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MULTIDISCIPLINARY APPROACH TO HORSE WELFARE IN DIFFERENT MANAGEMENT SYSTEMS

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ABSTRACT

From its domestication until nowadays, the horse has assumed different and important roles in human society. From meat production to its use as a means of transport and work, today this animal is involved in different kinds of activities, such as sports and animal-assisted interventions. Horses are not only farm animals but also companion animals. Because of their multiple roles and lack of precise regulation, different kinds of management systems have been developed over time for this species. This PhD research project aims to investigate horses' welfare in different management practices and housing systems, considering a multidisciplinary approach and taking into account biological function, naturalness and affective dimension.

The results are presented in five articles that evidence risk factors that can mine horse welfare, and examine the tools and parameters that can be employed for welfare assessment in this species. Our research shows the importance of considering the evolutionary history and the species-specific and behavioural needs of horses in their management and housing. Sociality, the possibility of free movement, diet composition and foraging routine are important factors that should be taken into account. Another factor that can affect horses' welfare is the workload that these animals undergo. Furthermore, this research has evidenced the importance of employing several parameters (e.g., behaviour, endocrinological parameters and immune activity) in welfare assessment and proposes the use of horsehair DHEA (dehydroepiandrosterone) as a possible useful additional non-invasive measure for the investigation of long-term stress conditions. Finally, our results underline the importance of the consideration of the affective dimension in welfare research. Its evaluation can be challenging in non-vocal animals, but recently Judgement Bias Tests (JBT), which are based on the influence on the decision-making process by affective states, have been widely employed in animal welfare research with promising results. However, our studies show several limitations in the use of spatial go/no-go JBT in horses and further research is needed to develop a specific test which pays attention to species-specific and individual characteristics.

Still today several management systems do not fulfil the species-specific needs of horses, thus the implementation of specific regulations could ameliorate horse welfare. A multidisciplinary approach to welfare assessment is fundamental, but it should be always remembered the individual and its own characteristics that can influence not only physiological, immunological and behavioural responses but also emotional and cognitive dimensions.

ABBREVIATIONS

ANS	Autonomic Nervous System
ASSI	Italian Agency For The Horseshow Activities
AVP	Arginine Vasopressin
BBP	Big Box Period
BCS	Body Condition Score
BOLD	Boldness
BPHS	Horses With Behavioural Pathologies
CM	Conventional Management
DA	Dopaminergic activity
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone sulfate
E-BARQ	Equine Behaviour Assessment and Research Questionnaire
EC	European Commission
ES	Ethological Stable
ETS	Easy To Stop
EU	European
FAWC	Farm Animal Welfare Council
FC	Faecal Cortisol
FEI	International Equine Federation
FISE	Italian Federation for Equestrian Sports
HC	Horsehair Cortisol
HHs	Healthy Horses
HPA	Hypothalamic-Pituitary-Adrenal
HPQ	Horse Personality Questionnaire
HSC	Human Social Confidence
IBI	Interval between consecutive heartbeats
Ig	Immunoglobulin
ISES	International Society For Equitation Science
JBT	Judgement Bias Test
M	Middle

MASS	Motivational Affective States
N	Negative
NB	Natural Boarding
NHSC	Non-Human Social Confidence
NM	Natural Boarding
NN	Near Negative
NP	Near Positive
OIE	Office International Des Epizooties
OT	Oxytocin
P	Positive
PAW	Positive Animal Welfare
PBS	Phosphate-Buffered Saline
PNS	Parasympathetic System
PP	Paddock Period
QoL	Quality Of Life
REM	Rapid Eye Movement
RIA	Radio-Immuno-Assay
RID	Rideability
rMSSD	Square root of the mean of the sum of the squares of differences between consecutive IBIs
RSPCA	Royal Society For The Prevention Of Cruelty To Animals
SAM	Sympathetic-Adrenal-Medullary
SECs	Situation Evaluation Checks
SIH	Stress-Induced Hyperthermia
SNS	Sympathetic System
SWS	Slow Wave Sleep
TR	Trainability
TS	Traditional Stables
US	United Sates
WC	Working Compliance
WOAH	World Organisation For Animal Health

1. INTRODUCTION

1.1 HORSE BEHAVIOUR

1.1.1 Evolution and domestication of the horse

Extant living species of equids belong to the *Equus* genus, family *Equidae, and* order *Perissodactyla.* This order comprises also *Tapiridae* and *Rhinocerotidae*, which, together with *Equidae*, constitute a group of odd-toed mammals, highly specialized in herbivore diet and running (Steiner and Ryder, 2011).

Inside the genus *Equus*, the caballine and the non-caballine lineages can be distinguished. The caballine lineage includes both domesticated and Przewalski horses (*Equus ferus caballus* and *Equus ferus przewalskii*), which are part of the same phylogenetic clade. The non-caballine lineage encompasses two phylogenetic clades: 1) zebras, which comprises three species (*Equus zebra*, *Equus burchelli*, *Equus grevyi*); 2) asses, which include African and Asian wild asses (*Equus africanus, Equus kiang, Equus hemionus*), and domestic donkey (*Equus africanus asinus*). In addition, the variety of the genus *Equus* is enriched by hybrids, such as mules and hinnies (Cucchi et al., 2017; Steiner and Ryder, 2011).

Because of its important role in human history, such as in agriculture, transport, warfare, and sport, and thanks to the well-preserved nature of the fossil, the evolution of the horse is deeply studied (MacFadden, 1992; Janis, 2007). It was characterized by progressive morphological adaptations that follow environmental changes. Therefore, ancestral equids, adapted to a forest environment, evolved into modern animals, which belong to a single relatively recent genus, and adapted to seasonal, arid grasslands and savanna conditions (Janis, 2007). Initially, the evolution of horses was considered a “straight-line evolution”, where ancestors evolved in their descendants following a linear pathway. However, from the early twentieth century, palaeontologists understood that the evolutionary history of horses resembles more of a complex branching tree, with many genera and species overlapping in time, multiple originations, and frequent extinctions (Fig. 1) (MacFadden et al., 2012; MacFadden, 2005).

