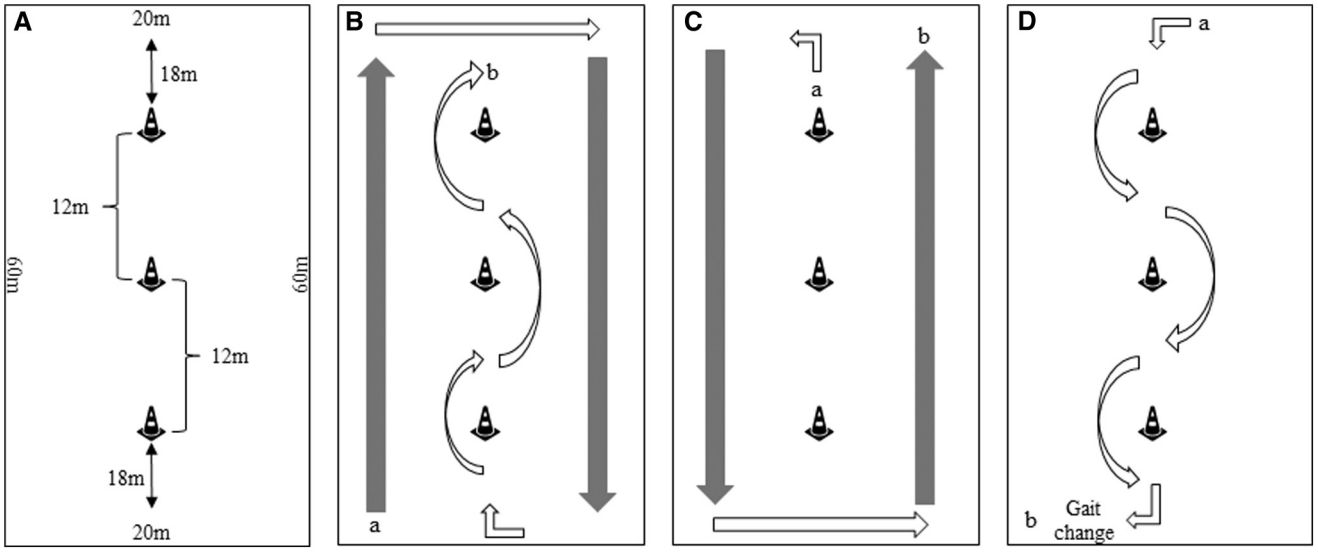


Figure 1. Training arena dimensions (A) and exercise scheme (B, C, and D). The exercises were performed from B to D at each gait, following the walk-trot-canter order. The direction is indicated by the white arrows from "a" to "b". The gray arrows represent the "shoulder-in" exercise and its directions.

Statistical analysis

Statistical analysis was carried out using SAS 9.4 software copyright© 2002-2012 by SAS Institute Inc. (Cary, NC). The significance threshold was classically fixed at 5%. The statistical analysis was conducted with two factors: session and rider.

For stride frequency at trot (SFT), stride frequency at canter (SFC), regularity at walk (RW), regularity at trot (RT), HR, and VLF, the assumption of normality of the data was tested using residual diagnostics plots with the UNIVARIATE procedure and was verified. Homoscedasticity was then tested with Levene's test using the GLM procedure and was also verified. Comparisons involving both factors were performed with the general linear mixed model using the MIXED procedure.

For stride frequency at walk (SFW), the assumption of normality of the residues of the model was verified using the residual diagnostics plots with the UNIVARIATE procedure, although the homoscedasticity was not verified according to Levene's test using the GLM procedure. Consequently, comparisons of the two factors were carried out with the general linear mixed model using the MIXED procedure. Owing to the heterogeneity of the variances, the GROUP=rider option was specified in the REPEATED statement of the MIXED procedure.

For looking around frequency (LAF) and PCB, the assumption of normality of the data was tested using the residual diagnostics plots and the UNIVARIATE procedure, although it was not verified. However, the probability density of the distribution of the observed variable suggests Poisson's equation applies to all these behaviors. Thus, comparisons of the two factors were made with the generalized linear mixed model using the GLIMMIX procedure and the Laplace method to estimate model parameters.

For regularity at canter (RC) and LF/HF, the assumption of normality of the residues of the model was tested using the UNIVARIATE procedure and was verified after a Box-Cox transformation of the data. Homogeneity of variances was verified with Levene's test using the GLM procedure. Comparisons involving both factors were performed with the general linear mixed model using the MIXED procedure.

Finally, when significant differences were found ($P < 0.05$) for any of the studied variables, multiple comparisons were analyzed with the Tukey test using the LSMEANS statement.

Results

Physiological data from one horse were not recorded due to technical issues (low battery), and the biomechanical data of nine horses were not recorded due the weakness of the internet signal in some of the facilities. These individuals were removed from the statistical analyses of the affected parameters.

Biomechanical data

Significant differences in SFW were observed between sessions (DF = 1; F =5.64; $P = 0.024$). More specifically, SFW was significantly higher in session 2 than in session 1 (Table 4). No significant

Table 1
Biomechanical parameters

Variable	Definition provided by the manufacturer
Stride frequency at walk (SFW)	Frequency of horse's strides per minute at walk
Stride frequency at trot (SFT)	Frequency of horse's strides per minute at trot
Stride frequency at canter (SFC)	Frequency of horse's strides per minute at canter
Regularity at walk (RW)	Regularity of the stride frequency at walk (score of 0-10)
Regularity at trot (RT)	Regularity of the stride frequency at trot (score of 0-10)
Regularity at canter (RC)	Regularity of the stride frequency at canter (score of 0-10)

Table 2
Ethogram used for video analysis

Variable	Definition
Looking around frequency/minute (LAF)	Head movement toward a stimulus when no cue was provided by the rider
Playing with/chewing the bit duration (PCB)	Mouth movements (e.g., mastication or licking) on the bit

Table 3
Adapted description of heart rate variability variables from Stucke et al., 2015, and Usui and Nishida, 2017

Variable	Definition	Interpretation
HR (beats per minute)	Heart rate mean	Overall variability of cardiac activity
VLF (%)	Very-low-frequency	Slow recovery component of the HRV (vagal tone)
LF/HF	Low-frequency/ high-frequency	Representation of the balance between the sympathetic (LF) and parasympathetic (HF) nervous systems

difference was observed in the rider effect (DF = 1; F = 0.10; P = 0.752) or in the interaction between session and rider factors (DF = 1; F = 0.77; P = 0.388).

Significant differences were observed for SFT in the interaction between session and rider (interaction session*rider: DF = 1; F = 14.02; $P < 0.001$). More precisely, SFT was significantly higher in horses ridden by the professional rider than by the amateur rider in both sessions. In addition, SFT was significantly higher with the amateur rider in session 1 than in session 2 (Figure 2).

Significant differences in SFC were observed between riders (DF = 1; F =8.44; P = 0.006). More specifically, SFC was significantly higher in horses ridden by the professional rider than in those ridden by the amateur rider (Table 4). No significant difference was observed in the session effect (DF = 1; F = 0.00; P = 0.950) or in the interaction between session and rider factors (DF = 1; F = 0.58; P = 0.453).

Significant differences in RW and RT were observed between sessions (RW: DF = 1; F = 9.20; P = 0.005; RT: DF = 1; F = 6.16; P = 0.019). More specifically, RW and RT were significantly higher in session 1 than in session 2 (Table 4). No significant difference was observed in the rider effect (RW: DF = 1; F =1.08; P = 0.306; RT: DF = 1; F = 0.31; P = 0.583) or in the interaction between session and rider factors (RW: DF = 1; F = 0.72; P = 0.402; RT: DF = 1; F = 0.40; P = 0.532).

Significant differences were observed for RC in the interaction between session and riders (interaction session*rider: DF = 1; F = 10.23; P = 0.003). More precisely, RC was significantly higher in horses ridden by the amateur rider than in horses ridden by the professional rider in session 2. In addition, RC was significantly higher with the professional rider in session 1 than in session 2. (Figure 3).

Table 4
Biomechanical data: descriptive statistics

Parameter	Session/rider	Mean ± SE	Median	Minimum	Maximum
SFW	Session 1	54.4 ± 0.7[a]	53.8	48.6	63.3
	Session 2	55.1 ± 0.6[a]	54.5	47.4	61.8
	Amateur	54.6 ± 0.7	54.7	47.4	63.1
	Professional	54.9 ± 0.5	54.4	48.6	63.3
SFC	Session 1	99.7 ± 0.9	98.9	91.3	111.2
	Session 2	99.8 ± 1.0	98.5	90.7	114.3
	Amateur	97.4 ± 0.7[b]	97.8	90.7	106.8
	Professional	102.0 ± 0.9[b]	102.1	93.4	114.3
RW	Session 1	4.0 ± 0.2[c]	4.0	0.5	6.5
	Session 2	3.5 ± 0.2[c]	4.0	0.5	6.5
	Amateur	3.9 ± 0.2	4.0	1.0	6.5
	Professional	3.5 ± 0.2	4.0	0.5	6.0
RT	Session 1	7.3 ± 0.1[d]	7.5	6.0	8.5
	Session 2	6.9 ± 0.1[d]	7.0	4.5	8.5
	Amateur	7.0 ± 0.2	7.0	5.0	8.5
	Professional	7.2 ± 0.1	7.0	4.5	8.5

[a,b,c,d] Significant differences between the marked conditions (a, d: $P < 0.05$; b, c: $P < 0.01$).

Behavioral data

Significant differences in LAF were observed between sessions (DF = 1; F = 15.78; $P < 0.001$). More specifically, LAF was significantly higher in session 1 than in session 2 (Table 5). No significant difference was observed in the rider effect (DF = 1; F = 0.76; P = 0.386) or in the interaction between session and rider factors (DF = 1; F = 0.65; P = 0.423).

Significant differences in PCB were observed between sessions (DF = 1; F = 7.60; P = 0.007). More specifically, PCB was significantly higher in session 2 than in session 1 (Table 5). No significant difference was observed in the rider effect (DF = 1; F = 0.45; P = 0.506) or in the interaction between session and rider factors (DF = 1; F = 0.32; P = 0.572).

Physiological data

Significant differences in HR were observed for the interaction between session and rider (interaction session*rider: DF = 1; F = 6.07; P = 0.019). More precisely, HR was significantly higher in session 2 in horses ridden by the professional rider than in horses ridden by the amateur rider. In addition, HR was higher with both riders in session 1 than in session 2. (Figure 4).

A trend was observed for LF/HF in the interaction between session and riders was not significant (interaction session*rider: DF = 1; F = 3.59; P = 0.066). LF/HF was lower in session 2 than in session 1 (Table 5) with the amateur rider (2.3 times lower) and with the professional rider (1.2 times lower).

VLF was higher in session 1 than in session 2 (DF = 1; F = 3.97; P = 0.054) (Table 5). No significant difference was observed in the rider effect (DF = 1; F = 0.03; P = 0.857) or in the interaction between session and rider factors (DF = 1; F = 0.08; P = 0.784).

Discussion

The results confirmed that the sessions produced the expected modifications to the horses' biomechanics. The shoulder-in exercise was expected to produce a decrease in regularity in session 2 because this exercise is a lateral movement performed in a straight line (Denoix, 2014). A general decrease in regularity was observed (0.5 at walk; 0.4 at trot) between sessions. At canter, a decrease was observed with the professional rider only (decrease of 1). Decreases in regularity were expected because it is harder to maintain a regular stride frequency while performing a lateral exercise than while performing longitudinal exercises (De Cocq et al., 2010; Symes and Ellis, 2009).

Horses ridden by the amateur rider had a higher RC score than those ridden by the professional rider in session 2. Professional riders should be less disturbing to the horse's biomechanics, as their body posture and stability should facilitate the maintenance of the stride frequency and thus maintain the regularity (de Cocq and Van Weeren, 2013). Nevertheless, these results should be interpreted carefully because the SFC was higher with the professional rider than with the amateur rider. The lower SFC with the amateur rider would facilitate the rider's balance and maintain RC scores. In addition, the SFC with the amateur rider was lower than the normal SFC, which is considered to vary between 99 and 105 strides/minute (Back and Clayton, 2013). It is difficult for inexperienced riders to maintain balance when riding a horse at canter, since canter is a three-beat gait (Symes and Ellis, 2009). Therefore, the decreased experience of the amateur rider compared with the professional rider might be associated with the imprecise provision of cues/aids (Symes and Ellis, 2009). The lack of cue precision from the amateur rider might induce increased behavioral and physiological

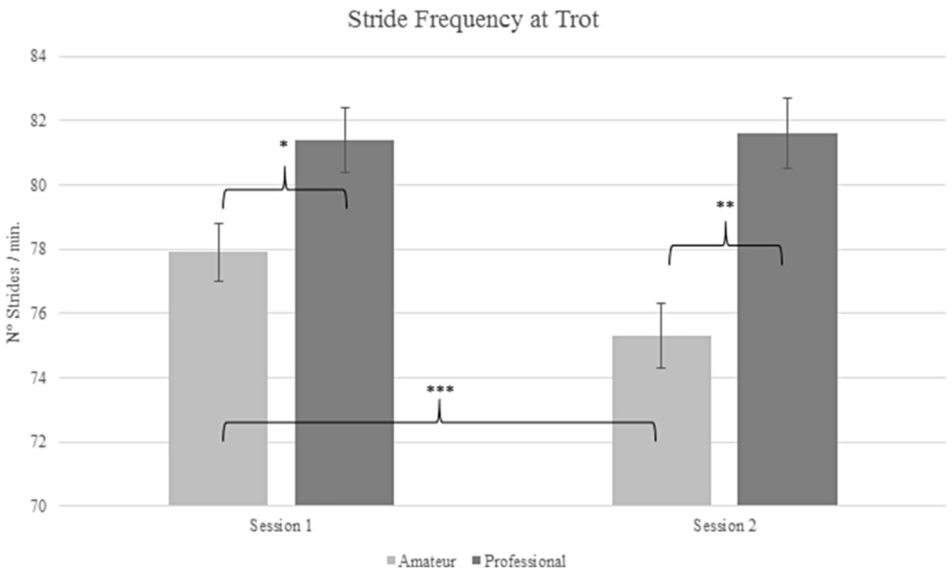


Figure 2. Effect of session and rider on stride frequency at trot: mean ± standard error. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

responses of the horse related to its misunderstanding of what it should perform.

Looking around has been used to study the attention of horses (Fleming et al., 2013). In the present study, the horses did not often look around; however, horses that performed this behavior looked around at least once every two minutes in session 1, and the LAF was reduced by at least two times in session 2 (performed once every five minutes). The riders could have prevented head movements through the reins; however, they were previously instructed and trained to allow head movements, either lateral or vertical in both sessions. Playing with/chewing the bit is considered a normal and desirable behavior, associated with relaxation during physical activity (Fenner et al., 2016; Smiet et al., 2014). In addition, playing/chewing the bit performance decreases when tightened nosebands, which were associated with conflict behaviors, are used (Fenner et al., 2016, 2017). In the present study, noseband tightness influence on these behaviors was prevented by keeping at least 1.5 cm of space between the animal's nose and the noseband as recommended by the ISES and the FEI. Strunk et al. (2018) suggested that riders' level in equitation has minimal effects on behavior and kinematics of ridden horses. Results concerning behavioral parameters in the present study are in line with previously published studies (Strunk et al., 2018), suggesting that the rider has little if any influence on the horses' behavioral parameters. The results concerning LAF and PCB between the two sessions suggest that lateral exercises, as performed in session 2, may be useful to increase attention and decrease tension, independent of the rider.

A limitation in the interpretation of these behaviors could be the lack of information on rein tension, which was not assessed in the present investigation. Nevertheless, rein tension seems to have no

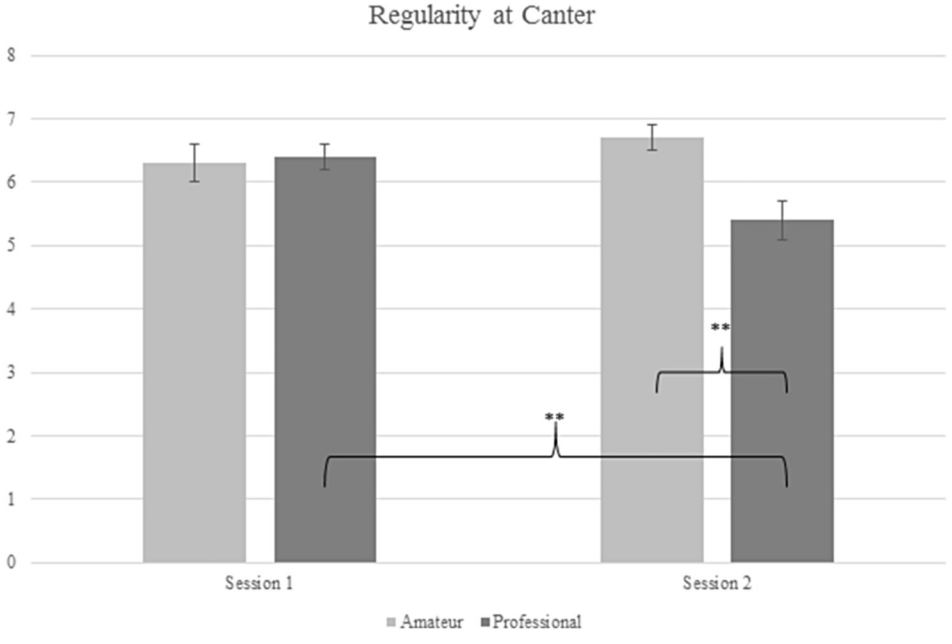


Figure 3. Effect of session and rider on regularity at canter score: mean ± standard error. $^{**}P < 0.01$.

Table 5
Behavioral and physiological data: descriptive statistics

Parameter	Session	Rider	Mean ± SE	Median	Minimum	Maximum
LAF (times/minute)	Session 1	Amateur	0.5 ± 0.3[a]	0.0	0.0	6.0
		Professional	1.1 ± 0.6[b]	0.0	0.0	10.0
	Session 2	Amateur	0.2 ± 0.1[a]	0.0	0.0	2.0
		Professional	0.2 ± 0.2[b]	0.0	0.0	3.0
PCB (%)	Session 1	Amateur	11.8 ± 4.6[c]	0.5	0.0	64.0
		Professional	20.7 ± 6.4[d]	6.5	0.0	92.0
	Session 2	Amateur	27.0 ± 6.6[c]	17.0	0.0	86.0
		Professional	36.7 ± 9.0[d]	12.0	0.0	100.0
LF/HF	Session 1	Amateur	15.2 ± 3.3	13.3	0.1	50.4
		Professional	11.1 ± 3.5	5.3	0.5	67.5
	Session 2	Amateur	6.6 ± 1.1	6.4	0.3	16.7
		Professional	9.1 ± 1.7	7.1	1.3	30.8
VLF (%)	Session 1	Amateur	2.2 ± 0.3	2.2	0.0	4.9
		Professional	2.2 ± 0.4	1.8	0.1	8.2
	Session 2	Amateur	1.6 ± 0.3	1.3	0.0	4.1
		Professional	1.7 ± 0.2	1.6	0.9	3.2

[a,b,c,d] Significant differences between the marked sessions (a, b: $P < 0.001$; c, d: $P < 0.01$).

influence on the performance of chewing the bit (Fenner et al., 2017). The use of different bit and bridles or even not using any of them can influence rein tension and welfare (Mellor and Beausoleil, 2017); however, little influence on the studied behavioral responses could be expected (Fenner et al., 2017). Moreover, changing the bit or bridle of a horse in this experiment would have probably created novelty and influenced behavioral responses. Therefore, the choice of keeping the usual bit and bridle for each horse was made.

Decreases in HR and LF/HF have been associated with an increase in parasympathetic nervous system (PNS) activity (Stucke et al., 2015; Evans and Young, 2010). Baseline or at-rest results of HRV (not shown) were not included in the statistical analysis of this study because the difference in the HRV parameters of resting and working horses could influence the differences between the studied sessions. The results of the present study suggest that a significant activation of the sympathetic nervous system (SNS) occurred during the longitudinal exercises in session 1 (higher values of HR and LF/HF), and predominant PNS activity was observed with the lateral exercises in session 2 (lower values of HR and LF/HF). Concerning the differences between riders, the lower HR observed in horses ridden by the amateur rider than in those ridden by the professional rider may be explained by the low stride frequencies applied by the amateur rider. Regarding LF/HF, even if no significant differences were observed, this variable was 2.3 times lower with the amateur rider and 1.2 times lower with the professional rider in session 2 than in session 1 (Table 5), which represents a substantial difference for this parameter (Mendonça et al., 2019b; Bachmann et al., 2003). This means that all horses were more balanced in terms of autonomic nervous system (ANS) activity in session 2 than in session 1 and that horses ridden by the professional rider were more stable in terms of the ANS activity between sessions.

Usui and Nishida (2017) suggested that VLF results should be interpreted carefully because decreases in VLF have been associated with increases in SNS activity, while high values of VLF have been associated with delayed recovery. Higher values of VLF were observed in session 1 (longitudinal exercise) than in session 2, which could indicate a delayed recovery after the effort of session 1. Nevertheless, in this study, no data concerning recovery were

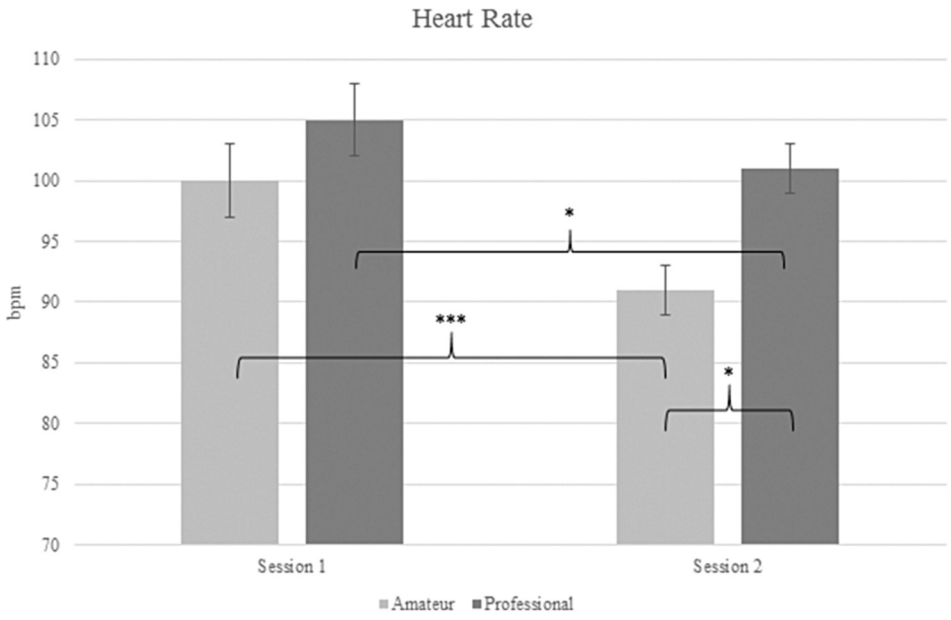


Figure 4. Effect of session and rider on heart rate (beats per minute): mean ± standard error. *$P < 0.05$; ***$P < 0.001$.

collected in any of the sessions, so no conclusion about the long-term effects of an increase in VLF could be proposed. Further research is needed to thoroughly investigate the long-term effects.

An imbalance in ANS activity with predominant SNS activity (higher values of HR and LF/HF) was observed in session 1, in which horses were more distracted (higher values of LAF) by the environmental stimuli and less relaxed (lower values of PCB). Predominant PNS activity was observed in session 2 (lower HR and LF/HF values), in which horses were more relaxed (higher PCB duration), which suggests that playing with/chewing the bit may be related to functional ANS responses. In addition, the modifications in the ANS activity were particularly significant with the amateur rider, which suggests that lateral exercises are an important tool that unexperienced riders could use to reduce stress-related events during training, as suggested in the equestrian literature.