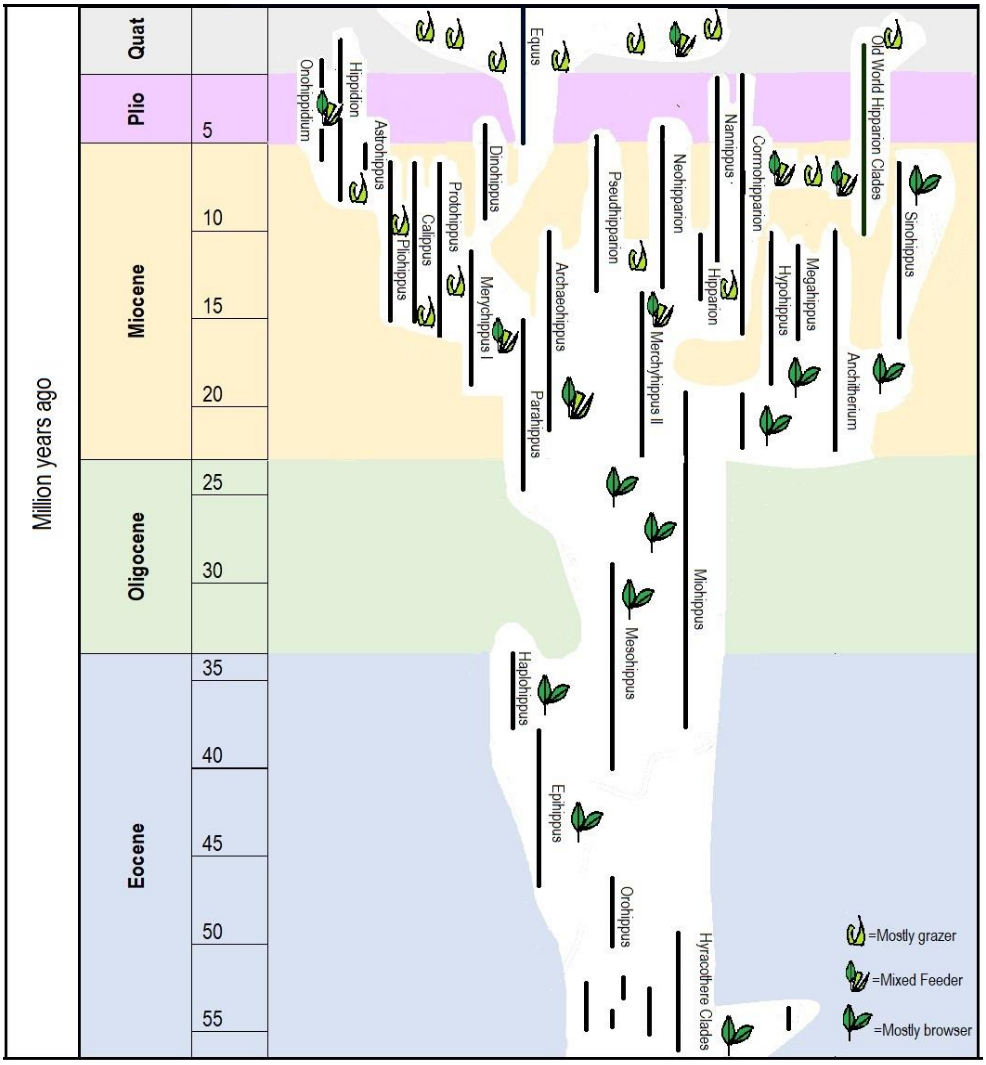

Figure 1. Evolutionary tree of equids and their diet modifications. Plio=Pliocene; Quat=Quaternary (Modified from McFadden, 2005)

During the evolution of horses, three subfamilies can be distinguished: the Eocene *Hyracotheriinae*, the Eocene to Miocene *Anchitheriinae*, and the Miocene to Recent *Equinae* (Janis, 2007). The hyracoteriine genera were *Hyracotherium, Orohyppus, and Epihippus.* The first is recognized as the most ancient ancestor of the domestic horse. It lived in Europe and North America about 58 million years ago and it is called also *Eohippus* (Bailey and Brooks, 2020). This animal was fox-sized (25-45 cm height at withers), with four-toed front feet and three-toed hind feet, footpads, and low crowned (brachydont) molars, characterized by bumpy chewing surface and fairly simple in form (bunodont). These morphological features suggest that they inhabited primaeval forests, mostly feeding with leaves, barries, and buds ("folivorous-frugivorous" diet) (Janis 2007, Zeitler-Feicht, 2004). Furthermore, paleontological findings indicate that they probably lived alone or in small groups (Zeitler-Feicht, 2004). In the Middle and Late Eocene, we found *Orohippus* (50-47 million years ago) and, later, *Epihippus*. Because of the temperature decline, these genera have less access to non-fibrous food, like buds and berries. This change in diet led to a progressive dental modification, and the molarization of premolars allowed them to process more leaves. Moreover, the development of larger third toes suggests that these animals were forced to wider their range of action to find food (Janes, 2007; Bailey e Brooks, 2020).

While *Hyracoteriinae* became extinct in Europe during the late Eocene, when the climate was colder and more seasonal, *Mesohippus*, the first of *Ancitheriinae,* spread in North America (Janis, 2007; Zeitler-Feicht, 2004). During the late Eocene and Oligocene, when evolved *Mesohippus* and, later, *Miohippus*, North America presented subtropical to temperate woodlands, more than tropical forests. Therefore, *Anchiteriinae* develop fully lophed and molarized teeth, adapted to a diet composed mostly of leaves. In addition, they had an enlarged and elongated third metapodial with a larger hoof and functional tridactyl forelimbs, suggesting an adaption to long-distance locomotion (Janis, 2007) In the late-early Miocene, about 20 million years ago, three radiations of anchiteriine can be found a) *Anchiterium* (with the species *Kalobatippus*), a large specialized browser that, during the early Miocene, migrated from North America to Eurasia via Beringia; b) *Archeohippus*, that had a smaller size; c) *Parahippinae* (genus *Desmatippus* and *Parahippus*) (Groves e Ryder, 2000; Janis 2007). In particular, *Parahippus* had high-crowned cheek teeth, which suggests an integration of grass in their diet, and a substantial reduction of the side toes (Janis, 2007).

In the late early Miocene, the savanna habitat of North America led to the appearance of *Merychippus*, the first component of the subfamily *Equinae* morphologically adapted to life in plains. *Merychippus* was characterized by hypsodont teeth that acquired cement, an enlarged chewing surface, and other modifications of the skull, such as the elongation of the face and a larger masseter, suggesting its adaptation to a more fibrous and coarse diet (grass). In addition, the great development of foot suspensory ligaments and the middle toe, the acquisition of unguligrade posture, and the loss of footpads made the locomotory system more prepared for fast movements and running, useful against predators (Franzen, 2010; Zeitler-Feicht, 2004). Probably also social structures changes, because the composition of a large group gives more survival chances to animals living in open and semi-open areas (Zeitler-Feicht, 2004). About 16 Mya, there was a split in the subfamily *Equinae* into two tribes: Hipparionini, which remain tridactyl, and Equini (*Pliohippus*, *Astrohippus*, *Dinohippus*, and *Equus*), which became monodactyl. From the start of the Pliocene less productive prairie took the place of savanna habitat, because of a general cooling and drying, and only *Equus* survived into the Pleistocene (Janis, 2007). About 10,000 years ago, the genus *Equus* became extinct in the New World, probably because of different causes, such as climate change and hunting, but they survived in the Old World (MacFadden, 2005). *Equus* migrated towards the Old World about 3.7 Mya, and here they had successful radiation, which led to the evolution of horses, asses and zebra. These three groups, which evolved and adapted to different environments, can be distinguished by both morphological, behavioural and genotypic (e.g., different number of chromosomes) characteristics (Bailey & Brooks, 2020).

Modern horses (*Equus caballus*) are the result of domestication and breeding selection. The earliest evidence of horses' domestication dates back to around 5500 years ago in Kazakhstan, where Botai employed horses for milking, harnessing, and corralling. However, these horses were not genetically related to the modern horse but belonged to the Przewalski horse lineage (Orlando, 2020). Therefore, the exact geographic origin of the modern horse is still unknown. About 5000 years ago, lots of different lineages of *Equus* were present in Eurasia, but, most of them became extinct and only a limited number of stallion lineages participated in the domestication process. Probably, this and the relatively recent increasing influence of Arabian-Oriental bloodlines have contributed to the lack of genetic diversity of the modern horse (Orlando, 2020). Throughout domestication, horses were selected not only according to their appearance (e.g., coat colour) but also for their locomotion characteristics, such as speed and gaits, especially ambling (Orlando, 2020; Librado et al., 2016). Domestication of horses was not only a source of meat and milk, but provide humans with a rapid means of transportation, contributing to an early process of globalization, and gave a great contribution to work, such as in agriculture, improving productivity (Librado et al., 2016).

1.1.2 Senses

The survival success of some free-ranging horse populations demonstrates how horse behaviour has remained almost the same since the beginning of the domestication process (Goodwin, 2007). Horses are prey, so their primary goal is to reach a successful escape from predators. To do that, they use and combine different sensorial cues (visual, auditory, and olfactory), and their senses are adapted to identify and avoid threats (Prendergast et al., 2016). Indeed, an animal acquires information from its surroundings through its senses, which represents the first biological filter. Smell, vision, and hearing acquire the same importance for prey animals. The second filter is the brain, which has the role to interpret this information according to different influential factors, such as the animal's previous experiences and mood, weather conditions, environmental situation, and time of the day. Finally, the interpretation of information determines the behaviour (Fig. 2) (Beaver, 2019).

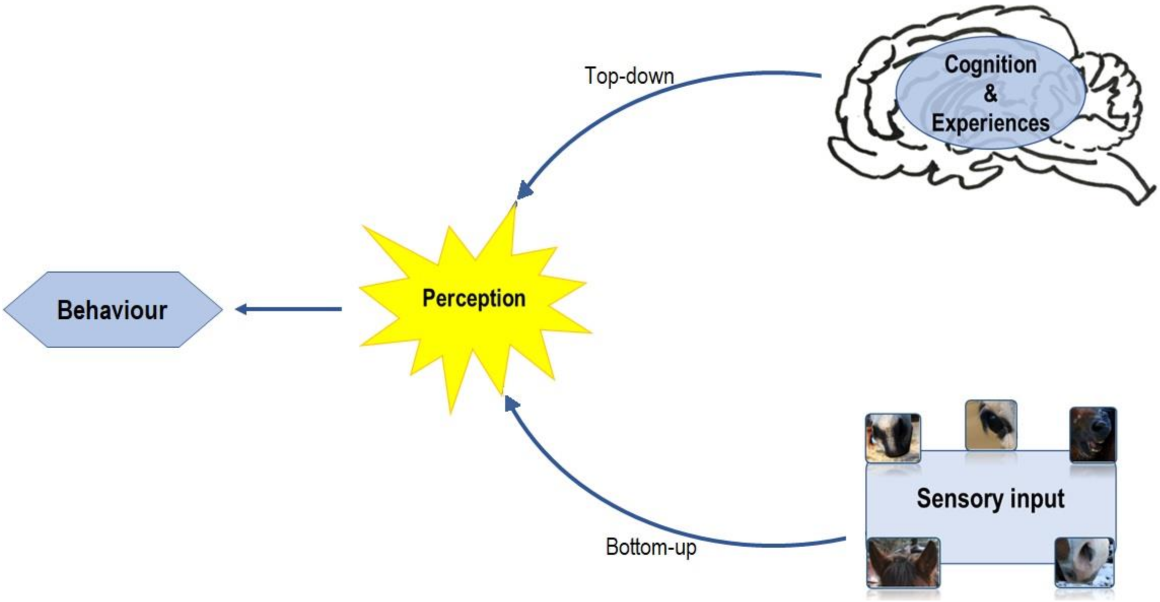

Figure 2. The horse receives and processes sensory inputs that derive from vision, olfaction, taste, touch and hearing (the bottom-up process of PERCEPTION). This information is organized, interpreted and elaborated into the brain, according to the cognitive abilities and experiences of the subject (the top-down process of PERCEPTION). The perception of the surroundings determines the horse's behaviour (Modified from Rørvang et al., 2020)

Vision

Considering visual capabilities, horses do not need to capture food, which is immobile and easy to identify, but they need to recognize potential threats. Often predators adopt camouflage strategies, and the colour becomes almost useless to distinguish them from the surroundings, while motion, brightness, depth, distance, orientation, and texture are more important (Beaver, 2019; Miller and Murphy, 2016). Therefore, horses can detect better moving objects than stationary ones, have a wide visual field to scan the environment, adapt well both to diminished and bright light conditions, and have dichromatic colour vision (Miller and Murphy, 2016). They have the largest eye among terrestrial vertebrates, and the horizontal and rectangular pupil extends the area of visual perception (Fig. 3) (Hall, 2007).

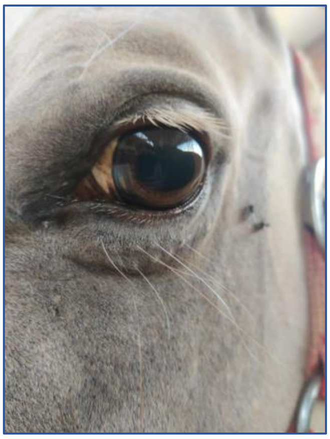

Figure 3. Eye of a horse (Source: picture of Martina Benfenati)

The visual field is almost spherical. Thanks to the anterolateral position of the eyes, the uniocular field of view ranged from 215°-228°, while the binocular one is 55°-65°, predominantly below the head, extending down ∼75°. Vertically, the visual field covered approximately 178°. Blind areas can be identified, perpendicular to the forehead, below the nose and direct to the rear (Fig. 4) (Beaver, 2019; Hanggi and Ingersoll, 2012).

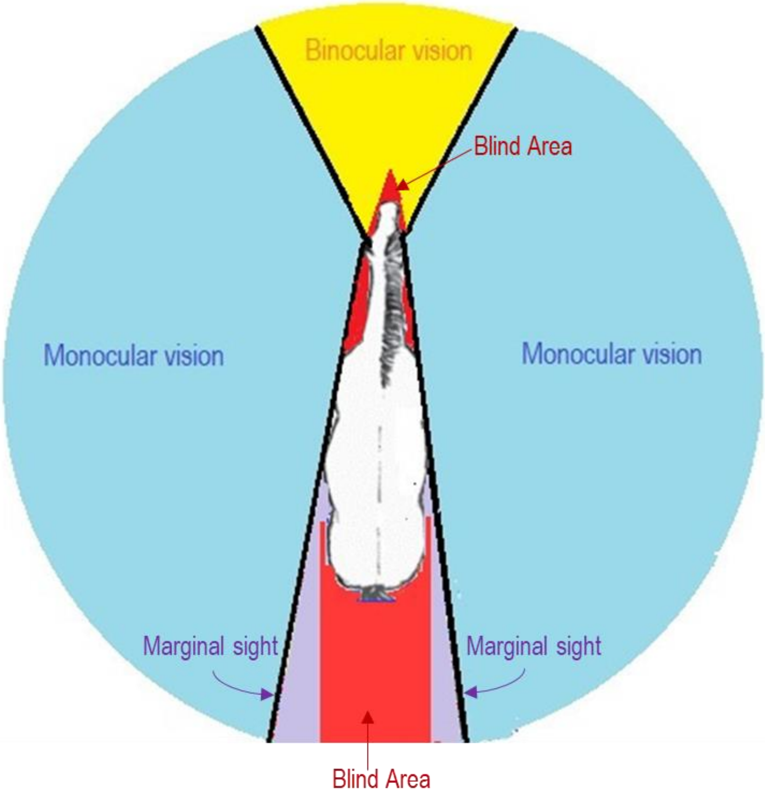

Figure 4. Visual field of horses (Modified from Beaver, 2019)