Conclusions

Lateral exercises were associated with higher parasympathetic activity and greater relaxation than longitudinal exercises, while longitudinal exercises were associated with higher sympathetic activity and distraction than lateral exercises. These results suggest that lateral exercises may be used for the management of stress-related responses during training sessions, especially with lower-level riders, which is in line with the equestrian literature (de la Guérinière, 1733).

Further research investigating the potential of lateral exercises as a tool for solving equine behavioral problems related to equestrian activities would be useful.

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Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Ethical considerations

This project was approved by the Research Institute in Semiochemistry and Applied Ethology (IRSEA) Ethics Committee (C2EA125) and the French Ministry of Research (APAFIS Process number 11949).

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2.3. Discussion

The shoulder-in exercise produced, as expected, a general decrease on the symmetry and regularity of the different gaits, except for the canter, in which no differences were produced with the amateur rider.

Looking around was previously associated with distraction while playing/chewing the bit was associated with relaxation and was considered a desirable behaviour. These behaviours showed higher frequencies for looking around and lower frequencies to playing/chewing the bit in the longitudinal session. No rider effect was observed for behavioural parameters.

HR, LF/HF and VLF parameters of the HRV analysis were higher in the longitudinal session. Concerning LF/HF, no significant differences were observed, however, this variable was 2.3 times lower with the amateur rider and 1.2 times lower with the professional rider in the lateral session.

In conclusion, horses were more distracted and disturbed by the environment with the longitudinal exercises, as showed by behaviours and HRV, and more relaxed and balanced with the lateral exercises; Lateral exercises performed by amateur riders may allow to balance equine emotional responses through training.

Athlete horses involved in this study are physically prepared and trained to perform these activities. However, different activities are performed with horses that had no training to perform them, as in equine-assisted therapy (EAT). Consequently, a new study with horses involved in EAT was designed to study equine's perception of these interactions.

Chapter 3: The impact of equine-assisted therapy on equine behavioural and physiological responses

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3.1. Introduction

Equine-assisted therapies involve different activities. Some of these activities involve petting, brushing, cleaning the horse's feet, watering and feeding the horse. Then, follows the working session in which some patients will perform in-hand work, e.g. the approach and the initial contact with the horse, making the horse move forward or backward, leading the horse to cross obstacles or to pass parallel bars on the ground or even lunging the horse in a round pen, while other patients will perform horseback riding activities. The choice of the activity the patient will perform is usually made by the therapist according to the patient condition.

Equine-assisted therapies have been used to improve many medical conditions in human patients. Medical conditions of these patients vary between physical and/or psychological aspects and any of these cases appear to benefit from these therapies. Many studies have demonstrated the benefits of these therapies to human patients, but few studies have been done on the equine perception of these interactions.

Some authors suggested that equine-assisted therapies do not produce negative emotions or outcomes in horses, though the absence of negative emotions does not mean that horses experience positive emotions. The aim of this study was to investigate whether equine-assisted therapy creates negative or positive emotions in horses and the influence of patients' therapy expectations on horses' emotional responses.

Nine horses and fifty-one patients were involved in this investigation. The patients were divided into two groups according to their own expectations of the therapy: improvement of psychological aspects (PSY) or improvement physical and psychological aspects (PHY+PSY). The study was made under real conditions by observing regular equine-assisted therapy sessions. The observations were divided into two phases: preparation of the horse and working session. Behavioural and physiological parameters were collected and compared between the phases of the therapy (preparation and working phases) and the groups (PSY and PHY+PSY groups).

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Article

The Impact of Equine-Assisted Therapy on Equine Behavioral and Physiological Responses

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Simple Summary: Equine-assisted therapies (EATs) are often applied to patients with different types of either mental or physical conditions. The increasing application of EATs has induced interest in the scientific community about equine well-being during these therapies. This study aimed to investigate whether equine-assisted therapy (EAT) creates negative or positive emotions in horses and the influence of patients' therapy expectations (one group of patients had physical and psychological expectations, and one group of patients had only psychological expectations) on horses' behavioral and physiological responses. We observed 58 pairs (patient–horse) during EAT sessions and when the horses were at rest. Then, we compared the horses' behavioral and physiological responses between the different periods of therapy and among the groups of patients. Our results suggested that the EAT in this study was neither a negative nor a positive event. EATs with patients who had both physical and psychological expectations were more challenging for horses than those with patients who had only psychological expectations. Further research on EAT should focus on providing horses with positive stimulation and reinforcement to understand whether a positive association with EAT can be created.

Abstract: Equine-assisted therapies (EATs) have been widely used in the treatment of patients with mental or physical conditions. However, studies on the influence of equine-assisted therapy (EAT) on equine welfare are very recent, and the need for further research is often highlighted. The aim of this study was to investigate whether EAT creates negative or positive emotions in horses, and the influence of patients' expectations (one group of patients had physical and psychological expectations and one group of patients had only psychological expectations) on horses' emotional responses. Fifty-eight pairs (patient–horse) were involved in this study. Behaviors and heart rate variability (HRV) data were collected during a resting phase, a preparation phase in which the patients brushed and saddled the horse, and a working phase. Behaviors and HRV were compared between phases and among the groups of patients. Our results suggested that the EAT in this study was neither a negative nor a positive event. EATs with patients who had both physical and psychological expectations were more challenging for horses than those with patients who had only psychological expectations. Further research should focus on providing horses with positive stimulation and reinforcement to understand whether a positive association with EAT can be achieved.

Keywords: behavior; emotions; horse; equine-assisted therapy; heart rate variability; welfare

1. Introduction

Animal-assisted interventions (AAIs) is a term that is commonly used to describe any modality using animals to improve the physical, mental, and social conditions of humans [1]. These interventions have proven effects in applications for autism spectrum disorder, Alzheimer's disease, behavioral problems, and emotional well-being [1–3]. Other medical issues in which this therapy has been applied include Parkinson's disease [4], heart failure [5], cerebral palsy [6], and neurological impairments and applications for children with developmental delays [7].

AAIs that involve equines are usually defined as equine-assisted therapies (EATs) or equine-assisted activities (EAAs) [8]. EATs have been used to improve social, emotional, and physical domains in patients suffering from anxiety, depression, autism spectrum disorder, multiple sclerosis, and spinal cord injury or to improve balance, muscle symmetry, coordination, and posture [6,8,9].

Animal welfare has become a major concern in all activities that involve animals, including animal production [10], sports [11], work [12] and even pet ownership [13]. The awareness of the welfare of the animals used in EATs is a focus of interest to the scientific community along with its applications [14]. The most commonly used physiological indicators of animal welfare focus on the negative impact of an activity on animal welfare, assuming the absence of a negative impact to be associated with well-being or "positive welfare" [15]. However, the definition of positive welfare includes not only the absence of negative experiences or feelings, but also the presence of positive experiences or feelings [16]. This means that the study of animal welfare should include both negative and positive indicators.

Equine-assisted therapy (EAT) activities that involve horseback riding appear to be no more stressful for horses than recreational horseback riding activities [14,17]. Additionally, in a previous study, interactions between patients experiencing post-traumatic stress disorder and horses assisting in their therapy were found to be equally stressful as the interactions between healthy humans and horses [18]. The authors of this study suggested that horses respond more to physical cues than to emotional cues from humans [18].

Many studies of EATs that have investigated behavioral and/or physiological aspects have suggested that EAT activities do not produce negative outcomes, such as stress, in horses [18,19]. Considering the absence of negative outcomes, some authors have suggested that EAT may produce positive outcomes for horses [14]. However, one study that investigated both negative (plasmatic cortisol concentrations and heart rate variability) and positive (plasmatic oxytocin concentrations) outcomes of EAT found no difference between the parameters [20]. Nevertheless, there are relatively few studies on EATs, and only one investigated positive outcomes of these activities.

Some authors have demonstrated that heart rate variability (HRV) is a suitable tool to study equine emotional responses in animals involved in EAT [21]. HRV analysis the autonomic nervous system (ANS) activity [22]. The frequency domain analysis of HRV studies the sympathetic (SNS) and parasympathetic (PNS) components of the ANS [23]. This analysis includes parameters, such as low frequency (LF), high frequency (HF), and very low frequency (VLF); these are influenced by the respiratory rate, which varies according to species; therefore, the frequency band widths should be adapted in this type of analysis [24,25].

Each of the described HRV parameters reflects different aspects of ANS activity [25]. Some authors have suggested that LF may represent sympathetic activity [22,26]. HF is commonly considered a marker of the cardiac vagal tone, and consequently, the parasympathetic system [22,23,27]. The LF/HF ratio reflects the SNS–PNS balance [22,23,27]. The VLF component of HRV has been associated with a slow recovery of the ANS balance after a mental or physical effort [28]. The analysis of the ANS activity provides an objective tool to study both positive and negative outcomes of EATs [16,29].

The emotional states are best assessed by a combination of behavioral and physiological measures [30]. Physiological and behavioral indicators should include both positive and negative outcomes for the assessment of equine emotional states and welfare [16]. Some behaviors have been associated with negative emotions [14,19], while others have been associated with positive

emotions [31]. Thus, these behaviors may be useful as negative and positive indicators of equine emotions in association with physiological indicators.

The aim of this study was to investigate whether EAT creates negative or positive emotions in horses and the influence of patients' therapy expectations on horses' emotional responses.

2. Materials and Methods

This project was approved by the Research Institute in Semiochemistry and Applied Ethology (IRSEA) Ethics Committee (C2EA125) and the French Ministry of Research (APAFIS Process number 11949).

General and specific information about the patients and the horses was collected through a questionnaire survey. Patients' medical conditions and expectations were collected with the agreement of the patients and/or their families. Nevertheless, to respect patient confidentiality, the diagnoses and expectations of individual patients were not reported; instead, data were classified into different categories.

This study was conducted in Avignon, France, between March and July 2018.

2.1. Population

This study included nine horses (10 ± 3 years old) and 51 patients (18 ± 11 years old) who participated in EATs.

The animals were recruited from two different equestrian facilities. Housing conditions were the same in both centers. Horses lived in paddocks that they shared with other conspecifics. The population included four females and five geldings (six grade horses, one Mérens, one Halfinger, and one Carmargue). These horses have been involved in EAT for: 9 years; 8 years; 6 years; 4 years; two horses for 3 years; 2 years; 1 year; and 6 months.

The patients involved in this study have been diagnosed with different medical conditions (see Table 1). From the patients' and therapists' perspectives, the expectations for improvement related to these therapies could be divided into two categories: psychological (PSY) or both physical and psychological (PHY + PSY) expectations.

Table 1. Disorders diagnosed in the included patients.

PSY	PHY + PSY
Autism spectrum disorder	Head injury with locomotor disability
Attachment disorder	Development deficit with locomotor disability
Schizophrenia	Pediatric spastic triplegia
Intellectual disability	Pitt–Hopkins syndrome
Anxiety	Post-traumatic physical rehabilitation
Attention deficit disorder	Trisomy 21 (Down syndrome)
Hyperactivity syndrome	
Depression	
Phobias with hallucination	

psychological (PSY) or both physical and psychological (PHY + PSY) expectations.

The statistical unit considered for this study was the pair (patient + horse). Some of the included patients had therapy with different horses on different days. Fifty-eight pairs of patient–horse were involved in this study.

2.2. Study Design and Data Collection

This investigation included a resting phase and two phases of EATs (preparation and working phases).

The resting phase consisted of the collection of HRV data in the normal living and husbandry conditions of the horses.

The preparation phase consisted of controlled interactions between the patient and the horse, such as petting, brushing, saddling, cleaning the horses' feet, and feeding or watering the horses. These interactions differed according to the patients' needs and abilities, and were selected by the therapists. Preparative sessions were performed near the paddocks.

The working phase followed the preparation phase. The patients could either ride or perform in-hand training with the horses. In-hand training included the following: the approach and the initial contact with the horse, making the horse move forward or backward, leading the horse to cross obstacles or pass parallel bars on the ground, and lunging the horse in a round pen. The therapists made the decision of whether the patient should ride or perform in-hand training. Working sessions were performed in riding arenas of variable size.

Each phase lasted between 20–30 minutes, and took place between 09:00–13:00 and 14:00–16:00 during the day.

Behavior data were collected for both the preparation and working phases by one observer through direct observation. The considered behaviors included "ears pinned back" (EPB), "head lateral movement" (HLM), "snort", and "defecation" (see Table 2) [14,31]. These parameters were assessed in terms of frequency.

Table 2. Ethogram used for behavioral observation [14,31].

Behavior	Definition	Emotions
Ears pinned back (EPB)	Backward placement of the ears	Stress, irritation, or frustration; negative emotions
Head lateral movement (HLM)	Turning the head to one side or the other when no command is provided	Stress, irritation, or frustration; negative emotions
Snort	Short, powerful exhalation from the nostrils	Relaxation; positive emotions
Defecation	Elimination of excrement	Stress, irritation, or frustration; negative emotions

HRV data were collected during all the phases with Polar V800 Equine® equipment (Kemple, Finland). Then, the collected data were transformed with Kubios® software (Kuopio, Finland), and only normal R-R intervals were considered for the HRV analysis. The described data included time domain (heart rate) and frequency domain analysis (see Table 3). Frequency band thresholds were established within each frequency interval using the following parameters: LF power = 0.005–0.07 Hz; HF power = 0.07–0.5 Hz; VLF power = 0.001–0.005 [25].

Table 3. Adapted description of heart rate variability (HRV) variables from Stucke et al., 2015 and Usui and Nishida, 2017 [23,28].

Variable	Definition	Interpretation
HR (beats per minute)	Heart rate mean	Overall variability of cardiac activity
VLF (%)	Very low frequency	Slow recovery component of the HRV (vagal tone)
LF/HF ratio	Low frequency/high frequency ratio	Representation of the balance between the sympathetic and parasympathetic nervous systems

2.3. Statistical Analysis

Statistical analysis was carried out using SAS 9.4 software Copyright © 2002-2012 by (SAS Institute Inc., Cary, NC, USA). The significance threshold was classically fixed at 5%.

2.3.1. Data

The duration of each phase differed according to the patient's responses to the therapy. For horse behaviors, the proportion of the frequency over the duration of the given phase was calculated to standardize this variable among different phases. Finally, the frequency of each behavior per minute (freq/min) was considered for the statistical analysis.

HRV data were analyzed as raw data to compare the differences between the two EAT phases and between the two categories of patients' therapy expectations. Then, the differences (Δ) between the preparation and resting phases and between the working and resting phases were calculated to study the evolution of these parameters from resting to the two different EAT situations. These parameters were defined as ΔHR, ΔVLF, and ΔLF/HF ratio.

2.3.2. Statistical procedures

For HR and the LF/HF ratio, the assumption of normality of the data was tested using residual diagnostics plots and the UNIVARIATE procedure. Comparisons between phases and between patients' expectations were performed with the general linear mixed model using the MIXED procedure (two-factor analysis). When significant differences were found, multiple comparisons were analyzed with Tukey's test using the LSMEANS statement.

For EPB, HLM, and VLF, the assumption of normality of the data was tested with the above-described procedure, although the assumption was not verified. Then, box-cox transformation was performed. The assumption of normality of the data was retested using residual diagnostics plots and the UNIVARIATE procedure. Comparisons between phases and between patients' expectations were performed with the general linear mixed model using the MIXED procedure. When significant differences were found, multiple comparisons were analyzed with Tukey's test using the LSMEANS statement.

For the differences in the cardiac parameters (ΔHR; ΔVLF; ΔLF/HF ratio), the assumption of normality of the data was tested with the above-described procedure, although the assumption was not verified. Then, the data were analyzed with the Wilcoxon Signed-rank's test (for paired samples).

For snort and defecation frequencies, data were transformed into binary variables, because the animals did not perform this behavior very often. In this case, the following code was adopted: 1 – the behavior was observed; 0 – the behavior was not observed. Comparisons between phases and patients' expectations were performed with the generalized linear mixed model using the GLIMMIX procedure. When significant differences were found, multiple comparisons were analyzed with Tukey's test using the LSMEANS statement.

3. Results

A few patients could not participate in one of the phases, as participation was not recommended due to their medical state, which lead to an absence of data. The lack of data concerned three patients in the preparation phase and one patient in the working phase.

One patient was highly disturbed by the presence of the observers during the preparation phase, so the therapists decided to remove the observers from the patient's visual field. Therefore, it was not possible to observe the horse's behavior during this phase.

One session occurred during feeding time. This caused a distraction for the horse, which was looking at the animal keepers feeding the animals, significantly increasing the HLM parameter. Therefore, the data concerning this parameter in the preparation phase were removed from the analysis.

In one working phase, a side walker was required to ensure the patient's safety. This unexpected situation clearly modified the horse's behavior. As some behavioral or HRV parameters were influenced by this condition, the authors decided to remove them from the statistical analysis (EPB, HLM, LF/HF ratio, and ΔLF/HF ratio).

Due to technical issues related to the heart rate monitor, the HRV data of two horses in the working phase were not recorded.

3.1. Behavior

There was no significant difference in EPB between the different phases, the two groups of patients' therapy expectations, or the interaction of phase*patients' therapy expectations (phases: DF = 1; F = 0.40; $p = 0.53$; patients' therapy expectations: DF = 1; F = 0.13; $p = 0.72$; interaction of phase*patients' therapy expectations: DF = 1; F = 1.64; $p = 0.21$).

Significant differences were observed for HLM in the interactions between phases and patients' therapy expectations (interaction of phase*patients' therapy expectations: DF = 1; F = 6.60; $p = 0.01$). More precisely, in the preparation phase, horses performed on average two times more HLMs with the PSY group than with the PHY+PSY group. Additionally, horses involved in therapies with the PSY group performed on average two times more HLMs in the preparation phase than in the working phase (see Figure 1).

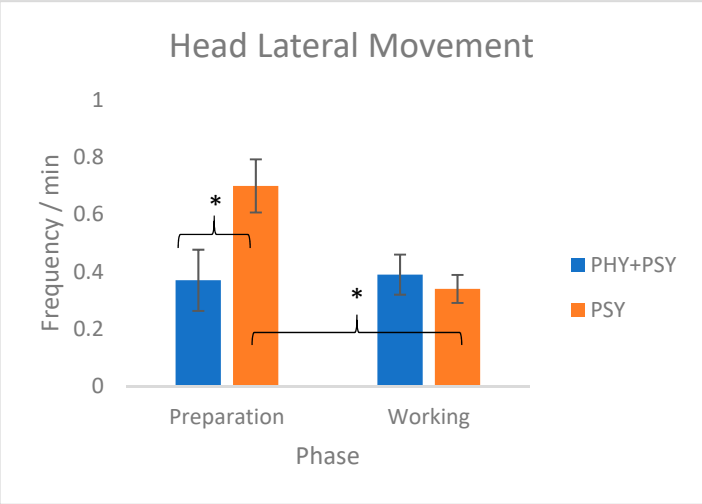


Figure 1. Equine-assisted therapies effect on Head Lateral Movement (freq./min.): effect of phase*patients' therapy expectations. Mean ± standard error. * $p < 0.05$.

There was no significant difference in snort concerning either the different phases or the patients' therapy expectations (phases: DF = 1; F = 0.05; $p = 0.82$; patients' therapy expectations: DF = 1; F = 0.05; $p = 0.82$; interaction of phase*patients' therapy expectations: DF = 1; F = 0.00; $p = 0.99$).

Defecation was only observed 11 times over a total of 111 EAT observations (preparation + working). For that reason, statistical analysis with a model was not applicable for this behavior.

3.2. HRV

HRV results concerning the interaction of phase*patients' therapy expectations were not significant for any of the studied parameters (HR: DF = 1; F = 1.46; $p = 0.23$; VLF: DF = 1; F = 1.27; $p = 0.27$; LF/HF ratio: DF = 1; F = 0.56; $p = 0.46$).

3.2.1. Phase Effect

Significant differences in HR, VLF, and the LF/HF ratio were observed between phases (HR: DF = 1; F = 76.67; $p < 0.001$; VLF: DF = 1; F = 4.59; $p = 0.04$; LF/HF ratio: DF = 1; F = 9.80; $p < 0.01$). More precisely, an increase of 10 beats per minute (bpm) on average in the horses' HR between the preparation and working phases was observed. VLF and the LF/HF ratio were significantly higher in the working phase than in the preparation phase (see Table 4).

Table 4. EAT effect on HRV: effect of phase.

Parameter	Phase	Mean ± SE	Median	Minimum	Maximum
HR (bpm)	Preparation	44.22 ± 0.95 ***	42.00	34.00	67.00
	Working	55.98 ± 1.62 ***	54.00	37.00	95.00
VLF (%)	Preparation	0.70 ± 0.19 *	0.30	0.03	7.44
	Working	0.74 ± 0.09 *	0.57	0.01	3.13
LF/HF Ratio	Preparation	2.71 ± 0.41 **	1.92	0.08	16.12
	Working	4.84 ± 0.57 **	3.39	0.22	15.13
ΔHR	Preparation	−0.46 ± 1.08	−3.00 ***	−11.00	31.00
	Working	11.40 ± 1.61	9.00 ***	−6.00	56.00
ΔVLF	Preparation	0.16 ± 0.21	−0.02 *	−1.08	7.19
	Working	0.21 ± 0.10	0.23 *	−0.98	2.21
ΔLF/HF Ratio	Preparation	−0.01 ± 0.46	−0.58 ***	−5.42	14.60
	Working	2.35 ± 0.58	1.52 ***	−6.64	17.07

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Significant differences in the ΔHR, ΔVLF, and ΔLF/HF ratio values were observed between phases (ΔHR: preparation: median = −3.000 VS working: median = 9.000; S = 570; $p < 0.001$; VLF: preparation: median = −0.020 VS working: median = 0.230; S = 264; $p = 0.01$; LF/HF ratio: preparation: median = −0.577 VS working: median = 1.516; S = 400; $p < 0.001$). More precisely, HR, VLF and the LF/HF ratio inversely changed from the resting phase to the other phases (see Table 4).

3.2.2. Effect of Patients' Therapy Expectations

Significant differences were observed in the LF/HF ratio between the two groups of patients' therapy expectations (patients' therapy expectations: DF = 1; F = 5.83; $p = 0.02$). More precisely, the LF/HF ratio was higher in the PHY+PSY group than in the PSY group (see Figure 2). There were no significant differences in HR and VLF between the different groups of patients' therapy expectations (HR: DF = 1; F = 1.74; $p = 0.19$; VLF: DF = 1; F = 0.42; $p = 0.52$).

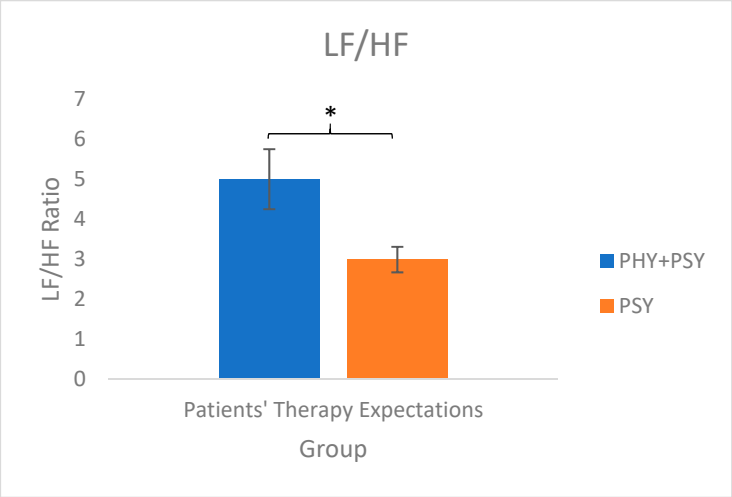


Figure 2. Effect of EAT on the LF/HF ratio: effect of patients' therapy expectations. Mean ± standard error. * $p < 0.05$.