Horses can detect depth both with the monocular and binocular view, but the first is five times less efficient than the second (Miller and Murphy, 2016). The lens can change its shape to allow the sharpest vision, but its accommodation has some limits with both distant and very close objects. For example, they cannot focus on things within 0.5m, so they might go back or move their head to see it better (Beaver, 2019). In the meantime, horses' visual acuity ranges from 20/30 to 20/60 on the Snellen scale, and it is better than that of cats (20/75 to 20/100) and some humans ("standard" human acuity is 20/20) (Hanggi and Ingersoll, 2012; Miller and Murphy, 2016). Indeed, by examining the distribution of retinal ganglion, a "visual streak" can be identified. The "visual streak" is a 1-2 mm high horizontal area in the tapetal zone, near the optic disc, characterized by a high density of nerve cells (6500 cells/ mm^2) (Evans and McGreevy, 2007; Hanggi and Ingersoll, 2012; Miller and Murphy, 2016). If an image enters near the bottom of the eye, it can land in this area and can be seen in the greatest detail (Miller, 1995). Therefore, a horse to see distant objects while it is running will need to raise its head and extend its nose, on the contrary, it will need to bend its head and neck down to walk over obstacles (Harman et al., 1999). The large dimension of the cornea and pupil and the presence of the fibrous tapetum lucidum favour the vision in a condition of dim light, while yellow-filtering pigments and the corpora nigra help horses to see in presence of bright light. The elongated shape of the pupil not only allows the light to enter the eye but also can close more completely than a round pupil and protect better the retina when the light is very bright. Therefore, the horse is a visual generalist, which can see under all lighting conditions (Miller and Murphy, 2016). However, when the horse moves from brightness to darkness, it takes about 30 minutes to accommodate its vision. This is because when the animal is in the dark, the rod photopigments, rhodopsin, continue to increase their sensitivity to light for a long period to adapt to this condition of light (Miller and Murphy, 2016; Beaver 2019). While night vision is important to provide safety and because horses eat all day long, colour vision is less significant. This is reflected in the proportion of rods, adapted for low light conditions as previously stated, and cones, important in colour vision, that in horses is 20:1, while in human beings is 9:1. Indeed, humans are not well-adapted for night vision and the colour vision is more important. Horses are dichromatic and have short and middle-to-long wavelength-sensitive cones for the detection, respectively, of 439–456 nm and 537–557 nm wavelengths. Therefore, they can see colours ranging from bluish to yellowish, as a red-green colour-blind person (Carroll et al., 2001; Hanggi et al., 2007; Roth et al., 2007; Timney and Macuda, 2001).

In horses, as in other species, the lateral position of the eyes has opened an interesting and recent field of research about visual laterality and its correlation with emotions. For example, some studies demonstrate that horses prefer the use of the left eye for the evaluation of the environment and objects with an emotional valence (Austin and Rogers, 2007; De Boyer Des Roches et al., 2008; Farmer et al., 2010).

Hearing

The great mobility of horses' ears is indicative of the importance of this sense. The audible range of the horse is from 55Hz to 33.5kHz, and its sensitivity peaks between 1 and 16kHz, the range of equine vocalization (Heffner and Heffner, 1983; Yeon, 2012). It is different from human beings (20Hz to 20kHz with its sensitivity peak at 1-3kHz), so they hear worse low-frequency sounds but have a good acuity at higher frequencies. This acuity is improved by the funnel shape of the ear, thanks to which the acoustic pressure gains by 10 to 20 dB. However, as in human beings, old age affects negatively the hearing abilities of horses. They can compensate for the progressive hearing loss with the other senses and by learning the routine, so detecting partial or complete deafness can be difficult. Nevertheless, this condition can partially compromise their communication both with humans and conspecifics (Rørvang et al., 2020). Deafness in horses can be congenital, usually due to a modification of the endothelin B receptor (EDNBR) gene. Often congenital deafness is related to the colour of the coat. Those horses with a splashed white or frame overo coat, a blend of this or with a tovero pattern, which are all typical coat colour patterns of paint and pinto horses, have a high risk. This risk can arise in those animals that have also blue eyes or extensive head and limb markings (Magdesian et al., 2009).

Similar to vision laterality, horses have also auditory laterality. In particular, they prefer the use of the right ear, so of the left hemisphere, when they have to listen to familiar neighbour calls. They do not show this laterality towards group members or strangers' calls (Basile et al., 2009). Horses also possess crossmodal recognition of familiar humans and conspecific. Therefore, they can integrate multisensory identity cues, and they can recognize familiar horses and humans also if they are deprived of one sense, for example, only using their vocalization without seeing them (Lampe and Andre, 2012; Proops et al., 2009).

Olfaction

The importance of olfaction in horses is suggested by the great development of olfactory organs and behaviour. Indeed, horses are microsmatic animals. The large dimension and the numerous folds of the olfactory bulbs in the horse's brain increase the area occupied by olfactory receptors, which receive information from the olfactory epithelium of the upper part of the nose through the olfactory neurons that are in turbinates. In addition, horses have a well-developed vomeronasal organ, which they exploit with the flehmen behaviour, to accurately detect non-volatile or poorly volatile compounds, often present in body secretion (Fig. 5) (Merkies et al., 2020; Saslow, 2002).

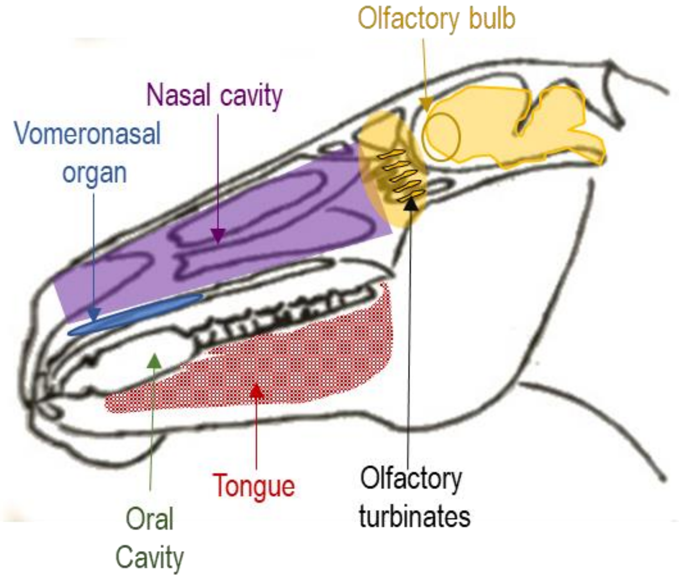

Figure 5. A simplified representation of nasal and oral cavities of the horse. The vomeronasal organ is located between the palate and the nasal cavity and it opens behind the upper front teeth. In the front part of the horse brain, there is the olfactory bulb that receives the projections of the sensory neurons of the olfactory epithelium, which covers the fold of olfactory turbinates (Modified from Rørvang et al., 2020)