4. Discussion

The results obtained with the different parameters showed that EAT may induce some behavioral and physiological changes in horses. Nevertheless, the results obtained regarding HRV, in particular LF/HF, were within the normal range in both phases [21]. Thus, these responses could not be linked to

any stress-related response in the present study. Therefore, our results are in line with previous studies that have suggested that EAT does not increase stress [14,19,20].

4.1. Behavior

Considering the studied behaviors, the HLM frequency was lower in the PHY+PSY group than in the PSY group during preparation, suggesting that horses searched more for visual information [14] with the PSY group than with the PHY+PSY group. This result could be explained by the curiosity of horses about unusual types of interactions. In addition, a decrease in the HLM frequency from the preparation to the working phase was observed in the PSY group, suggesting that interactions required more visual contact for the horses during the preparation phase than during the working phase. Nevertheless, the observed frequencies of all the behaviors in this study were very low. In general, in this study, horses performed EPB and HLM less than one time per minute. Snort was only performed in approximately half of the observations. Defecation was observed in 11 out of 111 observations.

EPB and defecation, as well as HLM during riding sessions, are behaviors that are commonly associated with negative emotions [14,19]. Snort was previously considered a behavior related to arousal [32–34], but recently, this behavior has been associated with positive situations [31]. The relationship between each of these behaviors and either a positive or negative situation or emotions is interesting, because such relationships may indicate that the EAT activities in the present study were neither positive nor negative for the horses, as none of the behaviors were frequently expressed by the animals.

4.2. HRV

In terms of the HR, an increase of approximately 10 bpm between the preparation and the working phases was observed. VLF was less than 1% in the preparation phase, and a small increase from the preparation to the working phase was observed, although VLF was still less than 1% after the increase. Concerning the LF/HF ratio, an increase of approximately 1.8 times between the preparation phase and the working phase was observed. Additionally, the LF/HF ratio of the horses involved in EAT with the PHY+PSY group was approximately 1.7 times higher than that of the horses involved in EAT with the PSY group.

Regarding the Δ values, a decrease in HR of 3 bpm from the resting phase to the preparation phase and an increase of 9 bpm from the resting phase to the working phase were observed. Moreover, the VLF showed a small decrease (0.02) from the resting phase to the preparation phase, and a higher increase (0.23) from the resting phase to the working phase. Finally, a decrease in the LF/HF ratio from the resting phase to the preparation phase and an increase in the LF/HF ratio from the resting phase to the working phase were observed. Nevertheless, values of LF/HF appear to indicate physiological changes, and usual LF/HF values in EAT horses could be considered to range between 2–5 [21]. Besides the observed variability, the results of the present study concerning the LF/HF ratio were all within the above-described range.

Horses involved in other equestrian activities may show an increase in HR that can exceed 100 bpm during working/training sessions [35,36]. The EAT activities in this study were performed at a low intensity level, which explains the low levels of the HR changes observed in this study. Declining trends in the VLF component of HRV have been related to slow recovery of the ANS balance, and have also been associated with heart disorders [28]. However, in our study, we observed a short decrease in VLF in the preparation phase, which was immediately balanced during the working phase. An increase in the LF/HF ratio represents an increase in sympathetic activity, while a decrease in the LF/HF ratio represents an increase in parasympathetic activity [26,28]. In our study, an increase in this ratio was observed in the working phase, which could be associated with attempts by the horses to cope with the situation. In addition, patients in the PHY+PSY group induced higher LF/HF ratios than the patients in the PSY group. This suggests that therapies with patients in the PSY group were less challenging to

the horses' emotional state than therapies with patients in the PHY+PSY group. However, neither of the groups represented a major stressor to the horses.

4.3. General Discussion

The benefits of EAT for people with mental and physical disabilities have been the subject of many investigations, which have demonstrated improvements in the muscle activity, social skills, and reductions in the anxiety of patients suffering from different medical conditions [6,8]. As EATs are getting popular, the welfare of horses involved in EATs has become a subject of increasing interest in the scientific community [14,19–21,37].

EAT activities are usually performed at low levels of physical activity [21], which was also observed in our study. Some authors have described HRV as a useful tool to study the emotional balance of horses involved in EAT and assess the horse–human partnership [21]. Indeed, our results support the use of HRV as an interesting tool to study equine perceptions of EAT because this analysis allows the study of the animal's response toward a stimulus and its capacity to subsequently recover.

Investigations about EAT have suggested that these activities are minimally stressful for horses and could produce positive outcomes or emotions in horses [14,18,19]. Nevertheless, a recent study suggested that EAT does not increase equine well-being [20]. The conclusions of that study relied on HRV and the plasmatic concentrations of cortisol and oxytocin before, during, and after EAT sessions. The assessment of oxytocin concentrations in equine plasma through enzyme immunoassay requires an extraction phase, as described by Bienboire-Frosini et al. [38]. This procedure was not performed in the previously cited study [20]. However, the results of HRV in that study were in line with the results obtained in the present study. The results of the present study concerning both behavior and HRV suggested that horses experienced neither negative nor positive emotions during EAT activities. In addition, horses rapidly balanced their ANS after these sessions. Hence, we suggest that the horses involved in EAT perceive EAT activities as neutral rather than negative or positive.

The positive or negative indicators of emotions could be considered one limitation in this study. Snort has often been described as an indicator of negative emotions [32–34]; however, recently, some authors have described this behavior as a positive indicator [31]. HLM was also described as an indicator of negative emotions [14], although this behavior could also be related to the natural curiosity of horses. These findings highlight the need for further research on the use of behavioral indicators to study equine emotions, especially positive emotions. Nevertheless, in the present study, HRV was also assessed to complement the behavioral assessment. This tool has been commonly used to study stress or negative emotions, but positive emotions could also be studied through this analysis, because positive emotions create modifications in the activity of the sympathetic and parasympathetic systems [16,29].

Another limitation to this study was the variability of the activities performed in each phase. Activities such as brushing and cleaning the feet, as well as riding the horse or performing in-hand work, may have induced different reactions. The variability in the horses' reactions to the different situations within each phase was not investigated in the present study, as the aim was to assess the overall reaction of horses to the preparation and working phases, and to assess whether EATs were perceived as positive or negative by the horses. Additionally, each group of patients in this study included patients with different illnesses, who may interact differently with the horses, producing negative or positive outcomes.

Previous studies on EAT have focused on the negative outcomes that EAT activities could represent for horses [14,19,20]. The results of these investigations suggested that there are no negative outcomes related to EAT activities for the studied animals. In addition, our results suggested that there were neither negative nor positive outcomes related to EAT for horses. Moreover, as described above, there were significant differences between patients in the PSY and the PSY+PHY groups, but these differences should be interpreted carefully, as the observed variables were within normal ranges [21]. These findings are of great importance, because they show that even if an event is not perceived as

negative or does not induce a negative outcome for horses, a positive association with the event or a positive outcome should not be expected.

5. Conclusions

In conclusion, EAT was neither a negative nor a positive event for the horses in this study, as demonstrated by the horses' behavior and HRV parameters. Behavioral and HRV changes were higher in horses involved in EATs with patients who had both physical and psychological therapy expectations than in horses involved in EATs with patients who had only psychological therapy expectations.

HRV is useful for measuring ANS activity, and thus assessing equine emotional balance during EAT.

Further research focusing on the positive aspects of these therapies for horses is required. This future research should focus on improving equine welfare in EAT, e.g., providing horses with positive stimulation and reinforcement, thus producing positive outcomes for the horses involved in EAT.

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3.3. Discussion

Behavioural and physiological changes were observed in this study. Some differences were observed in behavioural parameters, particularly in head lateral movement (HLM), between phases and groups. HLM was lower with the PHY+PSY group than in the PSY group in the preparation phase, which means that the horses searched more for visual information in this phase with the PSY group. This could be explained by the curiosity of horses about unusual interactions. Additionally, HLM decreased between the preparation and the working session with the PSY group, which means that the horses needed more visual contact with the patient in the preparation phase. Nevertheless, the frequencies of all the studied behaviours were very low, independently of the phase and group.

Physiological data, assessed through heart rate variability (HRV), showed some differences between phases and groups, particularly in LF/HF values. However, these values were kept within the normal range throughout the study. This means that even if behavioural and physiological changes were observed, there were no major stressful situations.

Equine-assisted therapy was neither a negative nor a positive event for the horses in this study. Behavioural and HRV changes were higher in horses involved in EATs with patients who had both physical and psychological therapy expectations. These results provide scientific knowledge on equine perception of EATs and unfold the possibility to improve these activities, perhaps using positive reinforcement, to help horses perceiving EATs as positive events with positive outcomes.

Equine-assisted therapies are performed at low-intensity levels of physical activity. However, different working activities, as the ones performed by the urban working horses in Chile, require endurance and strength, thus the equine's perception of these activities may differ from the perception of EATs. Consequently, this investigation proceeded with a new experiment to compare Chilean rodeo horses and urban Chilean working horses' emotional reactions to a handling test.

Chapter 4: Behavioral and physiological differences between working horses and Chilean rodeo horses in a handling test

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4.1. Introduction

In Chile, some horses still perform urban work like transporting different types of products, such as wood, sand and vegetable, while other horses are involved in Chilean rodeo. Chilean Rodeo is a sport in which the horses should herd a steer within a circular arena to finally press it against a wall with the horse's pectoral muscles. These activities, even if they seem very different at first sight, both require an important physical strength and effort from the horses to succeed. However, they differ regarding the horse's reactivity.

Chilean rodeo owners usually prefer horses that are more reactive to their instructions, while owners of urban working horses prefer horses that would not respond to environmental stimuli that could easily become a stressor. The aim of this study was to compare behavioural and physiological responses between urban working horses and Chilean rodeo horses when confronted with a handling test.

Twenty-six horses were included in this study (13 urban working horses and 13 Chilean rodeo horses). A bridge test, created by Wolff et al. (1997), was used to study equine emotional responses. In this test, horses should cross a bridge that was 1,5m wide and 2m long. Behavioural and physiological parameters were collected throughout the test to compare horses' emotional responses between the two groups.

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Communication

Behavioral and Physiological Differences between Working Horses and Chilean Rodeo Horses in a Handling Test

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Simple Summary: Animal welfare is a current societal concern, and non-invasive indicators are required to assess the welfare state of animals. The selection of horses for certain functions and individual differences could result in different strategies to deal with stressors. This is why in the present study we assessed behavioral and physiological responses of two types of horses (working horses and Chilean rodeo horses) to a handling test (bridge test). We evaluated five behaviors, the number of attempts, and the time required to cross a bridge. Heart rate and the variability of heart rate were registered with a polar system during rest and during the bridge test. Chilean rodeo horses displayed several active behaviors in order to avoid the bridge and required a higher number of attempts to complete the task, but physiologically responded better. On the other hand, all working horses crossed the bridge on the first attempt, without refusal behaviors, but physiologically did not respond as well as Chilean rodeo horses. Behavior does not always correlate with physiological data, and needs to be interpreted carefully when assessing horse welfare.

Abstract: Non-invasive measures are preferred when assessing animal welfare. Differences in behavioral and physiological responses toward a stressor could be the result of the selection of horses for specific uses. Behavioral and physiological responses of working and Chilean rodeo horses subjected to a handling test were assessed. Five behaviors, number of attempts, and the time to cross a bridge were video recorded and analyzed with the Observer XT software. Heart rate (HR) and heart rate variability (HRV), to assess the physiological response to the novel stimulus, were registered with a Polar Equine V800 heart rate monitor system during rest and the bridge test. Heart rate variability data were obtained with the Kubios software. Differences between working and Chilean rodeo horses were assessed, and within-group differences between rest and the test were also analyzed. Chilean rodeo horses presented more proactive behaviors and required significantly more attempts to cross the bridge than working horses. Physiologically, Chilean rodeo horses presented lower variability of the heart rate than working horses.

Keywords: equine; welfare; heart rate variability; behavior; working horse; Chilean rodeo horse

1. Introduction

Animal welfare is a current societal concern, accompanied with an increase in awareness on how animals are raised and used. This creates a challenge for scientists, who need to generate information

that can be useful to answer these concerns, but also contribute to the development of public policies based on science. For the assessment of animal welfare, this has resulted in the development of different evaluation protocols, based mainly on animal-based measures and also on individual differences that may arise as a result of different coping styles.

The term coping has become more common in the studies of the influence of environmental stressors on behavior and welfare [1,2]. Animals cope with environmental stressors through different mechanisms, among which behavioral and physiological adjustments, such as changes in heart rate variability (HRV), have been described [3,4]. Coping has been defined as the behavioral and physiological adjustments of an animal to master a situation [1,5]. A coping style is a coherent set of behavioral and physiological responses which are consistent over time and characteristic for a certain group of individuals [5]. Horses can then show specific coping strategies, revealing specific behavioral and physiological patterns to adapt to novel stressors [6].

On the basis of social stress research, two stress response patterns or coping styles have been suggested by Henry and Stephens [7]. The first type, known as the proactive (active) response, corresponds to the fight or flight response. Behaviorally, this response is characterized by territorial control, aggression, high resistance to restraint, and these individuals are more prone to take risks and form routines [1,5,8,9]. Proactive individuals respond physiologically with a strong sympathetic activation and an increase in noradrenergic stimulation [10]. The second type is known as the reactive (conservation-withdrawal) response characterized by behavioral immobility [11], in which the animal adopts a passive response, showing low levels of aggression if any [5]. These individuals respond to challenge with a strong hypothalamic-pituitary-adrenocortical (HPA-axis) and parasympathetic reactivity, increasing circulating glucocorticoids [10], and a higher baseline of non-enzymatic antioxidant capacity [12].

Since different coping styles involve particular responses of the autonomic nervous system (ANS), the use of variables associated with the variability of the heart rate (HRV) can be useful indicators [13]. From the HRV, the time domain analysis variables such as the mean beat-to-beat interval (mean RR), standard deviations of the RR intervals (SDNN), and root mean square of successive RR differences (RMSSD) have been proven to be a remarkable non-invasive measure of the ANS that can be applied in behavioral research to assess temperament and emotional states [13]. Moreover, frequency domain analysis can better discriminate between the contributions of the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS). This analysis includes two non-overlapping frequency bands [14]. The low frequency band (LF) of the HRV is influenced by the SNS, whereas the PNS is predominantly reflected in the high frequency band (HF). Considering this, it has been proposed that the low-to-high frequency ratio (LF/HF) is an index of the sympathovagal balance [15]. Moreover, the HF has been proven to be low during mental or physical stressful events [14].

Domestication has led to significant changes in the life of horses [16]. Housing and management can result in stress responses [17,18] when they do not meet horses' needs. This in turn can induce changes in production efficiency, product quality, and performance. The intensity of these changes varies according to the specific husbandry system in which equines are kept, usually associated with their function or activity (sports, draught work, pleasure). Horses used for different functions could also have different welfare problems and coping strategies. When owners or caretakers select horses according to certain behavioral traits or according to their function, they could also be favoring certain coping styles.

In Chile, it is still common to see urban working horses in the peri-urban areas of cities where they perform draught work, transporting different types of products (wood, sand, vegetables) [19]. These horses are usually mixed breeds with an average height to the withers of 143 cm [19]. On the other hand, Chilean rodeo is a national sport, where Chilean creole horses are used (average height to the withers of 142 cm). The main physical effort of this sport consists of herding a steer within a circular arena (medialuna), and then pressing the steer against a padded wall with the horse's pectoral muscles [20]. Chilean rodeo owners prefer certain behavioral characteristics that are usually associated

with a more proactive coping style [21]. These horses are subjected to routines [22], and owners prefer horses that respond more actively to commands and when working with a steer, whereas owners of draught horses prefer horses that react minimally and remain calm when confronted with a stressor (i.e., traffic) [21] and are subjected to a more flexible environment [19], characteristic of a reactive coping style.

In prior work, differences in basal levels of cortisol, oxidative stress indicators, and leukocytes, all parameters associated with stress response, were found between Chilean rodeo horses and Chilean urban working horses [21]. However, that study did not evaluate the differences between these two groups of horses when confronted with a potential stressor. The aim of this study was to investigate behavioral and physiological differences between Chilean rodeo horses and Chilean urban working horses when confronted with a handling test (bridge test).

2. Materials and Methods

2.1. Animals

A total of 26 horses, 13 working horses and 13 Chilean rodeo horses, were involved in this study. All horses were physically healthy, mares and geldings all over 5 years of age that had been actively performing either urban draught work (W-H) or Chilean rodeo exercise (R-H). All working horses were evaluated at the Veterinary and Animal Sciences Faculty of the University of Chile. All working horses arrived by trailer, and rested for at least 2 h before the start of the tests which were held between 10 a.m. and 4 p.m. All rodeo horses were assessed at the breeder's farm during the same hours. To test these horses, the same bridge was constructed on the breeder's farm. All procedures were approved by the institutional bioethical committee (certificate No. 12-2016).

2.2. Bridge Test (Handling Test)

The bridge test was designed as in Wolff et al. [23]. A bridge that was 1.5 m wide and 2 m long with a wooden floor and green iron rails at each side of 1 m height was used, and a starting line was set at a distance of 2 m before the bridge. The observer stood at a distance of 5 m (on the side) from where each horse was video recorded. An operator (the same for all horses) led the horse to the bridge and tried to make the horse cross it by pulling slightly on the rope if necessary. All horses were restrained and led with only a lead rope attached to a halter.

When the horse avoided walking on the bridge by going sideways or backwards, they were led back to the starting point and a new trial began. A trial was successful when the horse crossed the bridge with all four feet.

2.3. Behavioral Measures

All horses were video recorded during the bridge test. The behavioral events observed were retreat (backward movement with at least one foot), swerve (sideways movement), jump (over the bridge), snort (forceful quick exhalation), and neigh (loud, prolonged high pitch call). Videos were then analyzed using The Observer XT (2011) software (Noldus Information Technology, Wageningen, The Netherlands). Offline continuous recording was used, and all behavioral event occurrences, number of trials, and the total time to cross the bridge were registered.

2.4. Physiological Measures

Heart rate (HR) and heart rate variability (HRV) recordings were obtained using a Polar Equine V800 heart rate monitor system (Polar Electro Oy, Kempele, Finland). For each horse, a 5 min long data set during resting time was recorded [13]. For this, after placing the polar system, a habituation to the monitor for 30 min was performed Then, a 5 min data set was obtained and the horse was led to the area where the bridge test was performed. A second data set from the beginning of the bridge test

until its end was obtained. Additionally, heart rate was also recorded at the start of the bridge test (T1), when the horse crossed the bridge (T2), and 10 min after the bridge test (T3).

For HRV analysis, information from the Polar system was synchronized with the Kubios HRV software (Kubios standard 3.2.0, Biomedical Signal Analysis Group, Department of Applied Physics, University of Kuopio, Finland). Data were detrended using a smoothness parameter of 500 ms and artifacts were corrected by setting the custom filter to 0.3, according to Ille et al. [24]. Frequency band thresholds were established within each frequency interval using the following parameters: LF power = 0.01–0.07 Hz; HF power = 0.07–0.6 Hz [25–27]. The type of data extracted from each recording is shown in Table 1.

Table 1. Description of the time domain and frequency domain measures used in the present study.

Variable	Unit	Description
Time Domain Variables		
HR	bpm	Heart rate
RR	ms	R wave to R wave interval or inter-beat interval
SDNN	ms	Standard deviations of the RR intervals
RMSSD	ms	Root mean square of successive RR differences
Frequency Domain Variables		
LF	Hz	Low frequency band
HF	Hz	High frequency band
LF/HF ratio	–	Low frequency to high frequency ratio

2.5. Statistical Analysis

Descriptive statistics (mean and standard deviation) were calculated for both behavioral and physiological measures. For physiological measures, normal distribution was assessed using the Shapiro–Wilk test. Subsequently, differences between sampling periods (rest and bridge test) within groups were calculated using the paired *t*-test or the Wilcoxon signed-rank test accordingly. For differences between groups (R-H and W-H) for each sampling period, the two-sample *t*-test and Wilcoxon rank-sum test were used. A p-value of <0.05 was established for significant differences.

3. Results and Discussion

The bridge test is considered a restraint and human fear test. This test involves a combination of two potential stressors, namely, the procedure of handling and the fear towards humans [28]. Therefore, the demeanor and skill of the handler may make a significant difference, and the use of a familiar handler to the horse (owner, rider, or trainer) may have had a greater success in getting the horse to cross the bridge faster. In the present study, this method was used with the aim of comparing the behavioral and physiological responses between a group of working horses and a group of Chilean rodeo horses. The results suggest that the challenge of crossing an unknown bridge elicited different behavioral and physiological responses between the two groups.

3.1. Behavioral Measures

All working horses crossed the bridge on the first attempt, with a mean time of 9.77 ± 2 s. This group of horses did not display any of the assessed behaviors, with the exception of one horse that neighed before crossing the bridge. On the contrary, the Chilean rodeo horses required a significantly higher ($p = 0.007$) number of attempts to cross the bridge (1.77), varying from 1 to 4 attempts and an average time to cross of 19.81 ± 16 s ($p = 0.09$). Six rodeo horses required two or more attempts to cross the bridge, and these horses showed swerving and retreating when approaching the bridge and jumping once they started crossing it. Vocalizations such as snorts and neighs were present in three of these horses (Table 2). The time to complete the test was lower than the time reported by Wolff et al. [23], who reported times between 40 s and 10 min, although the distance from the bridge

and the length of the bridge used in the present study were the same. Most studies have applied this test to compare the emotionality of horses of different ages, sex, or training status [4,23,29] and not according to function as in the present study. The behavioral results suggested that Chilean rodeo horses displayed more active (proactive) behaviors attempting to avoid the bridge by removing themselves from the stressor [30]. On the other hand, working horses passively accepted the handling and crossed the bridge (reactive) [2,31].

Table 2. Exact time required for the bridge test, number of attempts, and frequency of occurrence of each behavioral event registered for each horse. Mean and standard deviation (SD) of each variable are also indicated.

Horse	Time (Seconds)		No. Attempts		Snort		Jump		Swerve		Retreat		Neigh	
	R-H	W-H	R-H	W-H	R-H	W-H	R-H	W-H	R-H	W-H	R-H	W-H	R-H	W-H
1	8	9.01	1	1	0	0	0	0	0	0	0	0	0	0
2	20	11.89	3	1	0	0	1	0	2	0	0	0	0	0
3	26	9.69	2	1	0	0	1	0	1	0	0	0	1	0
4	21.8	6.06	2	1	0	0	1	0	2	0	1	0	0	0
5	11.01	8.06	1	1	0	0	0	0	0	0	0	0	0	0
6	8.4	7.24	1	1	0	0	0	0	0	0	0	0	0	0
7	8.02	9.5	1	1	0	0	0	0	0	0	0	0	0	0
8	7.22	9.11	1	1	0	0	0	0	0	0	0	0	0	0
9	40.56	9.48	3	1	3	0	0	0	2	0	0	0	0	0
10	9.37	12.39	1	1	0	0	0	0	0	0	0	0	0	0
11	8.75	11.81	1	1	0	0	0	0	0	0	0	0	0	0
12	27.82	12.15	2	1	3	0	1	0	0	0	1	0	1	0
13	60.54	10.36	4	1	0	0	0	0	4	0	0	0	0	1

R-W, rodeo horse; W-H, working horse.