Odours are especially important for social purposes. Indeed, horses can recognize individuals, including competitors, through odour information that they can get from urine, faeces and body (Péron et al., 2014; Stahlbaum and Houpt, 1989). Moreover, studies about the volatile compounds of hair have suggested that each individual has its own odour profile but it is similar to related individuals, showing a certain degree of kinship (Deshpande et al., 2018). Even if domestication has partially changed the use of olfaction (for example domestic modern horses smell the nose and the body of a conspecific, while Prezwalski stallions sniff the genitals), olfactory individual recognition has maintained its evolutionary advantage (Beaver, 2019). Indeed, an individual odour profile can determine the outcome of an interaction, and this should be considered also in the communication between the horse and its handler (Rørvang et al., 2020). In addition, olfaction is important for predator identification, and some species can maintain the innate and adaptive response of flight and avoidance even if they live where there are no predators. It has been demonstrated that the vigilance of horses in presence of a predator odour increases, and this explains why horses in an environment where there are also predators can become more reactive and unpredictable (Rørvang et al., 2020). Similarly, during windy days when odours are difficult to identify, horses can be more vigilant (Beaver, 2019).

The developed olfaction of horses should be considered in their relationship with human beings. For example, horses can be conditioned to associate certain odours with positive outcomes (such as grooming, feeding or social comfort). This positive odour conditioning can be, subsequently, employed to calm the animal during stressful conditions, such as regrouping or transport (Rørvang et al., 2020). In the meantime, it has been suggested that domestication could have facilitated the recognition of heterospecific emotions through odours. Therefore, horses can react differently in front of body odours collected from happy or frightened people (Sabiniewicz et al., 2020).

Taste

The sense of taste is not well studied in horses. It is still unknown if, as humans, they can combine odours and taste and experience flavours. They can distinguish salty, sweet, bitter, and sour but it is unknown if they can differentiate umami (Rørvang et al., 2020). Horses haven’t got conical papillae, but, on the dorsum of the tongue, they present numerous filiform papillae, which have principally a mechanical function. Interspersed among filiform papillae, on the rostral and at the sides of the tongue, there are fungiform papillae. Fungiform papillae, together with two vallate papillae at the caudal part of the tongue, and a pair of foliate papillae near the palatoglossal arch, are associated with taste buds (Beaver, 2019). On the wall of the larynx, there are some taste buds that can be stimulated by different things, like water, carbon dioxide and probably some chemical compounds from the nasal cavity (Yamamoto et al., 2001). The stimulation of G-protein linked to receptors of taste buds by flavour molecules can cause sweet and bitter sensations, while salty is caused by the stimulation of receptors by alkali metal ions (Beaver, 2019).

Horses’ responses to different tastes can change among individuals, and there is a great variety of taste preferences. Flavour can affect the time of consumption and the avoidance or not of a certain food, but nutritional components seem to be more important than taste and odour in nutritional choices (Goodwin et al., 2005; van den Berg et al., 2016).

Tactile Perception

The largest sensory organ both in humans and in horses is the skin. Horses' skin has both mechanical and thermal receptors, and its sensitivity change according to the area of the body, which have a different density of sensory nerve receptors. For example, the muzzle, neck withers, cornets, shoulders, lower flank and rear are most sensitive. In particular, the facial area has a thinner epidermis and, around the muzzle and the eyes, there are the vibrissae, another tactile organ that differentiate from hair follicles because they have a greater enervation, are thicker and do not moult (Fig. 6). Therefore, the muzzle is particularly sensitive and the removal of vibrissae for esthetical purposes and the use of restrictive nosebands cause welfare concerns (Beaver, 2019; Rørvang et al., 2020).

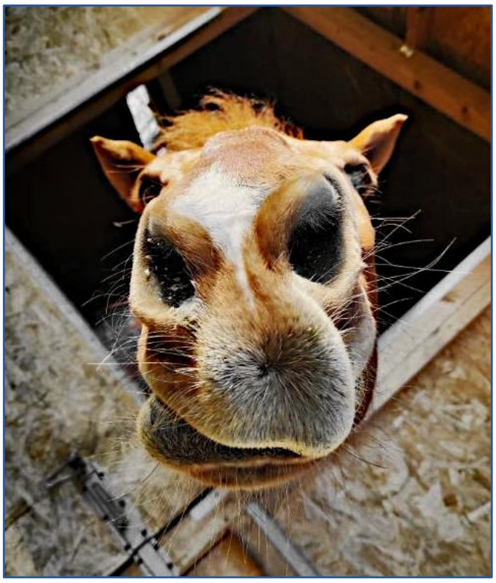

Figure 6. Horse's muzzle with vibrissae (Source: picture of Irini Kiumurgis)

Tactile stimulation can be a vehicle of communication with conspecifics and with humans. Grooming and mutual grooming both intraspecific and with humans is a positive behaviour. Indeed, mutual grooming between conspecific is used to reinforce social bonds, and researchers have shown how the grooming of particular areas, such as withers, can have a calming effect on the horse, lowering the heart rate (Feh and de Mazières, 1993; Normando et al., 2003). A positive tactile stimulation, such as a simulation of grooming and the scratching of itchy areas, has an innate reinforcing quality and can be exploited as a primary positive reward in human-horse communication. Also, massages can be considered a positive tactile stimulation and, as well as grooming, can induce relaxation (Rørvang et al., 2020).

There are also unpleasant stimuli that should be taken into account when tactile perception is considered. For example, the presence of biting flies can affect the feeding patterns of semiferal horses (Mayes and Duncan, 1986). Horses try to avoid unpleasant stimuli, such as flies, swishing the tail, stomping, shaking or flicking the ears (Saslow, 2002). In equestrian sports, the use of some devices, such as whips and spurs, should take into account the high tactile sensitivity of these animals, to safeguard their welfare (Rørvang et al., 2020). Indeed, these devices, as well as nose and ear twitching employed to immobilize animals, cause pain ("an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" (Raja et al., 2020)) to the animal. As a result of the activation of the complex neural mechanism that is below the pain sensation, the animal response includes not only reactive spinal reflexes but also affective and behavioural reactions (avoidance and escape) (Rørvang et al., 2020). Therefore, the knowledge of the tactile perception of horses is important to reconsider handling techniques employed with horses, taking into account also the individuality of tactile sensitivity (Rørvang et al., 2020).

1.1.3 Time budget

The behaviour of an animal species derives from its evolutionary process. Regarding horses, they have evolved to be plain and herd animals, and this influences their ingestive and locomotive behaviours and their sociality. In addition, they are flight animals and this justifies their response in front of threatening stimuli (Zeitler-Feicht, 2004). The ability of horses to adapt and succeed in different types of environments suggests that domestication has not influenced their behavioural repertoire, which has remained unmodified from that of their ancestors. Therefore, horses are still mentally and physically adapted to free-ranging life on open plains or mountains (Goodwin, 2007).