3.2. Physiological Measures

A significant increase in HR between T1 and T2 was found in both groups of horses ($p < 0.001$ in both groups), recovering initial HR by T3 (10 min after crossing the bridge), but no differences between groups were found within any sampling time (Figure 1). The mean HR during the bridge test had a small, but significant rise in both groups of horses, but no differences were found between groups, neither for the minimum and maximum HR (Table 3). Fast alterations of the mean heart rate can occur within 5 s as a response to fear or excitement in horses and are attributed usually with short-term effects [4,13,32]. A rise in HR can be caused by an increase of the sympathetic activity, a decrease in vagal regulation, or changes in both regulatory systems [13]. Thus, the use of HRV can provide a better understanding of how the individual is coping with a stressor, since psychological states may have an impact on the sympathovagal balance in the absence of changes in HR [33].

The mean RR had a significant decrease in both groups when comparing the resting period with the bridge test period, as expected, with no differences between groups (Table 3). Contrary to Visser et al.'s [4] findings, our results showed an increase of SDNN between rest and the bridge test in the working horses' group, and a significant difference between groups during the bridge test (Table 3). Thus, the overall variability of the HR during the bridge test was higher in the Chilean rodeo horses' group than in the working horses' group. Additionally, the working horses' overall variability of the HR (SDRR) was higher during the bridge test than at rest, whereas the Chilean rodeo horses were more balanced between rest and the bridge test (Table 3). On the other hand, RMSSD decreased during the bridge test in both groups, but was significantly higher during the bridge test in the Chilean rodeo horses indicating a higher activity of the parasympathetic nervous system (PNS) in this group. The PNS has been associated with adaptive responses to the environment [34] and could indicate a better coping capacity for the Chilean rodeo horses. Visser et al. [4] also propose that individuals

with a higher PNS activity would be more explorative and adaptive to environmental demands; our behavioral results would be in line with this since they displayed an active response in the bridge test.

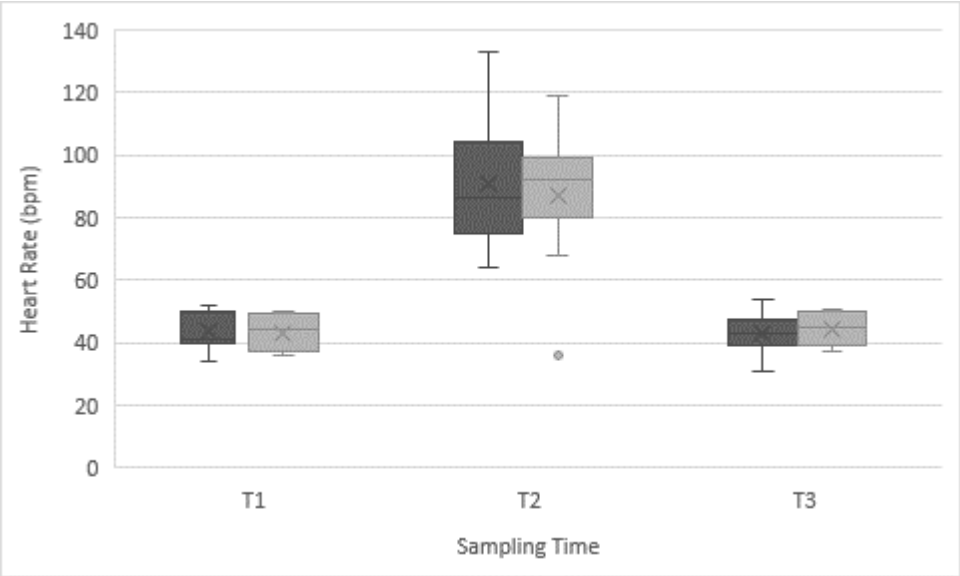


Figure 1. Box and whisker plot of the heart rate (HR) (bpm) of Chilean rodeo horses (dark grey) and working horses (light grey) at the start of the bridge test (T1), after crossing the bridge (T2), and 10 min after the bridge test (T3). Mean values, upper and lower quartiles, extreme values, and outliers are indicated.

Table 3. The mean, standard deviation (SD), and *p*-values for each variable are provided according to horse group and to sampling time (rest or bridge test).

Variable	Time	R-H	W-H	*p*-Value
Min HR (bpm)	rest	38.61 (4)	40.15 (5)	0.405
	bridge	43.23 * (10)	41.54 (5)	0.962
	p-value	0.045	0.097	
Max HR (bpm)	rest	52.31 (8)	55.62 (11)	0.286
	bridge	91.06 * (21)	87.07 * (20)	0.431
	p-value	*<0.001*	*<0.001*	
Mean HR (bpm)	rest	43.35 (5)	45.10 (6)	0.392
	bridge	57.05 * (15)	53.56 * (8)	1
	p-value	0.001	0.001	
Mean RR (ms)	rest	1402.58 (181)	1354.88 (189)	0.442
	bridge	1110.33 * (244)	1152.82 * (225)	1
	p-value	<0.001	0.00	
SDNN (ms)	rest	67.52 (28)	54.03 (15)	0.234
	bridge	83.11 [a] (31)	71.58 [b]* (18)	0.047
	p-value	0.142	0.001	
RMSSD (ms)	rest	73.97 (26)	59.50 (21)	0.208
	bridge	66.23 [a] (30)	54.38 [b] (17)	0.039
	p-value	0.208	0.393	
LF/HF ratio	rest	0.59 (0.6)	1.09 (0.9)	0.263
	bridge	1.46 * (1.9)	2.36 * (2.2)	0.064
	p-value	0.050	0.025	

* Indicates significant differences ($p < 0.05$) within groups (R-H and W-H) between sampling periods (rest and bridge test) for each variable. Different letters (a, b) indicate significant differences ($p < 0.05$) between groups (R-H and W-H) within a same sampling period (rest or bridge test) for each variable. R-H, rodeo horse; W-H, working horse; HR, heart rate; RR, R wave to R wave interval; SDNN, standard deviations of the RR intervals; RMSSD, root mean square of successive RR differences; LF/HF ratio, low frequency band to high frequency band ratio.

The LF/HF ratio obtained from the power spectrum analysis is considered as an index of the cardiac sympathovagal balance [26] and has been proven to be a useful indicator of the sympathetic activity during physical and psychological stresses [13,35]. The HF power is thought to reflect PNS activity, whereas the LF power component should reflect both the sympathetic nervous system (SNS) and PNS [27]. Our results suggest a higher SNS activity in the working horses' group than in the Chilean rodeo horses' group in the bridge test. These results are in line with the RMSSD findings, showing that Chilean rodeo horses had a higher activity of the PNS, hence a better balance in terms of the autonomic nervous system (ANS) than working horses during the bridge test. In a future study, it would be important to consider a recovery time period, after the bridge test, in order to fully understand the coping mechanism in both groups.

3.3. General Remarks

Working horses took less time and passed the bridge at the first attempt, without displaying any active (proactive) behaviors in order to avoid the task. Nevertheless, physiologically they showed signs of emotional unbalance and a lower capacity to cope with the test. These results are in accordance with Yarnell et al. [36] and Squibb et al. [30], where it is proposed that behavior may not accurately reflect the internal affective state of horses. This could be related to the fact that horses are prey species and may mask behavioral signs of stress [36]. As in Squibb et al. [30], it is possible that the working horses that crossed the bridge were experiencing higher levels of stress, but also a greater stimulus control [37] than the rodeo horses that displayed more refusal behaviors. This higher stimulus control could be related to the fact that the bridge test requires handling by a person and the use of a halter that could act as the specific stimulus inducing a desired response (in this case, crossing the bridge) [37].

The differences in the responses between the two groups of horses could also be the result of breed differences and management conditions. Lesimple et al. [18] reported that breed and housing conditions appear to be of major importance in determining horses' personality and remarked about the importance of considering how management practices can impact a horse's reactivity. Working horses are also confronted daily with different environmental conditions and they could be accustomed to this exposure to novelty and not express flight behaviors. If this was the case, it should also be reflected in the ANS response, as in Górecka et al. [38], where horses used to novelty showed a lower HR response.

The emotional state of working horses should also be evaluated under tests that do not require human handling, such as the open arena and a novel object test [28]. A second plausible scenario, that requires further study, is as follows: since working horses are frequently exposed to stressful situations, poor husbandry practices, a high incidence of painful conditions such as lameness, and poor training systems, they could lose active control of situations resulting in learned helplessness [39]. According to Squibb et al. [30], an animal experiencing learned helplessness "abandons its attempts to cope and develops a dullness related to a decline in motivation and emotional response". Although this study does not give evidence of an animal presenting learned helplessness, in situations where it does occur, this state would be a welfare concern, since individuals under this state have lost control of their environment.

4. Conclusions

Chilean rodeo horses displayed more active behaviors in order to avoid the bridge task. These behaviors were adaptive and efficient as physiologically these horses showed a better coping capacity. Working horses, that are frequently exposed to traffic, may have learned not to behaviorally respond to stimuli such as the bridge, creating an increase in reactivity in terms of their physiology. Our results are in accordance with other studies that show that an accurate interpretation of behavioral signs requires corroboration by means of physiological measures. This is of particular importance to take into account in current welfare assessment protocols where behavioral indicators are preferred as a non-invasive measure. Heart rate variability is an interesting, non-invasive tool, that provides physiological data.

Heart rate variability in association with behavioral indicators may improve the emotional assessment of equines.

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4.3. Discussion

Urban working horses crossed the bridge on the first attempt, with a mean time of 10 seconds, without displaying any of the other studied behaviours (retreat, swerve, jump, snort and neigh). Chilean rodeo horses required a significantly higher number of attempts to cross the bridge, with a mean time of 20 seconds, though the other observed behaviours were rarely displayed.

An increase in heart rate during the bridge test was observed in the two groups, but no differences were found between the groups. However, significant differences were observed between groups in SDNN, which is variable that represents the overall variability of heart rate in a given period. SDNN was significantly higher with Chilean rodeo horses during the bridge test. RMSSD, a parameter that represents the activity of the parasympathetic branch of the autonomic nervous system, was significantly higher with the Chilean rodeo horses. This means that the overall variability of the heart rate with Chilean rodeo horses was associated with a parasympathetic activity. This was confirmed by the LF/HF ratio results. The parasympathetic activity has been associated with adaptive responses to the environment.

This means that Chilean rodeo horses displayed more resistance to cross the bridge in the test, but this also means that their behaviour was adaptive and functional because it helped on balancing the autonomic nervous system activity of these horses. Urban working horses probably learned not to respond to different environmental stimuli, though this does not mean that the stimuli do not create emotional changes, as in this test, these horses did not show avoidance, but an unbalance of the autonomic nervous system activity was created, which means that they were emotionally challenged by the test.

These investigations provided insights on equine perception of different activities. They also provided information on equine emotional responses and how they differ between horses. Further research is needed to investigate how humans could improve equine welfare within each of the activities in which horses are involved.

Chapter 5: Investigating the oxytocinergic system: plasma measures of free VS total oxytocin fraction in two animal models

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5.1. Introduction

The analysis of oxytocin concentrations in plasma through EIA techniques required the validation of the available kits. Oxytocin is circulating under two forms in plasma: free and protein bound. The application of the EIA oxytocin kit originally designed to be used with human plasma was validated to assess free oxytocin concentrations in equine plasma (Bienboire-Frosini et al. 2017). However, due to the short half-life of free oxytocin in plasma (<10 min), normal levels are very low (at the pg/ml level), making difficult the measurement of plasma free oxytocin. Besides, some authors suggested that free oxytocin concentrations might be a confounding factor because free oxytocin concentrations can be modified by different factors, as e.g. age or drugs, which produce samples with non-detectable levels of oxytocin (Doneanu and Rainville 2013). Therefore, some authors developed a method to assess total oxytocin in plasma samples of dogs (Brandtzaeg et al. 2016), but nothing of this kind was performed for equine plasma samples. Moreover, it is unknown if a correlation between free and total oxytocin concentrations does exist for equines or dogs. The aim of this study was to apply the total oxytocin assay for equines plasma samples in order to subsequently investigate the correlation between free and total oxytocin concentrations in two species and to compare oxytocin (free and total) plasma concentrations between horses involved in competition and horses involved in equine-assisted interventions and between pet dogs and assistance dogs.

Methods: One healthy man (34 years old) was followed for 53 days. Daily saliva (awakening, +30, +45 min, evening) was used to quantify 7 steroid hormones. Blood was collected weekly over nine weeks to measure: (a) 19 immune and metabolic biomarkers, (b) 16 hematological parameters, and (c) 15 parameters of immune cell type composition assessed by flow cytometry. An allostatic load (AL) score was also computed. For each biomarker, stability was assessed by computing its coefficient of variation (C.V.) indicating % change over time.

Results: Neuroendocrine salivary biomarkers were on average twice more variable at night (mean 83%; range 47–166%) than at awakening (45%; range 26–67%) with cortisol (166%) being the most variable at night. Individual blood-based biomarkers including insulin, CRP, C-peptide, triglycerides, and estradiol were relatively stable (13%, range 1–38%). The average variability for hematological measures was 11% (range 1–57%). In contrast, immune cell subtype composition was variable (67%, range 29–230%), particularly the proportions of terminally differentiated effector memory (TEMRA) CD4 T-cells, activated CD4 and CD8 T-cells. AL ranged from 1 to 3.

Discussion: Overall, these high-temporal resolution data reveal substantial intra-individual variability for a wide range of salivary- and blood-based neuroendocrine, metabolic, and immune markers, as well as for AL theorized to represent cumulative dysregulation. These preliminary results highlight the potential value of repeated biomarker assessments.

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The impact of neuroeducational methods on telomere length

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Background: Psychological factors are known to modulate the aging process, and might also act on genetic level. Telomeres and telomerase are basic molecular features of cells genetic senescence that also contributes to various diseases. The aim of this study is to investigate the effect of neuroeducational methods on telomere length shortening.

Method: The study was conducted on 20 relatively healthy subjects aged 23–59 years old. Data summarizes the findings on telomere length of neuroeducational group and controls at baseline and after 6 months post-intervention. Ten persons had regular (20 h/month) neuroeducational sessions (stress reduction, mindfulness, art therapy with professional specialists). Control group consisted of 10 individuals, matched by demographic and health history criteria. HT-Q-FISH (LifeLength, Spain) was used to measure the median telomere length (TL).

Results: In neuroeducational group individuals, median TL over the 6 months period decreased 100 ± 27 base pairs (bp) – from 10480 bp to 10380 bp, whereas subjects in the control group lost 420 ± 80 bp telomeres – from 10920 bp to 10500 bp. The subjects of control group lost telomeres statistically significantly ($p = 0.02$).

Discussion: The findings of this pilot study show that neuroeducational sessions reflect to slower shortening of telomeres. We hypothesize that neuroeducation can impact some biochemical pathways involved in stress-related mechanisms (cortisol level, autonomous nervous system, etc.) and may contribute to a "decreased aging" phenotype.

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Investigating the oxytocinergic system: Plasma measures of free vs. total oxytocin fractions in two animal models

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Oxytocin measurement methodologies differ, resulting in discrepant published results. Recently, a method measuring the total oxytocin fraction (free and protein-bound) was developed, without elucidating its biological meaningfulness. This study aims to measure plasma levels of free (fOT) and total (tOT) oxytocin in dogs and horses, also assessing the influence of sex and social activities.

Pet ($n = 21$) and assistance dogs ($n = 20$) enrolled were 20 females and 21 males. Forty-eight horses were included (19 females; 27 geldings), with 17 horses involved in competition and 9 in equine-assisted interventions (EAI). fOT was measured according to Bienboire-Frosini et al. (2017) and tOT according to Brantzaeg et al. (2016).

fOT and tOT levels are weakly correlated in dogs (Pearson's $r = 0.34$; $p = 0.04$) but not in horses (Spearman's rho $= -0.03$; $p = 0.88$). In dogs, fOT was not significantly different between sexes (Student t-test; DF = 39; $t = 0.33$; $p = 0.74$) or between activities (Welch t-test; DF = 31.80; $t = -0.87$; $p = 0.39$). Similar results were obtained for tOT: sexes (Student t-test; DF = 38; $t = 1.02$; $p = 0.32$); activities (Student t-test; DF = 38; $t = 0.91$; $p = 0.37$). In horses, no significant difference was observed for fOT between sexes (Wilcoxon Two-Sample test, $Z = 0.18$; $p = 0.85$) but a trend between EAI and competition horses (Welch t-test; DF = 5.80; $t = 2.21$; $p = 0.07$) was observed. tOT was significantly higher in males than in females (Student t-test; DF = 24; $t = -2.36$; $p = 0.03$) but was not different between activities (Welch t-test; DF = 23.96; $t = 1.04$; $p = 0.31$).

fOT and tOT fraction levels, their relationships, and their link with sexes or sociality could depend upon the species. Further studies should clarify their respective biological significance, considering future clinical applications.

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5.3. Discussion

In this study, the correlation between free and total oxytocin concentrations was weak for dogs and no correlation was observed for horses. For dogs, no differences were observed considering sexes or activities. For horses, no differences were observed between sexes for free oxytocin, but a trend was observed for the activities. The opposite was observed for total oxytocin, with no significant differences between activities, while males displayed significantly higher total oxytocin concentrations in plasma than females.

These results are interesting because they suggest that free and total oxytocin may have different valences as welfare indicators, but this also means that their correlation is variable according to each species. Further research is still needed to disclose the implications of free and total oxytocin as welfare indicators.

GENERAL DISCUSSION

Animal welfare awareness has increased over the last years and ethical concerns around the use of animals by humans for different purposes have been highlighted and discussed. Whether the use of horses for human benefits, sport, and leisure is ethical or not is one of the main questions among the equine industry and lay population (Dashper, 2017). The most common answer to this question is that these activities are ethical if they consider the horse's interests before everything else (Jones and McGreevy 2010; McLean and McGreevy 2010). However, to understand if the animal's interests or welfare are being considered before everything else among the equine industry, scientific studies on equine welfare in such activities should be conducted. Until now, researches about equitation are often related to performance and biomechanics with only few studies focusing on equine positive welfare.

The assessment of emotional responses through measurable parameters is essential to understand how horses perceive the activities in which they are involved and how equine welfare is impacted by them, either positively or negatively. The present work intended to increase the scientific knowledge on equine emotional responses to some of the activities imposed by humans to horses. Equine emotional responses were studied through behavioural and physiological parameters under different conditions.

The first study, entitled "Equine activities influence horses' responses to different stimuli: could this have an impact on equine welfare?", intended to investigate whether equine emotional responses could differ or not according to the activities in which horses are involved. This study was important to assess whether horses would respond equally to a panel of stimuli, independent of the activity in which they were involved, or if there was a need for studying equine activities separately. Results from this study have demonstrated that horses' responses to different stimuli vary according to the activity they practice. There are some possible reasons for the variability of equine responses to different stimuli between adult horses involved in different activities. The development of behavioural responses is associated with learning

experiences, motivation and contexts (McGreevy and McLean 2010a; Hothersall and Casey 2012). Therefore, the observed differences in emotional responses is not surprising because horses are usually trained to perform different activities through distinct learning procedures and in different settings; however, some basic skills are common to every activity and learned using similar procedures (e.g. go, stop, turn, etc.; McGreevy and McLean 2010b). This means that some behavioural patterns could be expected in horses trained to perform a specific activity. Nevertheless, individual variability between horses involved in the same activity was also observed. This variability could be related to temperament characteristics of the tested horses.

Temperament traits are usually defined as individual differences in behaviour that are present early in life and are stable across various kind of situations and over the course of life (Goldsmith et al. 1987; Lansade et al. 2008). This means that some behavioural traits may be specific to a temperament profile. In the present study no temperament assessment was done, however, the reproduction or selection of horses to perform a specific activity over time has considered equine behaviour and might have created, unintentionally, different temperament profiles for each activity. Riders/handlers/therapists tend to prefer horses with a balanced temperament to decrease the risks of injury (Graf et al. 2014). Nevertheless, these professionals search different behavioural patterns according to the activity they are expected to perform (McGreevy and McLean 2010b; Borgi et al. 2015; McKinney et al. 2015; Malinowski et al. 2018), which might also be the reason for the differences in behavioural responses observed between activities in this study. Some authors validated a temperament test to assess temperament profiles in horses in controlled conditions, however, the experimental setting was created to test mostly equine reactivity and recovery after a stressing event, which was artificial and represent a risk of injury (e.g. stationary and rolling exercise balls) (Graf et al. 2014). Nevertheless, in practical terms, the use of temperament tests to determine if one horse would be suitable to perform a specific activity remains difficult, because riders and owners would not perform a test that might represent a risk of injury for both the animal and

the person involved, especially if the horse represents a huge economic value. In only one assessment (e.g. pre-purchase evaluation), some behavioural traits can be identified, although no conclusions can be made on how reliable these behavioural traits are to stablish a temperament profile. Regardless, temperament might have an influence on behavioural responses and might have an influence of the results of the present study.

Since an influence from the activity in which horses are involved was observed on their emotional responses to different stimuli, the following studies of this PhD work were conducted by investigating athlete horses, therapeutic and working horses separately. This decision was made regarding the economic value and the number of animals involved in each of these activities, the availability and willingness of the owners to have their horses participating in these investigations and the increase of the therapeutic and professional use of horses. Nevertheless, there are other activities that could not be considered for this investigation, but would probably benefit from further investigation, e.g. racing and endurance.

The second study, entitled “de la Guérinière was right: shoulder-in is beneficial for horses physical and metal states”, intended to investigate equine emotional responses to lateral vs. longitudinal exercises and to horseback riders with different skills in equitation. Some descriptive behaviours were studied to assess the horse’s attention and relaxation through the experiment. Looking around and playing chewing the bit are behaviours that were previously considered as good indicators for equine’s attention and relaxation in training, respectively (Fleming et al. 2013; Smiet et al. 2014). Additionally, heart rate variability (HRV) was used to assess the autonomic nervous system activity of the horses involved in this experiment. HRV parameters, like heart rate (HR), low-frequency – high-frequency ratio (LF/HF) and very low-frequency (VLF), were often used to assess negative emotional responses (Désiré et al. 2002; von Borell et al. 2007). However, these parameters could also be used to assess positive emotional responses (Boissy et al. 2007). Results from this study demonstrated an association of lateral exercises with relaxation and higher parasympathetic activity, while longitudinal

exercises were associated with distraction and higher sympathetic activity. Additionally, these results suggested that less experienced riders may benefit from lateral exercises to manage equine stress-related responses through training, as the difference in the HRV results between sessions was biggest with the amateur rider. Lateral exercises are often used by horseback riders to improve musculoskeletal aspects or simply to increase horses' musculature (de la Guérinière 1733; Oliveira 2006; Denoix 2014). These exercises are also used when horses become distracted or resistant to the riders' instructions with an empiric approach (Oliveira 2006; de Coux 2007). However, only biomechanics and kinematics were previously investigated with a scientific approach (Symes and Ellis 2009; De Cocq et al. 2010; de Cocq and Van Weeren 2013; Denoix 2014). Results from the present study added scientific information on the psychological aspects of these exercises.

These findings described equine emotional responses of horses to lateral exercises and different skilled horseback riders. Horses involved in this study have been trained to perform this type of exercises. Although, horses involved in other activities receive no training and constantly need to adapt, both physically and psychologically, to the tasks they perform. This is the case of equine-assisted therapy (EAT) horses or working horses.

The third study, entitled "The impact of equine-assisted therapy on equine behavioural and physiological responses"; intended to investigate equine emotional responses to EAT activities. These activities are very variable depending on the medical condition of the patient, which generally require different approaches. Usually, horses involved in these activities receive no training for their specific role, either in physical or psychological terms. The absence of training to perform these activities makes these activities very interesting for the study of spontaneous equine emotional responses during human-animal interactions. These interactions are unique given that, most often, these patients would not use the same communication skills that common horseback riders or handlers use, and that they differ between patients. This means that the therapeutic horse needs to continuously adapt to the

person with whom it will interact. In this study, equine emotional responses were investigated through behaviours and heart rate variability. Stress, irritation and frustration were studied through previously validated behaviours (Kaiser et al. 2006): ears pinned back, head lateral movement and defecation. Snort was previously associated with arousal (Bachmann et al. 2003; Christensen et al. 2008; Leiner and Fendt 2011), but recently this behaviour has been associated with relaxation (Stomp et al. 2018b). Snort was used to study positive emotions in the present study. Heart rate variability was used to assess both negative and positive emotional responses with the same parameters used on the precedent study: HR, LF/HF and VLF. Results from this study suggested that EATs are not perceived as either negative or positive events by the horses. However, even if these activities did not produce negative or positive outcomes, horses were more challenged by patients that had both physical and psychological therapy expectations than with patients who had only psychological therapy expectations.

The neutral perception of these activities by the horses is a very interesting finding because this means that these horses were able to cope with different challenges in this study, with no negative outcomes. Additionally, these horses were not trained to perform therapies, though they have demonstrated high levels of tolerance with persons that could not use the common communication skills, with which horses are familiarised. An improvement of the handling procedures and the therapies might modify the equine perception of these events, so they perceive them as positive situations. The ways to improve these therapies need further research. Thus, the use of positive reinforcement and the creation of a positive relationship between the therapist and the horse may help horses on perceiving these situations as positive. These horses may benefit from physical activities with experienced horseback riders, as these animals should carry persons with no skills or with low levels in equitation. The therapists involved in this study reported that horses involved in therapies are often treated for back pain, though in the scientific literature there is no information on back pain association with EAT. All the animals were submitted to a physical examination before this experiment and

all the horses were healthy. Then, the animals were frequently submitted to physical examination throughout this study by request of the therapists and back pain was only observed once. Nevertheless, training with experienced horseback riders would help increase horses back musculature and tone, thus, to avoid back pain.

Equine working activities, as equine athletic activities, are diverse and the physical activity associated with each working activity differ in intensity levels. Horses involved in EAT are considered therapeutic or working horses, however, the activities they perform are usually at low-intensity levels. At the contrary, Chilean urban working horses should perform draught work for example, which pushes horses to their physiological limits and in terms of intensity might be compared to athletic horses' activities. Chilean rodeo is a sport that consists of using a horse to herd a steer within a circular arena and then pressing the steer against the wall, using pectoral muscles. Chilean rodeo horses are also physiologically challenged by their activities. Working horses and Chilean rodeo horses' activities might be very different, but they both involve challenges to the horses' physiological capacities. Horses involved in these activities were thus an interesting model to compare equine emotional responses of working and athletic animals to a common situation.

The fourth study, entitled "Behavioral and physiological differences between working horses and Chilean rodeo horses in a handling test", intended to investigate the differences of equine emotional responses between horses who were involved in two different activities (working horses and rodeo horses) to a handling test. Ethical concerns related to these activities are often discussed in Chili. Urban working horses are usually mixed breeds and owners select these animals regarding their calmness and low levels of reactivity, whereas Chilean rodeo horses' owners prefer horses that are more reactive and quickly respond to the commands. This means that owners are seeking for horses with opposite behavioural traits for these activities. In this study, the bridge test, designed by Wolff et al. (1997), was used to compare equine behavioural and physiological responses. This test is considered a restraint

and human fear test, thus a suitable test to stress emotional responses of horses to handlers. Chilean rodeo horses displayed avoiding responses to the test. Their behaviour was associated with a more balanced physiological response, which was assessed through HRV analysis. This means that avoidance was an efficient behaviour in balancing the autonomic nervous system of these horses, so this behaviour could be considered adaptive. Working horses are frequently exposed to different stimuli to which they should not react, however, this does not mean that they are not emotionally challenged when the stimuli are present. In this study, working horses did not perform any avoidant behaviours, which is what would be expected from them at working periods, however, heart rate variability parameters were highly modified during the test with an important increase of the sympathetic nervous system activity. This means that these horses' behaviour was less adaptive than the behaviour from the Chilean rodeo horses and that these horses had more difficulties in coping with the test, even if they performed it better.

Horses have previously learned to perform all the activities on the above mention studies. These activities, whatever their aims are, rely on the human-horse communication (Saslow 2002), either tactile, acoustic or visual. Horses learning success depends on humans training skills (Symes and Ellis 2009). Human with lower skills in communication may provide unprecise cues, eliciting undesired responses (Symes and Ellis 2009). This may conduct to frustration and lead to punishments or persistence of the demands from the human to the horse, instead of producing positive or negative reinforcement and learning (Maslow 1941; Mcgreevy 2004). Humans may experience ambivalent emotions at this point because they have a huge motivation to succeed but they are experiencing unsuccess and sometimes fear, because the horse is not performing as expected and humans do not know how to react, even if at the origin the reason is miscommunication (McGreevy and McLean 2009). The horse will be disturbed by the human's emotional state as well. Therefore, miscommunication may lead to a decline of the human-horse relationship and to poor welfare (Mcgreevy 2004). This is transversal to all activities as they all imply human-animal relationships. Regardless of the activity human-

horse relationships are important, especially in less experienced riders or handlers, whose cues are less precise. Riders and horses can, according to the findings of this PhD, benefit from the use of lateral exercises to improve their relationship. Patients involved in EAT, due to their physical and mental conditions, may experience more difficulties in providing precise cues to the horses. These horses may benefit from riding sessions with experienced riders to provide them with familiar situations, and they may benefit from the therapist guidance during therapeutic sessions as well.

Some physiological and behavioural parameters were studied in the different experiments of this PhD. This was the case for the HRV parameters and snort. HRV parameters represent modifications in the ANS activity between two situations, or two periods of time, experienced by the same horse (Bowen 2010). However, the normal range of these parameters is only defined for EAT horses (Gehrke et al. 2011). When physical activity is performed the modification of the ANS activity appears to have huge individual variability (Stucke et al. 2015), depending on the induvial response of the sympathetic nervous system, which can be influenced by many factors (e.g. emotional state, environment, physical preparation, exercise intensity, etc.). This means that the comparison between different horses, some at rest and others performing different intensity levels of physical activity, would not be accurate. Therefore, no comparison between the HRV results obtained in the different studies can be done.

Snort was used to study both negative and positive emotions in the different studies of this PhD. Snort was first considered as an indicator of negative emotions (Bachmann et al. 2003; Christensen et al. 2008; Leiner and Fendt 2011), then most recently was considered as an indicator of positive emotions (Stomp et al. 2018b). Thus, this behavioural indicator has become ambivalent and new investigations are emerging (Stomp et al. 2018a; Lesimple et al. 2019) to better understand the acoustic meaning of this behaviour. In the different studies of this PhD snort was not often observed, so no correlation between positive or negative emotions

could be done. Nevertheless, further research is still needed to understand if snort should be considered a negative or positive indicator of equine emotions.

A limitation of this PhD work is that only the ANS activity was assessed and emotional responses have implications on other physiological systems, as the hypothalamic-pituitary-adrenocortical (HPA) axis (von Lewinski et al. 2013). Many studies have investigated stress-related responses or emotional responses through the assessment of the HPA axis activity (Ayala et al. 2012; Malinowski et al. 2018). These studies used, in general, serum or plasma, salivary and faecal cortisol as the main parameters, however, cortisol concentration is variable and usually increases with exercise (Hughes et al. 2010; Ferlazzo et al. 2017; Janczarek et al. 2019). Additionally, some authors suggested that the HPA axis activity may be influenced by the hypothalamic-pituitary-thyroid (HPT) axis activity and that exercise and stress may influence both axes (Ferlazzo et al. 2017). The study of these axis through serum/plasma cortisol requires multiple blood sampling to provide interesting information, either on the animals' response to the potential stressor or the following recovery (von Lewinski et al. 2013). However, the blood sampling process is itself a stressor for horses (Peeters et al. 2011). In saliva only the free fraction of cortisol occurs, while serum/plasma samples assessment comprise both free and protein-bound cortisol is assessed (Schmidt et al. 2010). However, through the validation procedure of the salivary cortisol kit, Schmidt et al. (2010a) observed cross-reactivity from the antiserum with cortisone and several corticosterone metabolites and suggested that results concerning salivary cortisol should be interpreted carefully and cortisol immunoreactivity would be more a more appropriate designation of the results rather than cortisol concentration (von Lewinski et al. 2013). Additionally, cortisol concentrations in saliva appear to increase and come back to a baseline faster than in serum (Peeters et al. 2011), and are also influenced by exercise (Strzelec et al. 2011). Schmidt et al. (2010a) observed decreases in salivary cortisol in the 5 minutes following the stressing event (transport). Some authors suggested that increases in salivary cortisol occur during a stressing event (Contreras-Aguilar et al. 2019), while others suggested that increases in salivary cortisol would take 30

minutes after ACTH stimulation (Peeters et al. 2011). This means that different outcomes were observed in each of the different studies concerning cortisol concentrations or immunoreactivity in saliva. Therefore, the correlation between salivary cortisol and equine emotional responses is still unclear.

Faecal cortisol metabolites were assessed in 40 horses to study exercise and stress-related responses (Gorgasser et al. 2007). The authors observed interindividual variability and the concentration they have obtained differed from other studies. The circadian concentrations of faecal cortisol metabolites varied between sexes, being higher for mares than for stallions or geldings (Gorgasser et al. 2007). Additionally, the authors suggested that faecal cortisol metabolites concentration might be breed-specific or depend on environmental conditions, as housing (Gorgasser et al. 2007). Faecal cortisol metabolites increases are only observed 24 hours after a stressing event (Schmidt et al. 2010b), which means that to compare equine physiological responses, through faecal cortisol metabolites, to two different situations there is a need of at least 24 hours of interval between sessions. Additionally, cortisol metabolites concentration in faeces may increase or decrease according to feeding timing (Schmidt et al. 2010b), which means that different concentrations could be observed in different facilities due to their feeding schedules. Faecal samples should be fresh and frozen immediately (<30 minutes after defecation) (Palme 2012), which is a limitation on the field because often equine facilities are more than 30 minutes way of research facilities, making this freezing procedure impossible. Therefore, faecal cortisol metabolites are influenced by many factors and their association with emotional responses needs further research (Fureix et al. 2013; Pawluski et al. 2017).

Cortisol concentration on hair samples have been assessed as well, however these assays can only investigate chronic increases in HPA activity because they depend on hair growth that is usually 2cm/month (Duran et al. 2017). Additionally, the population of animals should be homogenised for age, sex and reproductive state and the hair should be tested to ensure

that no external contaminations could have influenced the results (Heimbürge et al. 2019). Finally, Pituitary Pars Intermedia Disease (PPID) induces increases in hair cortisol concentrations (Banse et al. 2019). PPID is a common endocrine disorder of aged (over 15 years-old) horses (Banse et al. 2019), however, subclinical horses, which means horses that have the disease but do not manifest clinical signs, were described in post-mortem exams (van der Kolk et al. 2004). This means that horses should also be tested to ACTH concentrations to ensure that the results would not be influenced by subclinical disease.

ACTH concentrations in plasma increase rapidly (5-10 minutes) with physical activity in Thoroughbred horses involved in running activities (Hada et al. 2003). Horse's heart rate increases in fearful situations, but ACTH concentrations appear to be lower when horses react actively (behavioural responses) to stressful stimuli than when they react passively (no observable behavioural response) (Budzyńska 2014). Fazio et al. (2016), described highest increases in ACTH concentrations in horses at the third day of the second week of training (dressage training) and suggested that it would be difficult to determine if these horses were "stressed" or "unstressed", which means that the relationship between ACTH increases and stress could not be determined. Additionally, ACTH responses in horses with more experience are lowest, which might be related to a reduction of the emotional impact of novel situations (Cayado et al. 2006). This means that horses populations in research experiments should be homogenised according to their experiences to investigate ACTH responses. However, horses experiences in terms of situations/conditions producing emotional responses is difficult, especially when horses live in different facilities.

Regarding the influence of exercise and circadian rhythm variations on the activity of the HPA axis, the influence of the HPT axis on the HPA axis, and the different limitations in the previously described methodologies to assess the different parameters, the decision to assess HRV in these PhD studies was made. This analysis appeared more reliable, and no stress or at least low stress would be induced by the application of this methodology because horses

are familiarised with this type of material, so the lowest influence on equine responses could be expected. In the previously discussed studies of this PhD thesis the aim was to investigate emotional responses and for that there was a need for using previously validated physiological indicators of emotional responses. Nevertheless, the investigation of the HPA parameters relationship in previously validated stressful or emotional challenging situations may be interesting as well to discern the implication of these parameters in emotional responses. Additionally, novel parameters, as oxytocin, which implications in equine emotional responses are yet under investigation might be interesting.

Oxytocin is “a mammalian peptide hormone, produced in the brain, with wide-ranging effects on numerous aspects of animal physiology and behavior, including reproduction and social interactions” (Ondrasek 2019). Oxytocin is usually assessed through plasma or cerebrospinal fluid (CSF) samples (Winslow et al. 2003; Lansade et al. 2018). Some authors suggested that plasma oxytocin concentrations do not reflect brain concentrations (Neumann 2008), while other authors have associated lower human oxytocin plasma concentrations with attachment and positive emotions (Jobst et al. 2014) and higher human oxytocin concentration with stress and fear (Onaka et al. 2012). Oliva et al. (2019) results suggest an association between a situation in which dogs were less stressed/aroused and lower plasma oxytocin concentrations. Additionally, Lansade et al. (2018) suggested that horses in a general state of well-being would have lower plasma concentration of oxytocin as well. These results suggest that oxytocin might be an interesting biomarker for the assessment of positive emotions in horses. Therefore, a new investigation on oxytocin implication in equine emotional states and emotional responses was designed. The fifth study of this PhD have produced the first results of this line of investigation.

The fifth study, entitled “Investigating the oxytocinergic system: plasma measures of free VS total oxytocin fractions in two animal models”, intended to apply the newly developed method (Brandtzaeg et al. 2016) assaying the total oxytocin fraction to equine samples and

investigate the correlation of the two fractions of oxytocin in equine and dog plasmas. As in horses, no correlation was observed, the comparison of both free and total oxytocin between sport horses and therapeutic horses and between genders was performed. The results of this study suggest that free plasma oxytocin may vary according to the activities (sport or therapy), i.e. a non-physiological or “environmental/social” condition, while total oxytocin variations would be more related to the gender of the animal, i.e. a physiological or “innate” condition. These results are interesting because they suggest that free and total oxytocin may be interesting indicators with different meanings for the study of emotional responses of horses. However, further research is needed to clarify their respective biological meaningfulness.

While in this investigation attention was centred in oxytocin, other biomarkers appear to be interesting for the study of equine emotional responses. Serotonin low plasma concentrations were associated with stress and aggressiveness (Westergaard et al. 2003; Ayala et al. 2012) while higher concentrations were associated with stabilisation of the HPA axis activity (Bush et al. 2003). However, there are many factors to take into account for the investigation of serotonin implications in equine emotional responses, as the age (Ferlazzo et al. 2012), circadian rhythm (Bruschetta et al. 2013), social relationships and maternal care (Bruschetta et al. 2017) and physical activity as well (Alberghina et al. 2010b; Bruschetta et al. 2014). Nevertheless, serotonin implications in equine emotional responses are still unclear and new researches are needed, e.g. the influence of gender in serotonin plasma concentrations or the diet effects as well. These researches are needed to disclose all the possible factors influencing in serotonin plasma concentrations and to ensure that experimental designs are created to study modifications on these concentrations due to emotional responses and not to any external factor.

The different studies of this PhD included some of the activities that humans impose to horses and were carried out in two branches of the equine industry to which the human-animal relationship is fundamental (sports, working/therapeutic animals). Regardless of the activity

that humans impose to horses, in the different studies of this PhD, these animals appeared to be highly tolerant and to create adaptive coping strategies. These strategies varied between horses who were involved in different activities.

This research provides original information about horses' perception of different situations. These findings are important because they have demonstrated an influence of the activity horses perform in their behavioural and physiological responses, which means that different emotional responses may be expected in horses involved in different activities. This does not mean that horses would rather prefer to perform one specific activity above the others, but that their strategies to cope with different situations/contexts differ, perhaps due to their learning processes and what reactions are accepted or not by humans in each activity. However, according to the results obtained in these studies negative emotional responses might be managed with exercises that horses already perform (e.g. shoulder-in), by using them in potential stressful situations. This is interesting because these observations allowed to conclude that some exercises already performed in horses' routines and activities might be the solution for negative emotional responses created in the context of these activities. Positive outcomes of different activities could not be observed, possibly because the positive indicators of equine emotional responses used in these studies are ambivalent. Nevertheless, improvements of equine emotional responses (behavioural and HRV responses) were observed in different situations of the different studies (with lateral exercises in the second study; with different groups of patients in the third study). These results suggest that the tools (exercises or contexts) to improve equine welfare are already available, though, their interest is probably unknown. Finally, behavioural indicators alone should not be considered to assess equine emotional responses, because horses that tended to be more reactive in the different studies had lower modifications in HRV and horses that were apparently calmer had higher modifications in HRV. Therefore, horses that may appear to cope well with a task may be more emotionally challenged than horses who refuse to perform it.

These findings disclosed some of the influences that the activities imposed by humans have on equine emotional responses and welfare. Additionally, this work provides information on some aspects that can be used or improved to achieve positive welfare conditions within the equine industry. Finally, regarding ethical concerns, in this work, equine activities were not associated with negative welfare, which means that the use of these animals by humans has the potential to create a mutual benefit for the two species if positive situations/conditions for are created for both. By identifying the stimuli to which horses show more difficulties to cope with or which exercises can be used to reduce stress-related responses opened new perspectives. Further research is needed to increase the scientific knowledge about the different activities that are imposed to horses and most importantly to understand how equine welfare can be improved in the different activities. The aim of novel investigations should be the disclosure of solutions to improve equine welfare. New research on how to turn working/therapeutic activities into positive situations using equitation exercises or physical therapy would be interesting. Comparing horses trained with positive reinforcement techniques with horses that receive classic training with negative reinforcement when they face a potential stressful situation/context, as work, therapies or competition would be interesting as well. Some lines of investigation or new ideas are proposed in the perspectives section.

OVERALL CONCLUSIONS AND PERSPECTIVES

Conclusions

This work produced new findings with different implications. The main conclusions of this research are the following:

1. The investigation of emotional responses in horses is a complex process that requires multidisciplinary approaches.
    a. Emotional responses in horses are not always associated with behavioural responses. Thus, their study should be performed by investigating both behavioural and physiological parameters.
    b. Heart rate variability is a non-invasive tool that allows the study of the autonomic nervous system. The analysis of heart rate variability is suitable for the investigation of both negative and positive welfare conditions, though this methodology should be combined with a behavioural assessment.
    c. Behavioural indicators of negative or positive welfare should be interpreted carefully, as some behaviours (e.g. snort, head lateral movement, looking around) appear to occur both in negative and positive welfare conditions. These indicators in equine species need further research.
    d. Plasma concentrations of free and total oxytocin implications in equine behaviour and emotional responses require further research as this investigation disclosed the absence of correlation between these parameters. The two parameters may be indicators of different conditions, that still need to be clarified, and may probably be interesting as welfare indicators.

2. The activities imposed by humans modify equine emotional responses. This modification can produce negative, positive or no outcomes. Emotional responses were studied in this work and the conclusions were the following:
    a. The modification of these responses is expressed by behavioural and/or physiological responses and differ from horses who are involved in different activities.
    b. In training, humans influence on horses' emotional responses can produce positive outcomes thanks to lateral exercises. These exercises are particularly beneficial to horses ridden by unexperienced horseback riders.
    c. The state of positive welfare should not be assumed when negative or poor welfare was ruled out. Equine-assisted therapy horses in this work were neither in a negative nor a positive welfare situation.
    d. Most of the activities that humans impose to horses need these animals to learn not to respond to environmental stimuli. The absence of response or reactivity to those stimuli does not mean that no emotional response is produced. Chilean urban working horses, who have learned not to respond to different stimuli, performed correctly a bridge test, though their physiological responses described an important emotional activation.

3. Ethical concerns about equine use from humans were approached throughout this research. Ethics is a subjective discipline; however, some legislation is created based on ethics. This means that scientific research should help on the decision whether an activity is acceptable or not before the creation of new legislation.
    a. This work provides information on the perception of equines of the activities that humans impose to them.
    b. The different studies of this work were made under optimal conditions with professionals from the different branches of the equine industry. The

absence of negative welfare conditions observed in the present studies does not mean that no negative welfare conditions can occur. Nevertheless, these findings mean that positive welfare conditions can be achieved by possibly improving the way human use horses so that the use of these animals may benefit both species.

Perspectives

This work disclosed new perspectives concerning equine welfare and the use of equines at sports, work and therapies by humans.

New lines of research, to disclose novel welfare indicators of positive and negative welfare, are needed. Studies on the different parameters (e.g. oxytocin or serotonin) suggested that these are promising welfare indicators with different valences. New investigations on the influence of social contact, intra- and interspecific, in plasma oxytocin or salivary concentrations would be interesting. The assessment of oxytocin concentration in horses presented to behavioural consultation for aggressiveness at the time of the consultation, during and after behavioural modification therapies would be interesting as well.

Clinical studies comparing plasma/serum or salivary serotonin concentrations in mentally health horses with horses presented to behavioural consultation for impulsive/aggressive behaviours would produce interesting information on how behavioural problems modify this biomarker. The follow-up of these cases with the assessment of serotonin concentration would produce novel information on whether behavioural modification therapy modifies this biomarker.

The use of lateral exercises improved equine emotional responses in the present study, though these horses were only included in the experiments when no behavioural problems were diagnosed or described by the handlers, trainers or riders. Thus, the use of these exercises in horses displaying behavioural problems as part of a behavioural modification

therapy would be an interesting subject of investigation. Additionally, the improvement of equine welfare using positive reinforcement in equine training, especially in equine-assisted therapy activities, would be an interesting subject of investigation.

Horse riders usually describe a side preference on their horses. Laterality may have an impact on equine emotional responses, as horses are asked to equally perform on both sides. This represents a challenge concerning the physical aspects and many studies have described this phenomenon. However, little information exists on the influence of laterality in equine emotions. An investigation on equine emotional responses comparing training on the preferred side with training in the opposite side would be interesting.

Equines are trained with varied methodologies. Some equine trainers focus their training on equine biomechanics aspects while others focus their training on exercises that will be performed in competitions. There is no information on whether on method or the other is more performant or has impact on equine emotional responses and welfare. Therefore, new investigations comparing the two methods, using previously validated emotional biomarkers (HRV), pain-related scales and performance scales would be interesting.

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ANNEX

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Working Smarter Not Harder: Oxytocin Increases Domestic Dogs' (*Canis familiaris*) Accuracy, but Not Attempts, on an Object Choice Task

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The neuropeptide oxytocin (OT) has been shown to enhance dogs' ability to perform an object choice task (OCT) involving the use of human pointing cues, when delivered intranasally. This study aimed at further investigating whether OT enhances task performance by increasing choices made, or by increasing correctness of choices made, and to compare these treatment effects to dog appeasing pheromone (DAP), known to balance emotional activation in dogs. Hence, we compared OCT performance between three groups of dogs: (i) dogs administered OT and a sham collar, (ii) dogs administered a saline placebo and a DAP collar, and (iii) control dogs administered a saline placebo and a sham collar. All three groups consisted of a combination of male and female pet dogs and assistance-dogs-in-training currently living with a volunteer carer. The study also evaluated the effect of intranasal OT and/or DAP on plasma levels of OT, and prolactin; which has previously been linked with anxiety in dogs. The dogs' emotional state was measured using the Emotional Disorders Evaluation in Dogs (EDED) scale. The owners'/carers' degree of anxious- and avoidant-style attachment to their dogs was accessed using the Pet Attachment Questionnaire (PAQ). Interesting descriptive data appeared for both treatment groups. Particularly, in OT group, we obtained significant results demonstrating that intranasal OT enhances OCT performance in dogs compared to control, by increasing the percentage of correct choices, but not the number of choices, made. Results also support that the mode of action of intranasal OT is via direct access to the brain and not via the blood, since no elevation of plasma OT (or prolactin) levels were observed after intranasal administration in this study. Similarly, DAP application did not significantly alter OT or prolactin peripheral concentrations. Several differences were observed between fostered and pet dogs, namely: fostered dogs demonstrated higher levels of serum prolactin, made more choices on the OCT compared to pet dogs but were not more likely to be correct, and were fostered by carers with higher avoidant attachment scores than pet dog owners. These findings implicate consideration of potential carer and training consequences for assistance dogs.