Starting considering ingestion behaviours, the horse is a preferential grazer, even if in some environments it can also browse trees and bushes (Beaver, 2019; Goodwin, 2007). Horses spend 70-80% of the day on eating behaviour (about 16 hours). They prefer grazing during the first half of the morning and late in the afternoon, but they eat during both daylight and night (Goodwin, 2007; Zeitler-Feicht, 2004). The eating pattern can change according to the period of the year, and it is influenced by weather conditions and insect presence. For example, during the summer, due to the presence of insects and flies, horses prefer to eat in the early morning rather than in the afternoon, and they usually rest during the warmer hours of the day (Beaver, 2019). They eat with lowered head position, as an antipredator strategy, because this allows them to detect the entire surroundings (Fig. 7). In addition, while they graze, they slowly move, following a trajectory that is parallel to the wind direction, to maintain also olfactory vigilance (Beaver, 2019). This slow locomotion is a fundamental daily activity for horses (Zeitler-Feicht, 2004). In addition, the importance of respect for foraging motivation in these animals is evidenced by the fact that stabled horses that have a restricted possibility of turnout and that are usually fed with infrequent high concentrate meals can develop abnormal oral behaviours and behaviours in anticipation of the meal as an expression of frustration. This problem can be partially relieved with *ad libitum* feeding, giving the possibility of expressing their species-specific behaviour (Hothersall and Casey, 2012).

Figure 7. Horses that are grazing on a pasture with lowered head position (Source: picture of Martina Lamanna)

Horses are selective grazers and they use principally olfaction to select food. Therefore, if it is possible, they defecate in separate areas and avoid eating where faecal odour is present (Beaver, 2019). The creation of separate latrines reduces the possibility of parasite ingestion (McGreevy, 2012). Elimination behaviours (defecation and urination) have not only the aim of excreting waste substances from the organism but also of social communication in both stallions and mares (Beaver, 2019; McDonnell, 2003; McGreevy, 2012). For example, stallions can defecate near some strategic positions, such as water points or where the herd has to transit, or on the faeces of other horses, creating dung piles. This marking behaviour can help to avoid numerous conflicts (Beaver, 2019; McGreevy, 2012).

After foraging, the second most frequent behaviour is standing, which approximately occupies 25-36% of the daytime. Of the time spent in a standing position, 60% is alert and 40% is resting (Beaver, 2019). Resting can occur several times during the day and the night, and horses select those resting sites that allow them to easily detect possible threats and escape, and, secondarily, to protect themselves against insect or weather conditions (McDonnell, 2003). Horses rest while standing or in recumbency, and, taking advantage of living in a group, while they rest or sleep, usually, some members of the herd remain vigilant as sentinels against predators (Beaver, 2019; McDonnell, 2003). The process from wakefulness to deep sleep is gradual, and horses have also an intermediate phase that occurs before drowsiness. During drowsiness, which is considered the first phase immediately before sleep, the horse is inattentive, but it can quickly change its status if stimulated (Dallaire, 1986). However, true sleep occurs with a state of unconsciousness and can be divided into the SWS (slow wave sleep), which is closest to consciousness and is the first phase of the sleep, and the REM (rapid eye movement) or paradoxical sleep that is the deep sleep, during which it is very difficult to awaken the horse. A single sleep cycle goes from the onset of REM sleep to the next and lasts 15 minutes (about 6.4 minutes of SWS and 4.2 minutes of REM). Paradoxical sleep is always preceded by SWS sleep, and between sleep cycles, there are drowsiness and an intermediary phase, which is similar to drowsiness but has an arousal threshold more similar to that of wakefulness. Usually, horses' sleep sessions last 30-40 minutes. Sleep sessions can occur every time during the day, but especially during the night (Belling, 1990; Dallaire, 1986). During the 24 hours of the day, horses are awake and conscious for 18-19 hours, to avoid predators, while drowsiness lasts about 2 hours, and sleep 3 or 4 hours, of which REM sleep occurs for no more than 1 hour (Beaver, 2019). The electroencephalographic recordings are different for the different phases (Fig. 8):

- wakefulness is characterized by beta waves, which have a low amplitude (10-30 μV) and a fast frequency greater than 12 cycles per second (cps), up to 40cps;
- intermediate phase and drowsiness present alpha waves, which have a medium amplitude (150 μV) and a lower frequency (8-12 cps);
- SWS recordings show delta waves, with a large amplitude (up to 250μV) and a frequency of occurring at 1–4 cps;
- REM sleep has a pattern with beta and theta waves (medium amplitude waves that occur at 4-8 cps) (Dallaire, 1986; Williams et al., 2008).

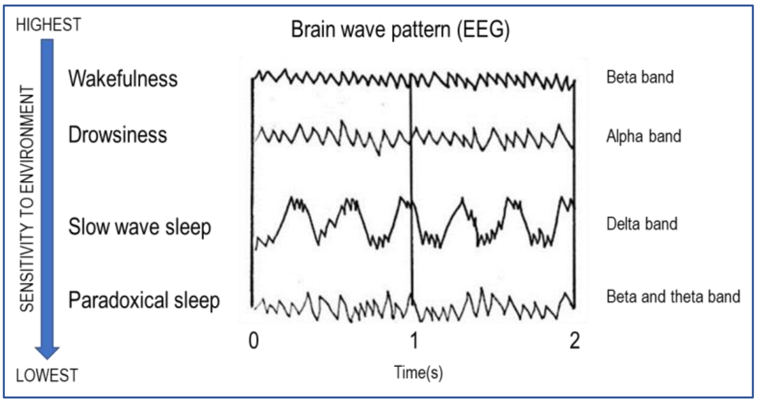

Figure 8. Schematic representation of the electroencephalographic tracings that shows the wave patterns for different levels of vigilance (Modified from Beaver, 2019 and Dallaire, 1986)

SWS and drowsiness can occur both in recumbency and in a standing position, with lowered head, partly closes eyes and one hind leg flexed. On the contrary, REM can occur only in lateral recumbency (Fig. 9), or, if it is impossible, the horse needs to rest the head and body against a surface. Recumbency occurs for 15% of the 24 hours, and lateral recumbency is about 3% of the time in adult free-roaming horses (Beaver, 2019; Dallaire, 1986). Stabled horses spend about 5 hours into recumbency, usually during the night. 1 hour of those 5 hours is spent in lateral recumbency. In pastured horses, the time spent lying decreased to 3 hours, with about 30 minutes of lateral recumbency. Horses need at least 30 minutes of REM sleep per day (Beaver, 2019; Williams et al., 2008). However environmental factors, such as social and environmental insecurity (social isolation or new environment) or inadequate size or bedding of the stall, and chronic pain can influence negatively the quality and quantity of sleep. This can cause sleep deprivation, and the impossibility of lying down can result in a partial collapse of the standing animal (Belling, 1990; Bertone, 2006; Burla et al., 2017).

Figure 9. Pony in lateral recumbency (Source: picture of Giovanna Marliani)

As previously stated, horses evolved in open plain habitat, therefore movement and the possibility of escape is fundamental (Beaver, 2019; Zeitler-Feicht, 2004). Locomotion occupies 5-8% of the activity budget of an adult horse. Usually, trot and gallop are occasional and for short periods, and most of the time spent in locomotion is constituted by walking. Locomotive behaviours include the four natural gaits (walking, trot, canter and gallop), but also jumping, group movements and swimming. They move slowly while they graze, and they can cover several miles, for example, to reach a water point or an area where they can rest (Beaver, 2019; McDonnell, 2003). The neck has an important role in balancing the body weight during movement, indeed 60-65% of body weight is carried on the forelimbs (Beaver, 2019). Also foal, after several minutes from its birth, can stand and look for its first meal, and soon it becomes able to follow its mother for a short distance (Waring, 2003). For the development of the musculoskeletal system locomotor activity is fundamental in foals. In particular, cantering, which is typical of young foals, especially during the first month, and play activities have an important role in this sense because both cause a high loading intensity of musculoskeletal structures. Thus, keeping foals at pasture with the possibility of exercise is central (Kurvers et al., 2006).

The frequency of playing is high in foals and decreases with increasing age, even if also adults show play behaviours, especially bachelor and harem stallions. The play has several functions: it improves not only motor skills and musculoskeletal and cardiovascular fitness but also favours the knowledge of the environment and the development of survival, social and communication skills (Cameron et al., 2008; McDonnell, 2003). It can be distinguished by solitary (object and motor play) or social play, during which horses simulate fight or reproductive behaviour. Differentiating serious situations from play can be difficult, however during play vocalizations are rare (McDonnell, 2003). Colts usually more frequently initiate the play and are generally more engaged in social play than fillies, because they need to develop fighting skills to enhance their reproductive success. Thus, social roles can affect the type and frequency of play, which is also fundamental in the construction of social bonds (Cameron et al., 2008; Hartmann et al., 2012; McDonnell, 2003). Another factor that influences the amount of play is food availability (an increase in nutrition causes an increase in energy and, consequently, in the amount of play). Therefore, foals play more when their mothers' investment, which can be reflected in a loss of their condition, is higher. Individuals that played more, survived more and developed better physical conditions and social skills (Cameron et al., 2008).

Another important behaviour for foals is exploration. Investigative behaviours start soon after the birth and foals begin to investigate visually and orally, first of all, to explore their mother to nurse and then the environment. The mother, initially, regulates the social contacts and exploratory range of the foal (Beaver, 2019; Waring, 2003). Adult horses show explorative behaviours too, but they are more cautious and the distance that they maintain during the exploration can be indicative of their state of apprehension. Exploration towards a new object, odour or sound, or towards another subject is done employing all the senses, looking, smelling, mouthing, pawing, and tasting, and it lasts until the horse decides to go away or to interact. Explorative behaviour is fundamental for the acquisition of awareness about the surrounding environment, avoiding threats and identifying the resource, and helps behavioural development (McDonnell, 2003; Waring, 2003).

Exploration and play are considered, together with grooming, self-rewarding behaviours because they cause the release of endorphins, such as endogenous opioids and dopamine, allowing the animals experience short-term satisfaction. Therefore, animals are highly motivated to perform these behaviours, not only for their biological function. For the same reason, these are those behaviours that are prone to develop into stereotypies in conditions of frustration, chronic stress or boredom (VanDierendonck and Spruijt, 2012). Grooming can be distinguished as self-grooming, grooming using inanimate objects and allogrooming. This behaviour has several biological functions, such as protection against insects, parasites' control, distribution of body oil, care of skin and hair and social facilitation (Beaver, 2019). Self-grooming is performed using a part of the body to groom another (such as nibbling, rubbing against the forelimb or scratching with the hind hoof), shaking, or rolling (Beaver, 2019; Crowell-Davis, 1987). The rate of grooming decreases with illness, except for the infestation by ectoparasites when self-grooming increases (Crowell-Davis, 1987). On the other side, allogrooming is defined as when a horse nibbles a partner in preferred areas that cannot otherwise be reached, often the whiter is the preferred part (Beaver, 2019). Allogrooming has been identified, together with play, as an “ethological need” for horses (VanDierendonck and Spruijt, 2012). Indeed, this behaviour has a fundamental affiliative social function and, as stated before, activates the self-rewarding system. All individuals of the species regularly perform this behaviour, and both allogrooming and play start within the first and second week of life. This behaviour identifies a preferential long-lasting bond between two individuals, and it has long-term advantages in terms of social cohesion, safety, and cultural transmission (VanDierendonck and Spruijt, 2012). Moreover, the prevention from both play and allogrooming seems to induce chronic stress and, once the individual can interact again with a conspecific, both of this behaviour are over-expressed, probably as a rebound effect (VanDierendonck and Spruijt, 2012).

1.1.4 Social organization

Feral horses, as social animals, form cohesive social bonds within their family and bachelor bands (Mills and Nankervis, 1999). However, in the modern husbandry system, most of the time domestic horses are kept solitary or are in a group that does not reflect the composition of that of feral horses. Domestication and selection process usually influence mostly the fear and reactivity of animals, but not (or only little) their sociality. Indeed, domesticated horses that return to become feral maintain their social behaviour and the capability to organize in a well-defined social group (VanDierendonck and Spruijt, 2012). Therefore, in welfare science, there is an increasing interest in deepening the research into the capability of coping within non-voluntary domestic groups, to ameliorate the introduction of horses and their formation (Hartmann et al., 2009). Most of the owners' concerns about keeping horses in a group are the possibility of injuries and aggression toward new individuals during their introduction into an established group (Hartmann et al., 2009). On the other hand, deprivation of social contact causes welfare impairment, and horses that cannot interact with conspecifics during ontogeny are highly predisposed to develop impaired social skills and can have several difficulties in coping with social challenges (Hartmann et al., 2009). Keeping horses in groups fulfils their ethological needs and favours movement, enhancing gastrointestinal and musculoskeletal health. In addition, group-kept horses are easier and safer to handle than solitary-kept ones (Hartmann et al., 2017).

Feral horses have a highly complex social system that is based on the formation of stable breeding social groups called bands or harems, composed of one stallion, one or more unrelated mares and their off-springs (foals, yearling and 2 years-old off-springs). The total number of horses in a band varies between 4 and 17. In addition, sometimes a bachelor stallion can ally with the stallion of a band. In this case, he can occasionally breed with some mares but the key point is that there are several advantages in terms of defence of the harem (Beaver, 2019; Cameron et al., 2009). The band membership among adults is stable but, to avoid the breeding of closely related members, both male and female offspring disperse from the natal band between 4 and 6 years old and can be actively moved away by both mares or stallions (Cameron et al., 2009; McGreevy, 2012). Fillies tend to leave the natal band later than male foals. They remain affiliated with their mother and do not breed with the stallion. Once they have left the natal band, they will form another harem or, if the number of stallions is scarce in the territory, they can aggregate together. On the other hand, young males usually aggregate in bachelor groups, which sometimes include also stallions that have lost their role. Bachelor groups remain nearby the natal band, and males wait for forming their harem, joining other females or defeating another stallion (McGreevy, 2012; Waring, 2003). The territory occupied by a band is called the home range and its size varies according to the availability of resources. Horses usually are loyal to their home range but tend to defend more the social members of the group than the territory. Therefore, home ranges of several bands can overlap, and a herd of horses that occupy a certain territory can be composed of breeding and non-breeding groups, bachelor groups, sub-adult group of females, young solitary males, and mares with their offsprings (Beaver, 2019; Waring, 2003).