Keywords: oxytocin, DAP, dog, attachment, object choice, pheromone, cognition, OCT

INTRODUCTION

Dogs have remarkable abilities to communicate with humans (Hare and Tomasello, 2005), and we now specifically breed dogs for the unique purpose of placing them in working roles that assist humans. Nowadays, dogs are used by humans for various working roles, including assisting, guiding, herding, detecting, racing, and guarding (Cobb et al., 2015). The ability of dogs to successfully work in these roles is dependent on a wide variety of factors depending on the job, but for assistance (guiding, hearing, and service) dogs (for definition see, Bremhorst et al., 2018), this includes their ability to cope in stressful situations, and to form affiliative bonds with their human counterparts. This is no small feat as it is often necessary for these dogs to forge several human relationships over their lifetime, each time detaching from the previous to allow the new relationship to form. If they turn out to be mismatched to their handler, they can be re-homed up to eight times (Lloyd et al., 2016) and must detach and attach every time. Cobb et al. (2015) has estimated the current rate of success across a variety of trained working dogs to be only around 50%. The total cost of a guide dog's 8-year working life has been estimated to be US$40,598 with the majority of these costs (US$34,972) incurred by the training school during the dog's first year of life (Wirth and Rein, 2008). Hence, research to close this gap between steep costs and low success rates is clearly warranted.

Dog-human attachment should not be considered in one direction only – the ability of humans to attach to a working dog is also very important to consider. Following the work of Ainsworth et al. (1978) who classified human infants as securely, anxiously or avoidantly attached to their primary caregiver, Zilcha-Mano et al. (2011) found that these styles of attachment can also be applied to the way in which adult owners attach to their pets. The fact that assistance dogs are raised by volunteer carers who inevitably must give them back to the assistance dog association in which they were born may cause carers to form more insecure (anxious and/or avoidant) attachments with the dogs they care for, compared to companion dog owners. Avoidant adult attachment styles have been associated with owning dogs with separation-related disorders (Konok et al., 2015), and this could be quite problematic in a foster care situation where there would be an even greater need for the carer to guard against an inevitable loss. While previous work by Mariti et al. (2013) showed that there was no difference in behaviors indicating an attachment bond between pets and search-and-rescue dogs who live with their "handlers," these differences have not been investigated in pets versus fostered dogs.

Insecure attachments have been shown to impact dogs' ability to use human social gestures. For example, Oliva et al. (2016a) showed that owners who scored high for anxious attachments to their dogs, according to the Pet Attachment Questionnaire (PAQ) (Zilcha-Mano et al., 2011), owned dogs that were more likely to perform poorer on an object choice task (OCT) in which pointing cues were used to indicate the location of a hidden food reward. For dogs whose job requires the use of human pointing gestures or the ability to read human non-verbal communication in general, this could be a critical problem. Interestingly, this relationship disappeared following the intranasal administration of synthetic oxytocin (OT) (Oliva et al., 2016a). Oxytocin is known for its role in mammalian bonding (for a review see, Lim and Young, 2006) and social cognition in humans (for a review see, Bartz et al., 2011). In dogs, intranasally applied OT has been shown to increase positive expectations (Kis et al., 2015), decrease friendliness in response to a threatening person (Hernádi et al., 2015), increase affiliation toward owners and conspecifics (Romero et al., 2014), increase play behaviors toward conspecifics (Romero et al., 2015) and increase mutual gaze with their owners (Nagasawa et al., 2015). It has also been found to enhance performance on OCTs (Oliva et al., 2015; Macchitella et al., 2017). It remains unknown exactly how and where OT exerts its OCT-enhancing effects. With regards to "how," two possibilities exist. The first is that OT *directly* improves social cognitive function, and the second is that it *indirectly* enhances cognitive function by modulating emotions which may be inhibiting social cognition. Indeed, in humans, OT has been shown to have anxiolytic properties (Heinrichs et al., 2003; de Oliveira et al., 2012). With regards to "where," it has long been believed that intranasally administered peptides gain access to the brain as increased levels of the peptide can be measured in cerebrospinal fluid (CSF) following administration (Born et al., 2002; Gossen et al., 2012; Neumann et al., 2013; Striepens et al., 2013; Dal Monte et al., 2014; Freeman et al., 2016; Rault, 2016; Lee et al., 2018). However, as discussed in their review, Leng and Ludwig (2016) explain that the increases in CSF are only modest in comparison to the amount administered. However, the authors also acknowledge that central OT may be largely degraded in brain tissue and therefore only enter the CSF in minimal amounts. Nevertheless, the relatively large rise in peripheral measures that sometimes follows intranasal application has cast doubt in these researchers' minds that the administered peptide is primarily acting at the level of the brain (Leng and Ludwig, 2016). Furthermore, it has recently been suggested that the neuro-behavioral effects of intranasally administered OT may stem from a peripheral mechanism of action, following results from a study by Lee et al. (2018) which demonstrated highly variable timings and extents that both intranasally and intravenously administered OT reached the CSF of monkeys. Temesi et al. (2017) and Romero et al. (2014) also demonstrated an increase in OT measured in dog blood following intranasal application, however, as these studies used varying methods, doses, and small sample sizes, the current study will extend upon these findings in a larger sample of dogs using recently validated methods (Bienboire-Frosini et al., 2017; MacLean et al., 2017), as stated hereunder in the "Materials and Methods" section.

Unfortunately, central OT function is difficult to measure in a minimally invasive way. Indeed, OT is produced in the hypothalamus and released from magnocellular neurons that project to the posterior lobe of the pituitary where it is naturally secreted into the bloodstream and acts as a hormone (Lim and Young, 2006). Oxytocin is also directly secreted into central brain regions from hypothalamic magnocellular neurons (Knobloch et al., 2012) which also release the peptide

into the CSF from their somas and dendrites, where it has the potential to act as a kind of slower-acting and longer-lasting "hormone" in the central nervous system (Ludwig and Leng, 2006). Extrapolating from peripheral measures is problematic because the central and peripheral release of OT from magnocellular neurons of the hypothalamus are not necessarily time-locked; OT can be released independently at different neuronal sites, in response to the same stimulus. Indeed, there are reports of dendritic release being delayed by more than 1 h, compared to the more immediate release at the terminal bouton, and exerting its effects for much longer (Ludwig and Leng, 2006). Furthermore, certain social stressors in rats have been shown to induce central, but not peripheral secretion (Engelmann et al., 1999). Conversely, studies in guinea pigs show that peripheral OT increases in response to suckling, without an accompanying increase in the CSF [measured by immunoassay with prior solid phase extraction (Amico et al., 1990) and High-Performance Liquid Chromatography (Robinson and Jones, 1982)]. As neuropeptides do not readily pass the blood–brain barrier (Vorherr et al., 1968; Mens et al., 1983; Robinson, 1983; Veening et al., 2010), it follows that levels in the brain and in the blood may be substantially different at any one time.

Thus, while extrapolating from peripheral measures to draw conclusions about real-time central OT function is dubious, peripheral levels of the peptide may be a possible reflection of the overall functioning of the oxytocinergic system of the organism. For example, in humans, plasma OT is positively associated with number of attachment figures (Jobst et al., 2014) and tendency to be emotionally open (Tops et al., 2007). For this reason, we are interested in investigating the association between plasma levels of OT and OCT performance in dogs, and the influence of intranasally administered OT, in the current study. We are also interested in measuring serum prolactin, firstly because its release can be stimulated by OT, for example in response to suckling, mating and ovarian steroids (Kennett and McKee, 2012), but also because it is released in response to situational stressors in humans (Jeffcoate et al., 1986; Armario et al., 1996) and rats (Torner and Neumann, 2002; Torner et al., 2004). In addition, serum prolactin has been associated with scores on the Emotional Disorders Evaluation in Dogs (EDED; Pageat, 1995) scale in a sample of anxious dogs (Pageat et al., 2007). The EDED assesses behaviors and physiologies of dogs that are modified by emotional disorders and scores them according to their severity.

An alternative method to potentially activate central OT is via the administration of pheromones believed to involve the release of OT in the brains of mammals (see reviews, Bielsky and Young, 2004; Wacker and Ludwig, 2012). In dogs, a synthetic analog of the natural maternal appeasing pheromone, known as dog appeasing pheromone (DAP) (ADAPTIL®; Ceva Santé Animale), which is naturally released by bitches to appease their young, has been identified. Processing of pheromones occurs within the medial amygdala (Salazar and Sànchez Quintero, 2009) and is likely to integrate information coming from both the main olfactory bulb and the accessory olfactory bulb, where OT receptors have been reported (Wacker and Ludwig, 2012). Behavioral effects of DAP are not dissimilar to the behavioral effects following intranasal OT administration. For example, studies have shown that DAP reduces behavioral indicators of stress in a shelter (Tod et al., 2005), owner-reported fear of fireworks (Sheppard and Mills, 2003) and fear and anxiety scores in response to a thunderstorm recording (Landsberg et al., 2015). It also alleviates behavioral and neuroendocrine perioperative stress responses (Siracusa et al., 2010) and increases relaxation and reduces anxiety (but not aggression) in a veterinary clinic (Mills et al., 2006). It has also been shown to reduce stress-related behaviors (Gaultier et al., 2008) and fear of novel people (Gaultier et al., 2009) in recently adopted puppies. Furthermore, there is evidence that DAP reduces undesirable behaviors related to dogs' separation from their owners, with a similar efficacy as using the psychotropic drug, clomipramine (Gaultier et al., 2005). In addition, it reduces separation-related anxiety signs during hospitalization (Kim et al., 2010), and reduces fear and anxiety in puppies during training and enhances socialization up to 1 year later (Denenberg and Landsberg, 2008). There are currently no known studies of the efficacy of DAP in enhancing "following" behaviors in response to human social cues or blood measures of OT or prolactin related to DAP use.

If both DAP and intranasally administered OT lead to increased levels of OT in the brain, exposure to both DAP and OT intranasal administration could have similar effects on consequent measures of blood neuro-hormone levels, and possibly on OCT performance. Hence, the aim of this study was to (i) compare the enhancing effects of DAP and intranasally administered OT on plasma levels of OT, and prolactin, and dogs' OCT performance, against control and (ii) to explore the predictive power of: dog gender (as Oliva et al. (2015) demonstrated that male dogs perform better on the OCT after saline, but females respond more to the oxytocin intranasal treatment), origin (pet or foster dog), dog weight, home location (inside/outside – ambient temperature is known to influence levels of prolactin in the body, which may have flow on effects for the individual (see review, Alamer, 2011), point-following ability (spontaneous/non-spontaneous – refer to Materials and Methods section), plasma OT levels, serum prolactin levels, owner avoidant attachment scores, owner anxious attachment scores, EDED scores, and treatment, on OCT performance. Finally, the study aimed to identify differences in pet owner versus puppy carer attachment, as well as pet versus foster dog prolactin levels. It was hypothesized that, (i) plasma levels of OT will increase and serum levels of prolactin will decrease following DAP and OT exposure and that OCT performance will be enhanced by both OT and DAP compared to placebo. We also expected (ii) higher levels of plasma OT to be present in better performing dogs and better performing dogs to be more likely to be male and to be pets, while higher EDED scores and levels of prolactin to be present in poorer performing dogs. Lastly, we expected that foster owners would demonstrate greater avoidant attachment toward their dogs compared to pet owners and that foster dogs would have higher levels of serum prolactin compared to pet dogs.

MATERIALS AND METHODS

Animals

A sample of 51 dogs and their owners or volunteer carers were recruited for the study. All dogs were more than 10 months old and recruited from either the assistance dogs association, Frédéric Gaillanne Foundation (FGF), or from owners that heard about the study via word of mouth, poster advertisements in pet shops, groomers or on the social media website, Facebook. Dogs who, at physical examination, were found to be pregnant, lactating, sensory impaired, or less than 2 kg were not included in the study. All entire females were tested on the OCT at least 2 months out of estrus. All dogs recruited from the FGF were in training and still living part time with the same human carer who had been assisting the FGF to raise them since they were between 8 and 10 weeks old. Henceforth these dogs shall be referred to as "foster dogs" and their volunteer carers as "puppy carers." Dogs were to be excluded from the study if they showed signs of physical illness or extreme social phobia or aggression. The assessment of the dogs' emotional status according to the EDED (refer to Materials section) scale did not reveal any emotional disorders which could have resulted in a non-inclusion. All dogs fell into the "normal" range, except for two pet dogs allocated to the DAP treatment group who fell into the "phobic" range. One foster dog was found to be pregnant at examination and was thus not included in the study. Two pet dogs displayed aggression in session 1 and were thus excluded. Two additional foster dogs changed puppy carers between sessions and were also excluded, thereby leaving a final sample of 46 dogs, 25 pets [17 Males (4 entire), 8 Females (2 entire)] and 21 foster dogs [9 Males (0 entire), 12 Females (2 entire)]. **Tables 1** and **2** show details of the population involved in the study. The study was approved by the IRSEA Ethics Committee, approval number AFCE_201605_02.

Materials

Twenty-four international units of OT (Sigma, St Quentin Fallavier, France) diluted in 0.2 ml of 0.09% saline, or 0.2 ml of 0.09% saline only (acting as a control) were administered to the nostrils of each dog, with a half-dose in each nostril. Treatments were delivered using a Mucosal Atomizer Device (Teleflex Medical SAS, La Pousaraque, France) connected to a 1 mL syringe. Treatments were prepared by a team member who did not take part in the experimental testing and were labeled as "A" or "B" to ensure that the researchers involved in the experiment were "blind" to the treatment conditions.

Adaptil® collars impregnated with DAP or identical collars without impregnated DAP (placebo) provided by Ceva Santé Animale (Libourne, France), were fitted to the dogs' neck in order to continuously expose dogs to DAP or placebo. Dogs were wearing the collar at least 1 day (24 h) before the OCT. Collars were also labeled as "A" or "B" by the same team member who did not take part in the experimental testing to ensure that the researchers involved in the experiment were "blind" to the treatment conditions. According to the combination of double-blinded intranasal and collar treatments that the dog received, it belonged to a treatment group called "A," "B" or "C," which the experimenters were completely blinded to as well, until after the data analysis was complete.

Two identical, opaque spaniel plastic bowls (19 cm base diameter, 11 cm rim diameter, 12 cm high, 8 cm deep) were used to conceal the food treats. Spaniel bowls were selected for their height and ability to conceal the treat from the dogs' vision. Two additional and identical spaniel bowls were placed underneath the two testing bowls and treats identical to those used in the experiment were hidden in the space between them. This method was used by Udell et al. (2008a) and Oliva et al. (2015) to ensure that both bowls smelled of the treats and the dog was consequently not able to rely on olfaction when making its choice between the bowls. The treats used were pieces of poultry and vegetable flavored Frolic brand dry treats. Scores were marked by the experimenter using pen and paper.

The PAQ (Zilcha-Mano et al., 2011) and EDED scale (Pageat, 1995) were completed using pen and paper. Dogs who obtain an EDED score between 9 and 13 are classified as having a "normal emotional state," dogs with a score of 14–16 are considered "phobic" and dogs scoring between 17 and 35 are considered "anxious." Dogs who receive a score beyond 35 are classified as having a thymic or mood disorder (Pageat, 1995; Pageat and Fatjó, 2013).

Procedure

The temporal unfolding of the overall procedure, comprising four steps (phone interview, session 1, session 2, and session 3), is

TABLE 1 | Study population descriptive data separated by treatment allocation group and origin.

	Origin		Age (years)		Gender		Point-following ability		Location			Weight (kg)		Time owned/in care (years)	
	Pets	Fostered	*M*	SD	Male	Female	Spontaneous	Non-spontaneous	Inside	Outside	Both	*M*	SD	*M*	SD
Oxytocin	8	6	3.5	2.9	8	6	5	9	6	2	6	25.9	10	2.9	2.3
Placebo	8	8	3.1	2.9	9	7	5	11	7	2	7	25.9	12.2	2.3	2.7
DAP	9	7	2.3	2.3	9	7	5	11	5	3	8	27.6	6.4	1.6	1.5
Pets	–	–	4.9	3.0	17	8	8	17	10	4	11	22.8	11.7	3.8	2.8
Fostered	–	–	1.1	0.2	9	12	7	14	8	3	10	30.9	3.7	0.9	0.1

M = mean, SD = standard deviation.

TABLE 2 | Breed differences between pet versus fostered dogs.

Origin of dogs			
Pet dogs		**Fostered dogs**	
Breed	***N***	**Breed**	***N***
Mixed	3	Bernese Mountain dog × Labrador (St Pierre)	14
Border Collie	3	Labrador	6
Welsh Corgi Cardigan	2	Labrador × Golden Retriever	1
Australian Shepherd	2		
German Shepherd	2		
Labrador	2		
Border Collie Cross	1		
Australian Shepherd × German Shepherd	1		
Bernese Mountain Dog	1		
Labrador × Boxer	1		
Malinois	1		
Samoyed	1		
Poodle	1		
Yorkshire Terrier	1		
Westie	1		
Chihuahua Cross	1		
Pinscher × Chihuahua	1		

N = sample size.

described in **Table 3**. The different tasks described within the steps' procedure are detailed hereunder.

Stratification Test

The dogs participated in a "stratification test" to determine their classification as "spontaneous point followers" or "non-spontaneous point followers," which would then inform their semi-random allocation to a particular treatment group. The setup of the stratification test consisted of three spots in a triangle shape on the floor, marked by small pieces of blue tape, 145 cm apart. The owner/puppy carer was asked to stand in the center of the triangle, connected to their dog by a leash. The owner/carer then randomly pointed five times at the spots, ensuring that each spot was pointed at once, without pointing at the same spot two times in a row. The behavioral veterinarian recorded how many times the dog correctly approached the pointed at spot. Dogs that followed their owner's/puppy carer's points correctly four or five times, out of the possible five were classified as "spontaneous point followers" while dogs that followed their owner's/carer's points three times or less were classified as "non-spontaneous point followers."

Following the stratification test, and according to their classification (spontaneous vs. non-spontaneous), their origin (fostered dog vs. pet) and their sex, they were then allocated into one of three treatment groups (DAP active and placebo = DAP, DAP sham and placebo = control/placebo, DAP sham and OT = OT) in a pseudo-randomized and counterbalanced way. Point-following ability was included in the counterbalancing of treatment groups so as not to have one group containing dogs with more of a "spontaneous" point-following ability. This was decided in line with previous reports of dogs exhibiting a wide range of individual variability of OCTs.

Blood Drawing

Blood was taken in sessions 2 and 3 for subsequent measurements of basal and "treatment-induced" OT and prolactin levels respectively. Owners were asked to keep their dogs indoors the night before both sessions 2 and 3 to minimize the effect of ambient temperature on neurohormone secretion. Blood was drawn by a veterinarian from the jugular vein or from the cephalic vein, depending on the preference of the veterinarian for the dog, with the help of an operator. Most of the time, the

TABLE 3 | Temporal unfolding of the procedure and actions performed in each of its steps.

Procedure steps	Time lapse	Actions
Phone interview	D0	Phone contact with the dog owners/carers to check the inclusion criteria
		Appointment scheduling for session 1
Session 1 (S1)	D1	Physical examination at the Clinical Ethology and Animal Welfare Centre (CECBA) to confirm dog inclusion
		Explanatory statement and consent form signing
		EDED scale completion
		Stratification test
		Allocation in one treatment group
Session 2 (S2)	D2 = D1 or any day in between until at least 1 day before D3 [range of +1 to +107 days (*M* = 16.67, SD = 19.10)]	Blood drawing
		24–48 h before S3, fitting dogs with the collar (follow-up phone calls were made to ensure that this was done at the correct time)
Session 3 (S3)	D3 = D1 + 47 days on average [range of +1 to +232 days (*M* = 47.17, SD = 48.64)]	Food deprivation 6–8 h before the OCT to enhance motivation toward the treats
		Intranasal administration of one of the treatments (saline or OT)
		Blood drawing 15 min after treatment administration
		PAQ completion, dog free to explore the testing room
		OCT beginning 45 min after treatment administration: warm-up phase, testing sessions

D = days.

dog was taken to a nearby room so that this was performed in the absence of the owner/puppy carer. Dogs received a treat immediately afterward in order to reduce stress responses. Up to 8 ml of blood was collected into pre-chilled pink EDTA-Aprotinin vacuum tubes (BD® tubes, Elvetec, Pusignan, France) and 1 ml into red vacuum tubes with gel separator (Vacuette® Greiner Bio-One, Alcyon, Paris, France), using either a 25G, 23G or 21G needles, depending on the size of the dog. The pink tubes were immediately transferred to an ice compartment where they remained until centrifugation while red tubes remained at room temperature for between 30 and 180 min. Samples were centrifuged at 1,600 × *g* for 15 min at 4°C. The plasma or serum was then transferred to a plastic tube and stored at −20°C.

Object Choice Task

Warm-up phase

The dog was first introduced to the bowls by the experimenter, which each contained one treat. The experimental set-up was similar to that of Virányi et al. (2008) and Oliva et al. (2015). The two spaniel bowls were placed 1.5 m apart and the experimenter knelt 30 cm behind the mid-point between the bowls. The dog sat or stood in between its owner and an operator, restrained by the operator by its collar, and faced the experimenter at a distance of 2.5 m. The owner/puppy carer, although present in the room at all times, did not participate in the task in any way, except in some cases to initiate participation in the task, as described in the scoring section below. The experimenter first got the dog's attention by calling its name. The dog was then shown a treat before it was placed in one of the bowls in the dog's full vision. The experimenter then said the release word "va" (the French word for "go," or something equivalent if it was more familiar to the dog). The operator then released the dog and allowed it to approach one of the food bowls. If the dog approached the bowl containing the treat, it was allowed to eat the treat before both bowls were collected by the experimenter; if the dog approached the empty bowl or the experimenter, they immediately collected both bowls and the dog did not receive a treat. The warm-up phase consisted of four trials regardless of the dog's performance, so long as the dog chose a bowl at least two out of the four trials. In cases where the dog did not choose a bowl at least two out of the four trials, the warm-up phase continued until two choices were made, or the dog was excluded. Dogs were excluded after 10 min failing to make two choices. After each trial, the experimenter stood up with both bowls and walked to the side of the room which was blocked from the dog's vision by a barrier where they took another treat in their hand, while the operator fetched the dog and brought it back to the starting position. Once the dog was at the starting position, the experimenter returned and placed the bowls in their position on the floor and started the next trial.

Testing session

The experimental set-up was the same as in the warm-up phase. The testing session comprised two blocks of fifteen trials (10 where a pointing cue was provided and five control trials in which no cue to the treat's whereabouts was provided). The control condition was used to verify that the dogs were not relying on scent to find the hidden food. Numerous studies have found that performance is at chance level (Hare et al., 2002; Soproni et al., 2002; Riedel et al., 2008; Udell et al., 2008a; Wobber et al., 2009; Macchitella et al., 2017), or below chance level (Oliva et al., 2015) when a control condition is employed. Each block comprised, in sequence: three control trials, five trials with a momentary distal pointing cue, two control trials and then another five trials with a momentary distal pointing cue, in accordance with the sequence in Oliva et al. (2015). Having only 15 trials per block was strategically designed to keep the dog motivated. Furthermore, the dog was allowed approximately 5 min break between testing blocks to avoid burnout. Position of the correct bowl (left or right) was predetermined according to the same pseudo-randomized chart used in Oliva et al. (2015) that did not allow more than two consecutive trials where food could be obtained on the same side. As in the warm-up phase, after each trial, the experimenter stood up with both bowls and walked to the side of the room blocked from the dog's vision by a barrier where they baited one of the bowls, while the operator fetched the dog and brought him/her back to the starting position. Once the dog was at the starting position, the experimenter returned and placed the bowls in their position on the floor and started the next trial.

Momentary distal point cue

The experimenter was kneeling, propped up on their toes, with their arms by their side. They got the dog's attention and then rose their ipsilateral arm and pointed (using their index finger) toward the correct bowl for 1–2 s, keeping their head straight, before lowering their arm back down to their side and saying "va" (or an alternative release word more familiar to the dog). The approximate distance between the experimenter's index finger and the rim of the baited bowl was 42 and 50 cm to the treat inside. The dog was then released and allowed to make a choice between the bowls.

Control condition

The kneeling experimenter, propped up on their toes, got the dog's attention, kept their head straight for 1–2 s, then said "va" (or an alternative release word) before the dog was released by the operator and allowed to make a choice in the absence of any cue.

Scoring of the OCT

Scores were recorded as correct responses out of 10 trials per block (20 per test). If the dog did not move within 5 s of being released, the cue was given again by the experimenter. The dog may also have been prompted once by the owner if instructed to do so by the operator. If the dog did not approach a bowl within 5 s, the score for that trial was "no choice." "No choices" were also recorded if the dog approached the experimenter instead of a bowl.

Hormone Analysis

Prolactin was assayed in serum using the Prolactin canine ELISA kit (Demeditec, Kiel, Germany) following the manufacturer's instructions. Plasma OT was assayed in the Oxytocin ELISA kit from Cayman Chemical (Arbor Inn, MA, United States) according to the manufacturer recommendations, including the plasma solid-phase extraction. Szeto et al. (2011), McCullough et al. (2013), and Christensen et al. (2014) have highlighted the

crucial importance of carrying out this step, due to the fact that studies which have been published using unextracted samples have yielded levels far higher than extracted samples, leading to discrepancies in findings. Because of OT levels inferior to the kit's sensitivity and in accordance with the manufacturer's suggestion, 1.2 ml of plasma was first extracted on C18 columns (Hypersep 1 g, Thermo Fisher Scientific, Illkirch, France) followed by elution in 98% acetone. After drying the samples by vacuum centrifugation, they were resuspended in 0.6 ml assay buffer, hence allowing a two times concentration factor. The available plasma volumes of four samples in sessions 2 and 6 samples in session 3 turned out to be too small to be assayed using this method. The global procedure of extraction/concentration and assay was first internally validated on seven Quality Control samples of dog plasma from an external study to assess the precision and the accuracy of the whole method through the kit's dynamic range: the mean precision was 12.0% and the mean recovery was 98.3%. Of note, some of the authors have recently published a validation of another Oxytocin ELISA kit from Enzo Life Sciences (Bienboire-Frosini et al., 2017) to assay OT in dog's plasma. This kit was initially used in this study but unfortunately lead to too many results under the limit of detection. Therefore, we decided to use the Oxytocin ELISA kit from Cayman Chemical with a lower limit of detection, such as in MacLean et al. (2017).

Statistical Analysis

Data analysis was realized thanks to SAS 9.4 software Copyright (©) 2002–2012 by SAS Institute Inc., Cary, NC, United States. Bilateral situation; the significance threshold was classically fixed at 5%.

Object Choice Task Performance

For the control conditions, correct attempts were totalled across the two blocks to give a score out of a possible 10 for each dog. Number of attempts made to the dog's left bowl and the dog's right bowl were also calculated. For the cued conditions, correct attempts for each of the testing blocks were combined to give a total raw score out of 20 for each dog, as well as a score for how many choices the dog made out of a possible 20. Then, a percentage was calculated for each dog as to the number of correct choices out of the total choices made. The same was done for the control conditions, whereby correct attempts for each block were combined to give a total raw score out of 10 for each dog. The data set was visually inspected for missing data and data entry accuracy. Two male pet dogs (1 entire, 1 neutered) were too scared to approach the experimenter/bowls in the warm-up phase of the OCT and so data pertaining to this part of the study could not be obtained in these cases. Furthermore, an additional 3 neutered male pet dogs were not motivated enough by the food (only by pets from the experimenter) to participate in the warm-up phase of the OCT and so OCT data could not be obtained from these dogs either. Hence the total sample size used in the analysis of the OCT was 41 dogs.

Validation of the experimental set-up

Validation of the experimental set-up was determined by firstly investigating side bias individually for each dog during the control and cued trials, based on the number of times a dog chose the right side and the number of times a dog chose the left side compared to the proportion 50% using a binomial test using the FREQ procedure. Secondly, validation of the experimental set-up was determined by analyzing whether dogs collectively performed the OCT above chance during the control trials i.e., in the absence of a cue (chance is 50%). Only dogs who made five or more bowl choices were included in this analysis, extrapolating the logic of including only dogs that made 10 out of 20 choices for the cut-off in the control trials explained below. Incidentally, after nine dogs were removed due to this cut-off, all remaining dogs made six choices or more. A one sample student *t*-test was realized using the TTEST procedure for this analysis.

Object choice task outcomes

To test for the effect of treatment group on OCT performance, two different outcomes were compared between the groups: (i) the number of "no choices" made and (ii) the percentage of correct choices made. Only dogs who chose 10 or more times (out of a possible 20) were included in the analysis pertaining to the second outcome. This was done because we believe a percentage calculated for a binary outcome (correct vs. incorrect), can be problematic because a certain amount of trials are needed to show if the performance is above chance level or not. For example, dogs who made only one or two choices could accidentally perform 100% correct or 100% incorrect, but this would not be a reliable reflection of their ability. Furthermore, using dogs who made less than 10 choices also makes them difficult to compare with previous studies which have generally used averages from 10 or more trials (Miklósi et al., 1998, 2005; Soproni et al., 2001; Udell et al., 2008b; Udell et al., 2010; Gácsi et al., 2009; Oliva et al., 2015). Hence, 7 pet dogs and 3 assistance dogs were excluded; 5 from the oxytocin treatment group, 1 from the placebo treatment group and 4 from the DAP treatment group. However, before comparing groups on the second outcome, we wanted to determine whether dogs in each treatment group (OT, placebo, DAP) and each recruitment group (pets and foster dogs) could perform the OCT above chance levels (50%). For this comparison, data analysis was performed by using a one sample Student *t*-test realized with the UNIVARIATE procedure. To compare performance between treatment groups, data analysis was performed using a one-way ANOVA by using the GLM procedure after testing that residuals were normal (verified with the UNIVARIATE procedure) and variances were homogeneous (verified with the GLM procedure). *Post hoc* multiple comparisons were carried out using the LSMEANS statement in PROC GLM using the Tukey-Kramer adjustment. For the data pertaining to the percentage of correct choices, the normality and the homoscedasticity of the data were verified, and a parametric independent samples ANOVA was then conducted. For the data pertaining to number of "no choices" made, normality and homoscedasticity was tested and found to be homogeneous between groups but not-normal. Hence, the non-parametric Kruskal–Wallis test was performed using the NPAR1WAY procedure.

Neurohormonal Parameters

Comparison of plasma oxytocin levels between sessions and treatment groups

To evaluate differences in plasma OT levels, plasma levels between sessions and treatment groups were compared. Conditions of normality and homogeneity of variances were verified with the UNIVARIATE and GLM procedures, respectively. Normality of data pertaining to plasma OT levels were found to be normal. Homoscedasticity was found to be verified. Given this, a General Linear Mixed Model was conducted using the MIXED procedure to evaluate the main and combined effects of treatment and session on OT levels.

Comparison of serum prolactin levels between sessions and treatment groups

Data pertaining to serum prolactin levels were found to be non normal. Other distributions were considered but were not found to be appropriate. Therefore, data was box-cox transformed but was still non normal after transformation. As such, only graphs can be presented to depict differences between sessions and treatment groups.

Comparison of serum prolactin levels according to origin

Average prolactin levels for each dog were calculated by calculating the mean serum prolactin value obtained from sessions 2 and 3. Data analysis was carried out in the following way: assumption of normality was verified using the UNIVARIATE procedure and homogeneity of variances using the TTEST procedure. Prolactin levels in pet and foster dogs were found to be skewed toward the lower range. An atypical value was also obtained in the foster dog sample. Variances were homogeneous, but due to the non normal distribution of the data a non-parametric Wilcoxon two-sample test was done, using the NPAR1WAY procedure, to compare levels between groups. The analysis was performed with and without the atypical value and it was not found to influence the results and hence, to maintain the integrity of the data the results including the atypical value is presented in the "Results" section.

Predicting Object Choice Task Performance

Backward deletion multiple regressions were conducted to identify significant predictors of OCT performance on the two outcome variables: (i) the number of "no choices" made and (ii) the percentage of correct choices made. For the same reasons described above in relation to the second outcome, only dogs who chose a bowl 10 or more times were included in this multiple regression analysis. Multiple regressions were realized for qualitative explicative variables: dog gender (female entire/female spayed/male entire/male neutered), origin (pet dog/fostered dog), weight, home location (inside/outside/both), point-following ability (spontaneous/non-spontaneous), session 3 plasma OT levels, session 3 serum prolactin levels, owner avoidant attachment scores, owner anxious attachment scores, EDED scores, and treatment group (OT/DAP/placebo). Homogeneity of variances was verified using the HOVTEST = LEVENE option in the MEANS statement of GLM procedure. Normality was verified on complete model residuals using the UNIVARIATE procedure and the complete model was done using the GLM procedure. After realizing the complete model, simplification of this was done using backward selection method. The aim of simplification was to find the best model for the data that maximizes the r^2 of the model. The GLMSELECT procedure with the SELECTION = BACKWARD option on the MODEL statement was used for this purpose. The selected model was then studied using the GLM procedure. Data pertaining to the percentage of correct choices was found to be normal and homogeneity was also found between groups.

Comparison of Human Attachment

Avoidant and anxious attachment to dogs were compared for pet owners and puppy carers. Data analysis was carried out in the following way: conditions of normality and homogeneity of variances was verified (with respectively the UNIVARIATE procedure and the TTEST procedure). Normality of data pertaining to owner/carer avoidant attachment scores toward their pet dogs and their foster dogs was found to be skewed toward the lower end in pet owners. Normality of data pertaining to owner/carer anxious attachment scores toward their pet dogs and their foster dogs was found to be skewed toward the lower end in both groups. Variances between groups were homogenous for both sub-scales but due to the non normal distribution of scores non-parametric Wilcoxon two-sample tests were employed to identify group differences.

RESULTS

Object Choice Task Performance

Validation of the Experimental Set-Up

Of the 41 dogs that were analyzed in the control trials, nine demonstrated a significant side bias, representing 21.9% of the population. Seven dogs (17% of the population) chose the bowl to their left side significantly more than the bowl to their right and two dogs (4.9% of the population) chose the bowl to their right side significantly more than the bowl to their left ($p < 0.05$). Of the 9 dogs, 6 were fostered dogs and 3 were pets, with 5/9 belonging to the OT group, 3/9 belonging to the DAP group and 1/9 belonging to the control group. Eight out of nine of these dogs demonstrated a bias for the side where they experienced their first food reward.

Of the 39 dogs that were analyzed in the cued trials (two dogs did not make any choices in the cued trials), 10 demonstrated a significant side bias, representing 25.6% of the population. Seven dogs (17.9% of the population) chose the bowl to their left side significantly more than the bowl to their right and three dogs (7.7% of the population) chose the bowl to their right side significantly more than the bowl to their left ($p < 0.05$). Of the 10 dogs, 8 were fostered dogs and 2 were pets, with 1/10 belonging to the OT group, 5/10 belonging to the DAP group and 4/10 belonging to the control group. Seven out of ten of these dogs demonstrated a bias for the side where they experienced their first food reward. Interestingly, only three dogs with a left side bias in the control trials also demonstrated the same left side bias in the cued trials, and only one dog with a right side bias in the control trials also demonstrated the same right side bias in the cued trials.

One sample student's *t*-test revealed the percentage of correct choices in the control trials, where no cue was given to indicate the location of the hidden treat, was not significantly different from chance (50%) ($M = 55.72$, 95% CI [49.85; 61.59], SD = 16.27; $t = 1.99$, $p = 0.056$), indicating that the dogs were performing at chance levels in the absence of a cue.

Outcome 1: Number of "No Choices" Made

Table 4 shows the average number of times dogs in each treatment group did not make a choice following a given cue and in the absence of any cues (control trials).

A Kruskal–Wallis test revealed a non-significant effect of treatment group on number of "no choices" made for trials where a cue was provided, X^2 (2, $N = 41$) = 1.96, $p = 0.374$.

Outcome 2: Percentage of Correct Choices Made

Table 5 shows the percentage of correct choices made by the whole population of dogs in the sample.

On the whole population, the highest mean percentage of correct choices and the lowest standard deviation are interestingly both obtained for the DAP group. Indeed, the standard deviations are quite large, particularly for the OT group. This is not surprising given that some of these dogs only chose a very small number of times, or in two cases, made "no choices" at all (refer to scores in **Table 4**) thereby obtaining a percentage of correctness that was based on 0–2 trials only (see **Table 6** for raw scores). Therefore, based on the logic explained in the "Statistical Analysis" section, it was decided to only include dogs that chose 10 times or more for inclusion in the ANOVAs in the following section. **Tables 6** and **7** show the pattern of performance of the dogs that chose at least once but less than 10 times and were therefore excluded from the ANOVAs.

What is immediately striking from **Table 6** is that none of the numerators are equal to the denominators, meaning that all these eight dogs experienced failure at some point during the task. The dogs in the OT group comprised 3 pet dogs and 1 foster dog while the DAP group comprised 2 pet dogs and 2 foster dogs, so origin does not appear to influence why these dogs made so few choices. To further investigate why some dogs might have stopped making choices, we looked at the rates of success or failure of the previous attempt before the dogs decided to stop. This was done for all dogs who started the OCT and either stopped completely (did not make any further attempts) or stopped and then started again, including both the control trials (10) and the cued (i.e., pointing) trials (20).

As can be seen from **Table 7**, almost half the dogs that made less than 30 attempts on the OCT started the task (which began with a control, i.e., un-cued trial) by not making a choice. Furthermore, dogs in all treatment groups stopped participating (i.e., did not participate in at least the next trial) more often after a failed attempt than after a successful one. This may suggest that their prior success (or failure) is influencing their decision to make a future attempt. Non-participation included anything that did not involve making a selection between the two bowls, so non-participation also included approaching the experimenter. While not considered participation in the task *per se*, this could still have been considered by the dogs as a potential strategy to obtain food. To investigate whether dogs were using this strategy more or less in a particular treatment group, percentages were calculated indicating how often this approach was used during a "stopping event" (refer to **Table 7**). By eye-balling these percentages, it appears that the OT group used this non-rewarding technique less than the other two groups.

Compared to chance

One samples *t*-tests were used to compare whether dogs in each treatment group and each recruitment group could perform the OCT significantly better than chance. As explained above, only dogs who chose 10 or more times were included in this analysis. Percentage of correct choices made were compared to the chance level of 50%. Dogs in all groups were found to perform the OCT above chance, as can be seen in **Table 8**.

TABLE 4 | Mean and median number of times dogs in each treatment group did not make a choice in the cued trials (out of 20) and in the control trials (out of 10).

Type of trial	Group	*N*	*M*	SD	Lower 95% CI	Upper 95% CI	Median	Lower quartile	Upper quartile	Min	Max
Cued	Oxytocin	13	7.69	9.22	2.12	13.27	2	0	18	0	20
	Placebo	13	2.62	5.69	−0.83	6.06	0	0	2	0	20
	DAP	15	5.27	6.88	1.46	9.08	1	0	1.46	0	18
Control	Oxytocin	13	3.23	3.49	1.12	5.34	1	0	7	0	8
	Placebo	13	1.54	2.47	0.05	3.03	1	0	2	0	9
	DAP	15	2.93	3.08	1.23	4.64	2	1	4	0	9

N = sample size, M = mean, SD = standard deviation, CI = confidence interval.

TABLE 5 | Mean percentage of correct choices made by all dogs in each treatment group.

Group	*N*	*M* (%)	SD (%)	Lower 95% CI	Upper 95% CI	Min (%)	Max (%)
Oxytocin	12	58.87	38.99	34.09	83.64	0	100
Placebo	13	57.05	22.74	43.31	70.79	0	90
DAP	15	63.15	16.28	54.14	72.17	33.33	94.12

N = sample size, M = mean, SD = standard deviation, CI = confidence interval.

TABLE 6 | Raw correct scores (out of total choices made by dog) for dogs that chose at least once but less than 10 times in the object choice task according to treatment group.

	Treatment	
	Oxytocin	**DAP**
	0/1*	1/2*
	0/2†	1/3†
	1/2*	3/5†
	0/1*	6/9*
Average number of attempts	1.5	4.75

*Pet dog, †fostered dog.

Comparison between treatment groups

An independent samples ANOVA revealed that there was a significant effect of treatment group (DF = 2, $F = 4$, $p = 0.030$). *Post hoc* multiple comparisons revealed that dogs that received OT chose the correct bowl significantly more than dogs in the control condition (Tukey, $p = 0.0248$). No other significant differences were found between groups.

Neurohormonal Parameters

Comparison of Plasma Oxytocin Levels Between Sessions and Treatment Groups

Mean levels of plasma OT according to session (2 or 3) and treatment (OT, DAP or saline) can be seen in **Table 9**, as well as mean levels of plasma OT according to session only (including a combination of dogs from each treatment group). A mixed model ANOVA revealed a significant main effect of session (DF = 1, $F = 5.36$, $p = 0.025$), an insignificant main effect of treatment DF = 2, $F = 0.92$, $p = 0.408$) and an insignificant session × treatment interaction DF = 2, $F = 2.13$, $p = 0.130$). These findings indicate that plasma OT levels were significantly higher in session 2 than in session 3 in the whole dog population, regardless of the treatment given to dogs.

Comparison of Mean Serum Prolactin Levels Between Sessions and Treatment Groups

Mean concentrations of serum prolactin between sessions and between treatments are shown in **Figure 1**. Visual inspection of **Figure 1** suggests no differences between sessions, or treatment groups as the error bars all overlap.

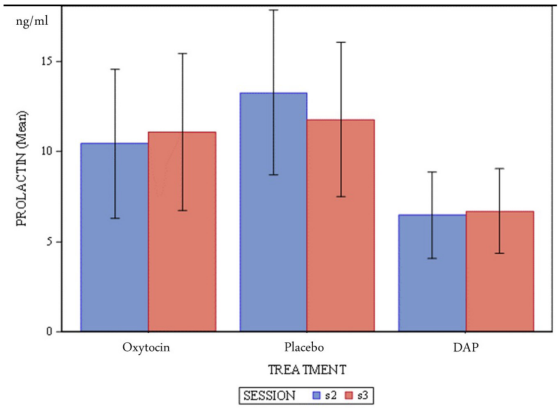


FIGURE 1 | Mean concentration (ng/ml) of dog serum prolactin and standard error according to treatments and sessions.

Comparison of Serum Prolactin Levels According to Origin

Levels of serum prolactin for session 2 and session 3 were averaged for each animal independently. The median of these values were then calculated for pet dogs and foster dogs separately, and are presented in **Table 10**.

A two-sample Wilcoxon Test showed that foster dogs had significantly higher levels of serum prolactin compared to pet dogs, Z ($N = 46$) = 2.27, $p = 0.024$.

Predicting Object Choice Task Performance

Predictors of OCT performance were evaluated separately for the two outcome variables: (i) total "no choices" and (ii) percentage of correct choices made. Backward deletion multiple regressions were conducted for the two separate outcomes variables using the following predictor variables: dog gender (female entire/female spayed/male entire/male neutered), origin (pet dog/fostered dog), weight, home location (inside/outside/both), point-following ability (spontaneous/non-spontaneous), session 3 plasma OT levels, session 3 serum prolactin levels, owner avoidant attachment scores, owner anxious attachment scores, EDED scores, and treatment group (OT/DAP/placebo). Means and standard deviations for the

TABLE 7 | Total number of dogs who made less than the total number of attempts set in the test (i.e., <30), the percentage of these dogs that started the OCT with a "No Choice", the total number of stopping events after successes and failures, and the percentage of these dogs who approached the experimenter at least once during their "Stopping Event."

Group	*N*	% started with "no choice"	Average number of choices not made in control trials (out of 10)	Average number of choices not made in cued trials (out of 20)	Total stopping events after success	Total stopping events after failure	Average % of times dogs approached experimenter as their "no choice"
Oxytocin	7	57.14	5.71	14.29	5	12	22.39
Placebo	5	40	3.20	7	3	8	36.55
DAP	9	44.44	4.44	8.78	9	16	39.26
Total	21	47.62	4.57	10.19	17	36	33.70

N = sample size.

TABLE 8 | Mean percentage of correct choices made by dogs (that chose 10 or more times) in each treatment and recruitment group compared to chance (50%).

Group	*N*	*M* (%)	SD (%)	Lower 95% CI	Upper 95% CI	Min (%)	Max (%)	*t* statistic	*p*-value
Oxytocin	8	82.05	16.70	68.09	96.01	50	100	5.43	0.001
Placebo	12	61.80	15.60	51.89	71.71	35	90	2.62	0.024
DAP	11	67.03	15.69	56.49	77.56	47.37	94.12	3.60	0.005
Pet	13	75.32	17.49	64.75	85.88	50	100	5.22	0.0002
Fostered	18	64.23	16.30	56.13	72.34	35	90	3.70	0.0018

N = sample size, M = mean, SD = standard deviation, CI = confidence interval.

TABLE 9 | Mean concentration of plasma oxytocin (pg/ml) in each session and in each treatment group separated by session.

	N	*M*	SD	Lower 95% CI	Upper 95% CI	Min	Max
Session							
2	40	29.27*	14.07	24.77	33.77	5.43	66.54
3	38	22.71*	13.29	18.34	27.08	5.09	56.60
Group and session							
Oxytocin S2	12	30.64	18.73	18.74	42.54	5.43	66.54
Oxytocin S3	12	27.17	16.55	16.66	37.68	5.09	56.60
Placebo S2	12	23.45	8.38	18.12	28.77	12.66	39.89
Placebo S3	11	21.44	13.57	12.32	30.56	7.89	50.23
DAP S2	16	32.61	12.86	25.76	39.46	14.20	53.56
DAP S3	15	20.08	9.71	14.70	25.46	8.26	35.31

*S = session, N = sample size, M = mean, SD = standard deviation, CI = confidence interval. *p = 0.025.*

categorical variables "gender," "origin," "home location," and "point-following ability" included in the analyses are shown in **Table 11**. Refer to **Table 8** for this information pertaining to treatment group.

For total "no choices", the model containing all the predictors was non-significant. Variables were removed from the model following a backward selection in the following steps: (i) gender, (ii) home location, (iii) treatment group, (iv) avoidant attachment scores, (v) weight, (vi) session 3 plasma OT levels, (vii) session 3 serum prolactin levels, (viii) anxious attachment scores, (ix) EDED scores, and (x) point-following ability. Step 10 resulted in the greatest improvement of the model and reached significance, DF = 1, $F = 11.93$, $p = 0.0015$, $r^2 = 0.27$, with "origin" explaining 27% of the variance in "no choice" scores. Regression coefficients for the significant model can be found in **Table 12**.

Because the significant variable left in the model was categorical, an independent samples ANOVA was then run to further study the selected model coming from the backward selection procedure, (i.e., using only the "origin" variable which was the significant predictor for "no choice" outcomes). The ANOVA revealed that pet dogs were significantly more likely not to make a choice than foster dogs, DF = 1, $F = 5.23$, $p = 0.028$.

For percentage of correct choices made, the model containing all the predictors was non-significant. Variables were removed from the model following a backward selection in the following steps: (i) gender, (ii) home location, (iii) anxious attachment scores, (iv) session 3 plasma oxytocin, (v) EDED scores, (vi) avoidant attachment scores, (vii) weight, (viii) session 3 serum prolactin, and (ix) origin. Step 9 resulted in the greatest improvement of the model and reached significance, DF = 3, $F = 3.89$, $p = 0.022$, $r^2 = 0.34$, with "point-following ability" and "treatment" explaining 34% of the variance in percentage correct scores. Regression coefficients for the significant model can be found in **Table 13**.

Because the significant variables left in the model were categorical, a two-factor ANOVA was then run to further study the selected model coming from the backward selection procedure, (i.e., using only "treatment group" and "point-following ability" as independent variables). In line with the previously presented independent samples ANOVA, the two-factor ANOVA revealed that percentage of correct choices made was significantly different according to "treatment group," DF = 2, $F = 4.86$, $p = 0.016$, and *post hoc* multiple comparisons revealed that dogs who were in the "OT" group were more likely to choose correctly than dogs in the "placebo" group (Tukey, $p = 0.014$). A trend was also observed for "point following ability" whereby spontaneous dogs were more likely to choose correctly than non-spontaneous dogs, DF = 1, $F = 3.82$, $p = 0.061$.

Comparison of Human Attachment

The range, and median scores for pet owner and puppy carer anxious and avoidant attachment scores can be seen in **Table 14**.

For avoidant attachment scores, a two-sample Wilcoxon Test showed that puppy carers were significantly more avoidantly attached than pet owners, Z ($N = 46$) = 3.03, $p = 0.0024$.

TABLE 10 | Serum prolactin levels (ng/ml) in pet dogs versus foster dogs.

	N	*M*	SD	Lower 95% CI	Upper 95% CI	Median	Lower quartile	Upper quartile	Min	Max
Pet dogs	25	6.05	8.84	2.31	9.78	2.17*	1.225	8.721	0.20	40.47
Foster dogs	21	14.73	17.43	6.79	22.67	8.39*	3.214	18.810	0.20	68.81

*N = sample size, M = mean, SD = standard deviation. *p < 0.025.*

TABLE 11 | Means and standard deviations for the categorical variables "Gender," "Origin," "Home Location," and "Point-Following Ability" included in the multiple regression analyses.

	Total "no choice"			% Correct		
	N	*M*	SD	*N*	*M*	SD
Gender						
Female entire	4	3.50	3.32	4	63.75	16.19
Female spayed	16	4.31	7.37	12	66.41	17.30
Male entire	3	6	10.39	2	57.50	10.61
Male neutered	18	6.22	8.16	13	74.49	18.48
Origin						
Pet	20	7.80	8.20	13	75.32	17.49
Fostered	21	2.71	5.91	18	64.23	16.30
Home location						
Inside	15	4.73	7.92	11	65.19	19.42
Outside	21	5.86	7.60	16	69.45	16.27
Both	5	3.80	6.38	4	76.75	17.99
Point-following ability						
Spontaneous	13	5	7.68	10	75.62	18.10
Non-spontaneous	28	5.29	7.53	21	65.67	16.58

N = sample size, M = mean, SD = standard deviation.

TABLE 12 | Standardized and unstandardized regression coefficients for each predictor in the significant model of the backward deletion multiple regression for "no choice" outcome.

Parameter	*B*	β	SE
Intercept	8.71	0	1.55
Fostered	−7.48*	−0.15*	2.17
Pet	0	0	–

*B = standardized regression coefficient, β = unstandardized regression coefficient, SE = standard error. *p = 0.0015, r^2 = 0.27.*

TABLE 13 | Standardized and unstandardized regression coefficients for each predictor in the significant model of the backward deletion multiple regression for percentage correct outcome.

Parameter	B	β	SE
Intercept	75.88	0	5.90
Spontaneous	0	0	–
Non-spontaneous	−13.91*	−0.40*	6
Oxytocin	13.54†	0.35†	7.24
Placebo	−7.42	−0.21	6.73
DAP	0	0	–

*B = standardized regression coefficient, β = unstandardized regression coefficient, SE = standard error. *p = 0.03, †p = 0.07, r^2 = 0.34.*

For anxious attachment scores, no significant differences were observed between groups analyzed with a two-sample Wilcoxon Test, Z (N = 46) = 0.30, p = 0.77.

DISCUSSION

This study aimed to further previous findings demonstrating that intranasal OT enhances dogs' performance on an OCT (Oliva et al., 2015; Macchitella et al., 2017) by comparing the effects of OT and DAP on (i) number of choices made on an OCT and (ii) correctness of choices made on an OCT, when compared to placebo. The study also aimed to investigate the effects of dog gender (female entire/female spayed/male entire/male neutered), origin (pet dog/fostered dog), weight, home location (inside/outside/both), point-following ability (spontaneous/non-spontaneous), session 3 plasma OT levels, session 3 serum prolactin levels, owner avoidant attachment scores, owner anxious attachment scores, EDED scores, and treatment group (OT/DAP/placebo), on OCT performance. Finally, the study aimed to identify differences in pet owner versus puppy carer attachment, as well as pet versus foster dog prolactin levels.

OCT Performance Compared to Chance

In line with previous findings (Oliva et al., 2015), all dogs that made more than 10 choices on the OCT were able to perform above chance. However, the difference in scoring between the two studies meant that more dogs in the current study made less than 10 choices. Indeed, when a dog did not make a choice in Oliva et al.'s study it was given a test of motivation, which involved two pre-training trials (one to each side). If the dog chose a bowl during this test of motivation, it was deemed to be motivated and thus an assumption was made that the previous "no choice" outcome was due to the dog not understanding the task and so was given a score of "incorrect choice" for that trial. In contrast, in the current study, the task purposely continued without a test of motivation, and a score of "no choice" was given to that trial. Another important difference in the study by Oliva et al. is that only two dogs did not pass the initial pre-training, in which the dog had to select the correct bowl four times in a row after being shown the treats being placed into the correct bowl. Inspection of Oliva et al.'s (unpublished) raw data revealed that, surprisingly, 42% of the 67 dogs who completed the initial pre-training needed more than four attempts to complete the initial pre-training, with 15% needing 10 or more trials. Furthermore, pre-training was repeated before each block, of which there were four per

TABLE 14 | Avoidant and anxious attachment scores in pet owners and puppy carers.

	N	*M*	SD	Lower 95% CI	Upper 95% CI	Median	Lower quartile	Upper quartile	Min	Max
Pet owner avoidant	25	1.57	0.61	1.32	1.82	1.20*	1.20	1.70	1	3.40
Puppy carer avoidant	21	2.08	0.67	1.77	2.38	1.90*	1.60	2.30	1.20	3.60
Pet owner anxious	25	2.34	0.99	1.93	2.74	2	1.60	2.70	1.20	4.80
Puppy carer anxious	21	2.42	0.92	2	2.84	2.20	1.60	3.30	1.40	4

*N = sample size, M = mean, SD = standard deviation. *p < 0.0025.*

testing session. While the number of dogs needing more than four attempts per session reduced over the blocks within each session, the number of dogs requiring more than four attempts for the initial pre-training before session two rose back up to 52%, however only 3 out of 63 dogs who completed session 2 needed 10 or more attempts. Therefore, it is fair to say that the dogs in Oliva et al.'s study received a lot more pre-training compared to the dogs in the current study which were given four trials only, regardless of the dog's performance, so long as the dog chose a bowl at least two out of the four trials. The reduced pre-training in the current study was a purposeful attempt to reduce the probability of dogs learning how to perform the task within the sessions, as was observed in Oliva et al. (2015). Still, the reduced pre-training in the current study did not appear to affect dogs' ability to perform above chance, in dogs that were willing to make more than 10 choices. It may, however, have reduced the number of dogs willing to make 10 choices.

The Influence of Oxytocin Versus DAP on OCT Performance

The hypothesis that OCT performance would be enhanced by both OT and DAP compared to placebo was only partially supported as the percentage of correct choices made in the OCT was enhanced by OT compared to placebo, but not DAP compared to placebo, in the sub-population of dogs who made at least 10 choices. These performance enhancing effects of OT are in line with previous studies (Oliva et al., 2015; Macchitella et al., 2017). In the current study we investigated performance in two ways (i) number of choices not made (based on the whole sample) and (ii) percentage of correct choices out of those made (based on the sample of dogs that made 10 or more choices, i.e., the "best choosers"). Our findings suggest that OT increases the percentage of correct choices made in the "best choosers" sub-population but not the number of choices made. This is an important finding, as previously it has been put forward that perhaps dogs are performing better due to a decrease in anxiety when performing the task (Oliva et al., 2015), however, the current findings do not suggest a willingness to "have a go" is the reason why dogs are performing better, as OT did not increase number of choices made. In fact, for the eight dogs that made at least one but less than 10 attempts when given the pointing cue, the four dogs belonging to the OT group demonstrated very low rates of choosing (1.5 choices on average) compared to the other four dogs belonging to the DAP group (who chose 4.75 times on average) (refer to **Table 6**). To investigate why this might be we looked at the sub-group of dogs (N = 21) who made less than 30 attempts (on both the control and cued trials) which revealed that dogs in all groups were more likely to stop performing the OCT (i.e., not participate in at least the following trial) after a failure than after a success (refer to **Table 7**). Hence, the very low number of attempts (<10) seen in the OT group may suggest that this group were more influenced by prior losses than the dogs in the DAP group. The reverse of this (i.e., being influenced by prior success) may have also been taking place in dogs who demonstrated a side bias, with the majority of these dogs developing a bias to the side where they first experienced a food reward. Interestingly, dogs in the OT group made up the majority of these dogs in the control trials at 56%, but reduced to the minority group in the cued trials at 10%, which may suggest that these dogs employed different techniques on the OCT, depending on whether a cue was offered or not. The OT group also contained the largest percentage of dogs who started the OCT (which always commenced with a control/no cue condition) by not choosing. It is possible that these dogs realized from the very beginning that they did not understand the task, or were being tricked that they had enough information to complete it correctly, and therefore made a beneficial decision to not perform – after all, if they don't perform, they can't be wrong. They also used the non-reinforcing strategy of approaching the experimenter (which resulted in the experimenter immediately picking up the bowls and walking away), less times than the other two groups. This may reflect a greater understanding in these dogs that this strategy would not result in a reward, food or otherwise, when performing this task, despite the fact that they might use this strategy to obtain food or attention from humans outside of the task. DAP has been shown to have effects as an emotional modulator in dogs (Sheppard and Mills, 2003; Gaultier et al., 2005, 2008; Tod et al., 2005; Mills et al., 2006; Denenberg and Landsberg, 2008; Kim et al., 2010; Siracusa et al., 2010; Landsberg et al., 2015). In this study, it did not significantly increase number of "no choices" made nor OCT performance in the "best choosers" population. However, it is noteworthy that on the whole population descriptive data, the DAP group displayed the lowest standard deviations/variability, suggesting a greater homogeneity in OCT performance for this group. This could be related to the general ability of appeasing pheromones in modulating/smoothing the cognitive-emotional responses among individuals, particularly in the context of cognitive tasks, as already described in horses treated with EAP (Equine Appeasing Pheromone, the homologous specific appeasing pheromone) by Mengoli et al. (2014). It could be possible that the "best choosers" dogs were already in this kind of balanced cognitive-emotional state, hence making us unable to conclude about DAP effects in this population.

An alternative explanation for the reduced choosing behavior observed in dogs treated with OT is that the putative "relaxing" effect of OT (Uvnas-Moberg and Petersson, 2005) may have caused them not to care to participate in the OCT since they were already in a kind of "rewarded" mental state of well-being and so did not need to work further to get a reward (refer to **Table 7**). It is also possible that the anorexigenic effects of OT impacted their motivation to perform a task where their efforts are rewarded by food (see reviews, Olszewski et al., 2010; Onaka et al., 2012; Spetter and Hallschmid, 2017).

The Influence of Intranasal Oxytocin on Neurohormonal Parameters

The hypothesis that plasma levels of OT and serum prolactin will change following DAP and OT exposure was not supported.

Treatment was not found to have any effect on plasma OT or serum prolactin levels. However, dogs in the OT treatment group demonstrated enhanced cognitive ability and so in relation to the modality of intranasal OT, our findings suggest that OT delivered intranasally reaches the brain directly, and not by a peripheral mechanism, as has been postulated (Lee et al., 2018). These findings are also discrepant with previous findings in dogs where an increase in levels of OT has been reported 15 min after intranasal application (Romero et al., 2014; Temesi et al., 2017). There are several reasons why our findings are discrepant with Romero et al. (2014). First, in their study 40 IU of intranasal OT was applied, vs. 24 IU used in the current study. As this is nearly double the amount, this could go some way to explaining the differences in findings between our study and theirs. Second, the number of dogs in their study was only five, compared to the 12 dogs that we were able to analyse in the OT treatment group. In addition to this larger sample size, we also observed a high rate of variability in our population, as evidenced in **Table 9**, which could have "hidden" any possible difference between the sessions and/or groups. Such wide variability in OT levels in plasma is not uncommon after intranasal administration and has also been reported in monkeys for instance (Lee et al., 2018). Indeed, a variability of approximately 50% of mean basal plasma OT concentrations has been previously reported within populations from several species, regardless of exogenous OT administration (Bienboire-Frosini et al., 2017). Crockford et al. (2014) precisely described and explicated the general variability of the oxytocinergic system within species. Third, the discrepancies could be due to differences in measurement procedures. For example, Romero et al. used radioimmunoassay (RIA) to assay OT in plasma, whereby the current study used EIA. With regards to Temesi et al. (2017) again, a small number of only six dogs was analyzed, and their methods to measure OT also differed from ours in an important way. For instance, they assayed OT in serum, not in plasma, and used a different ELISA kit, which has not been validated for use in dogs, to the best of our knowledge. Furthermore, the levels of OT they found after intranasal application (of 12 IU) is much higher than the one observed by Romero et al. with 40 IU intranasal application. Still, our lack of an OT increase in the blood is also inconsistent with findings from previous studies in rodents (Neumann et al., 2013), but may be explained by the fact that these changes take longer than 15 min to reach the blood. Indeed, Neumann et al. (2013) only observed a significant increase in blood 70 min following intranasal administration. This suggests that the OT first reaches the brain where it has measurable behavioral consequences and this may result in a downstream increase in the OT in the blood after 15 min. Conversely, other studies in primates and humans have showed that plasma OT could peak at 10–15 min after the intranasal administration (Striepens et al., 2013; Dal Monte et al., 2014). Future studies would need to be conducted to investigate this in dogs, using large samples and validated methods to measure OT in blood. Similar effects may also be observed in serum prolactin and future studies should take additional blood samples at various times to attempt to capture this.

Interestingly, in the current study, plasma OT levels significantly decreased from session 2 to session 3 in the whole population, with no influence of treatment, refer to **Table 9**. We can speculate that this decrease in OT between sessions reflects the dogs being less stressed/aroused during session 3. Indeed, previous authors showed that OT release in brain and plasma could be related to acute stress events (Wotjak et al., 1998; Onaka et al., 2012; Noller et al., 2013). Despite the veterinarians' attempts to reduce the stressfulness of the first blood sampling, it is possible that this handling could still trigger an increase in endogenous plasma OT levels to dampen the HPA axis activation, as already observed in mini-pigs (Marcet Rius et al., 2018) and beef heifers (Wagner et al., 2019). In session 3, the dogs would have been more familiar with the veterinarians and the testing location and therefore may have felt more comfortable with the environment. The opposite possibility is also true that they may have formed a negative association with the veterinarians due to the blood sampling. However, for the dogs that received OT intranasally in session 3, this may have acted as an anxiolytic as it has been successfully demonstrated in humans (Heinrichs et al., 2003; de Oliveira et al., 2012). Similarly, for the dogs that received DAP, this too may have acted as an emotional modulator as has been previously demonstrated (Sheppard and Mills, 2003; Gaultier et al., 2005, 2008; Tod et al., 2005; Mills et al., 2006; Denenberg and Landsberg, 2008; Kim et al., 2010; Siracusa et al., 2010; Landsberg et al., 2015). Alternative methods have shown promise in more accurately measuring total (bound and unbound) levels of OT, the bound fraction of which needs to be unbound before being measured (Brandtzaeg et al., 2016). Future studies may consider the use of these methods in assessing whether bound levels of OT have an effect on OCT performance in dogs.

Predicting OCT Performance

Contrary to our expectations, plasma OT levels did not differ in better performing dogs. In addition to our expectation that dogs with higher levels of plasma OT levels would perform better on the OCT, we also expected better performing dogs to be more likely to be male and to be pets, while higher levels of prolactin and EDED scores to be present in the poorer performing dogs. Our findings that gender did not predict performance are in contrast to Oliva et al.'s (2015) findings of an enhanced performance in male dogs compared to female dogs following intranasal saline administration, however, they are in line with their findings that gender no longer acted as a predictor following intranasal oxytocin administration. This may be explained by the significant treatment effect observed in our study. Interestingly, foster dogs were more likely to make a choice than pet dogs, but they were no more likely than pet dogs to choose correctly, refer to **Tables 12**, **13**. This highlights an important point that just because the dog is performing, does not indicate that it has a better *understanding* of *how* to perform than a dog that chooses not to. Furthermore, foster dogs' increased choosing behavior may not reflect greater willingness to have a go, but may be more reflective of their training and familiarity with following

orders, compared to pet dogs. Alternatively, this could reflect a change in the wiring of these dogs' brains, due to their particular upbringing. Previous studies have identified individual differences in OCT performance (Miklósi et al., 1998; Agnetta et al., 2000; Hare et al., 2002; Udell et al., 2008a,b; Udell et al., 2010; Virányi et al., 2008; Wobber et al., 2009; Oliva et al., 2016b). Oliva et al. (2016b) investigated whether this could have been due to differences in the OT receptor gene but were not able to demonstrate an association. Findings from the current study suggest that a dogs' early social and learning experiences may impact their ability to use human social gestures effectively, and this may be more influential than their genetic blueprint.

Human Attachment to Pet Versus Foster Dogs

In addition to having increased serum prolactin levels (refer to **Table 10**), foster dogs had carers with greater avoidant attachment toward them, compared to pet dog owners, in line with our hypothesis, refer to **Table 14**. We consider this to be one of the most important findings of this study because it highlights that foster dogs are being brought up by carers who are possibly reluctant to form a close attachment towards them. This is not surprising given that carers know they will eventually have to give the dogs away and so is a logical emotional defense mechanism. Avoidant attachment styles may require the dog to be more flexible from an emotional point of view and might adversely affect its development and ability to cope with subsequent rehoming experiences, which the dogs in the current study were yet to experience. Konok et al. (2015) has already demonstrated that avoidant styles of adult attachment have been associated with owning dogs with separation related disorders. While the dogs in the current study were not showing symptoms of emotional dysregulation, this could be a potentially important problem in assistance dogs given they are bred to be confident and calm. As, while they may have a natural propensity to be so, if their environment is not conducive to the development of these characteristics then consideration needs to be given as to how their environment can be enhanced and improved for their welfare and for the welfare of the humans that they are born to lead and assist.

Limitations and Future Directions

Limitations of the present study include the uncontrolled hunger levels between subjects which may have affected motivation to perform the OCT. We tried to control for this by instructing owners/carers not to feed their dogs for 6–8 h before testing but it is possible that these instructions were not adhered to by all participating owners/carers, or that food rewards were simply not motivating enough for some dogs. Interestingly, foster dogs made more choices on the task than pet dogs and we cannot rule out the possibility that this might be due to the fact these dogs were more motivated by food than pet dogs simply because they comprised of breeds known to be more motivated by food, refer to **Table 2**, (Raffan et al., 2015). Hence, future studies wishing to compare assistance dogs with pet dogs should use pet dogs of the same or similar breeds. Another limitation was the set-up of the room which was not 100% symmetrical – the food was placed into the bowls behind a black screen on the dog's left-hand side – the same side the majority of dogs with a side bias showed an overall preference for when choosing bowls. However, dogs with a significant side bias only represented a minority of the population, with 21.9% in the control trials and 25.6% in the cued trials. Collectively, in the control condition, all dogs (with and without a significant side bias) performed at chance level, which validates the experimental set-up in that they were not able to use the sense of smell to find the food, nor were they being influenced by potential subconscious "Clever Hans" effects (Pfungst, 1911) from the experimenter providing the cues. This relates to the importance of having a "blind" experimenter, a strength of the current study, so they are unable to unintentionally influence the dog's choices according to their treatment allocation. Lastly, we chose to be consistent with the timing and dose of Oliva et al.'s (2015) previous study, however, it is currently unknown what constitutes the optimal behavioral testing time after administration of OT in dogs, and how long the behavioral effects last. However, extrapolating from the findings of a human study investigating CSF following intranasal application of 40 IU and 80 IU of the very similar peptide, vasopressin (Born et al., 2002), and a pig study investigating CSF following intranasal application of 24 IU of OT (Rault, 2016), we can reasonably assume that OT is still active in the brain 100–120 min after administration, and potentially longer. Therefore, the cognitive effects in the current study were likely to have been maintained for the entire OCT, which normally lasted between 30 and 45 min, or 75 and 90 min post intranasal administration. Lastly, our final sample size for the percentage of correct choices made was reduced due to the exclusion of dogs that made less than 10 choices for this analysis, due to the interesting finding that a number of dogs chose less than 10 times. Therefore, future studies that plan to employ a similar low level of pre-training as the current study may wish to increase the number of dogs in their sample to account for this attrition.

CONCLUSION

The current study replicated previous findings that intranasal OT enhances performance on an OCT in a population of dogs that made more than 10 choices. The study furthered these findings by demonstrating that this enhanced performance is relevant to percentage correctness and not number of choices made. Furthermore, this study revealed that DAP does not have the same performance-enhancing effects. Findings that plasma OT does not increase 15 min following intranasal application of OT supports the notion that OT gains direct access to the brain, bypassing the bloodstream when administered intranasally in dogs. Neither plasma OT levels, nor serum prolactin levels were found to predict OCT performance, however, serum prolactin was found to be higher in foster dogs compared

to pet dogs. Additionally, fostered dogs were more likely to perform in the OCT in terms of making a choice, but these choices were not any more likely to be correct. Fostered dogs were also more likely to be cared for by humans with an avoidant attachment toward them. These findings highlight important considerations for current assistance dog foster and training situations.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

A sample of 51 dogs and their owners or foster carers were recruited for the study. Owners/carers read through an explanatory statement before the first testing session and signed a consent form allowing their dogs to take part. The study was approved by the IRSEA Ethics Committee, approval number AFCE_201605_02.

AUTHOR CONTRIBUTIONS

JO, MM, AC, PP, and CF designed the study protocol. JO, MM, and TM recruited participants for the study and executed the experiments. CC carried out the biochemical analysis. ET and CL carried out the statistical analysis. JO, MM, CL, and CF analyzed and interpreted the data. JO and CF were responsible for overall administrative management of the project. JO completed the principal drafting of this manuscript. AC and PP were responsible for the funding acquisition. All co-authors were involved in re-drafting the manuscript to completion.

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Conflict of Interest: The pheromone DAP used in this study was identified and patented by PP and licensed as the commercially available product, Adaptil®, by CEVA Animal Health Laboratories, who provided us with both the active and sham collars used in this study.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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