Living in a group means cohesion, which can be reached by the synchronization of behaviour and establishing predictable social relationships (Hartmann et al., 2017). As already stated, strong and long-lasting affiliative relationships involving preferred partners give stability to the group and can be established with several behaviours, especially allogrooming or resting together (Cameron et al., 2009; Waring, 2003).

Cohesion and coordination of behaviour as a group have several advantages, for example in feeding and anti-predator strategies, and the concept of leadership is born to identify the process of social influence by an individual that guides the action of the group (Smith et al., 2016). This concept in horse society has been widely discussed and it is not clear what characterized a leader, identified as the initiator of action. Indeed, the leader is not necessarily the older or highest-ranked subject, but anyone can act as a leader (Hartmann et al., 2017). Bourjade and colleagues (2009), studying movement in Prezwalskii’s horses, identified that the decision-making process before the movement can be shared by several individuals, and that probably it is driven by ecological causes and the consensus during departure can be dependent on social facilitation. At the same time, Briard and colleagues (2015) suggest that the success of the collective movement depends on the motivation of followers rather than on the characteristics of the initiator, which is only a trigger for a collective decision that has already been taken. In addition, affiliative bonds between individuals play a fundamental role in the joining process and preferential partners usually follow each other (Briard et al., 2015).

Another concept that should be discussed, speaking of social organization, is dominance. Dominance was defined by Drews in 1993 as: “An attribute of the pattern of repeated, agonistic interactions between two individuals, characterized by a consistent outcome in favour of the same dyad member and a default yielding response of its opponent rather than escalation. The status of the consistent winner is dominant and that of the loser subordinate. Dominance status refers to dyads, while dominance rank, high or low, refers to the position in a hierarchy and, thus, depends on group composition. Dominance is a relative measure and not an absolute property of an individual.” Therefore, in a stable group can be important to recognize an individual and have a memory of the outcome of previous conflict encounters. However, between two unfamiliar individuals, with no previous experiences, other features can be considered, such as morphological ones or the displays that correlate to the competitive ability of the opponent (Hartmann et al., 2017). The concept of dominance refers to the resolution of conflicts and access to resources, and it considers a complex pattern of interactions, even if most of the time this term is used to refer to agonistic behaviour (avoidance and aggression). However, once the order is established, horses can behave avoiding conflicts and aggressive behaviours (Hartmann et al., 2017). Hierarchy in horses is usually described as almost linear, but triangle often occurs. Indeed, horses can defend existing relationships with interventions between dyadic and this can change the outcome of a situation. Sex, age and body weight are not always predictors of rank and a stallion’s rank depends on the context and is not always the higher one. Harem stallions have less contact with the group than mares and their function is to maintain group cohesion and patrolling, remaining at the periphery of the band (Hartmann et al., 2017). However, the outcome of a contest depends not only on the rank but also on the motivation toward a resource and the cost associated with acquiring it (Drews, 1993).

The concept of dominance and the belief in the existence of a dominant mare or a leader stallion in a group is questioned, and research is trying to focus on more complex patterns to explain coordination and conflict resolutions in a social group (Hartmann et al., 2017). Indeed, as stated by Altmann in 1981, the concept of dominance is an abstraction made by humans to explain animals’ interactions, which consist of behaviours and have physical properties (“Dominance relationships are an invention, not a discovery […]. Do dominance relationships exist? Yes, in the mind and notebook of the human observer […]. With few exceptions, however, there is nothing in the agonistic behavior of animals that implicates an ability to make such abstractions. Are dominance relationships important? They surely are, but to the investigators, not to their subjects”).

1.1.5 Undesired behaviour, behavioural problems and stereotypies

Management practice and environmental inadequacies have led to the development of several behavioural problems in horses (Beaver, 2019; Hockenhull and Creighton, 2014). However, lots of the behavioural problems reported by owners can be more correctly identified as undesired behaviours, which can be normal behaviours for horses but that people cannot understand or cope with (Hockenhull and Creighton, 2014; Hothersall and Casey, 2012). An example is avoidance behaviours (e.g., not jumping toward an obstacle or not moving forward) that are identified by owners as “stubbornness”, but they are often shown by the horse to avoid an anxious or painful condition (Hothersall and Casey, 2012). Other undesired behaviours can be considered all the behaviours performed during pain conditions (e.g., aggressions), or accidentally reinforced by the person (e.g., kicking the door before food administration (Hockenhull and Creighton, 2014). The performance of these behaviours could be not necessarily a real problem for the horse, but the methods employed to solve them by people can create several problems. Indeed, horses can apply alternative avoidance strategies or can stop to respond because they learn that escaping from an anxious situation is impossible (Hockenhull and Creighton, 2014; Hothersall and Casey, 2012).

There are several categorisations of horses' behavioural problems by the owners. They can use the general term “vices”, especially for stereotypies, or they can recognise handling issues, frustration behaviour, abnormal oral/ingestive behaviour, aggression toward people, and locomotor stereotypies. However, these classifications are reductive and difficult to apply (Beaver, 2019). Indeed, behavioural problems are complex, with different causes and their expression changes among individuals. Among the behavioural problems reported fifty per cent are incidental or nuisance behaviours, as previously described, and the others are medically based problems or stereotypic behaviours (Beaver, 2019).

Stereotypies are repetitive and invariant behaviours without an apparent goal. They are performed as a result of motivational frustration and central nervous system dysfunctions and can become habits that are difficult to solve or rectify (Mason and Latham, 2004). Therefore, stereotypies can be indicators of poor welfare and suboptimal environmental conditions and management practices either currently or in the past (Hothersall and Casey, 2012; Mason and Latham, 2004).

Although the domestication process, behavioural patterns and needs of horses are not so different from those of their wild counterpart. They are social grazing animals that travel a lot and need to forage most of the daytime. However, often in domestic environments horses are kept solitary, their feeding routine is based on high-energy diets with lots of concentrates and relatively little forage provided twice a day, and the restricted environment where they live does not allow adequate movement (Sarrafchi and Blokhuis, 2013). Because of these environmental conditions, domestic horses' time budget is different from that of wild horses. Restrictions in diet, locomotion and social interactions cause chronic stress to the animals and can be considered risk factors for the development of stereotypies (McBride and Hemmings, 2009; Sarrafchi and Blokhuis, 2013). Another risk factor is weaning and its management. Indeed, foals that during post-weaning receive concentrates and live in a confinement environment than in pasture are more predisposed to develop stereotypies (Waters et al., 2002).

Stereotypies are considered by horse owners as "vices" often associated with health problems (McBride and Hemmings, 2009), and can be divided into oral (e.g., crib-biting, tongue flicking, wind-sucking) and locomotor (e.g., waving, box-walking) (Roberts et al., 2017; Sarrafchi and Blokhuis, 2013). The physical methods commonly employed by owners for preventing horses to show stereotypies are useless and can further compromise the welfare of the horse (McBride and Hemmings, 2009; Sarrafchi and Blokhuis, 2013). Indeed, considering that stereotypies could be strategies to cope with the stress of suboptimal enrichment, preventing these behaviours without solving the real causes can have a deleterious effect on horses' welfare. Therefore, it would be better to ameliorate environmental and management conditions, such as giving more roughage and hay and less concentrates, increasing the possibility to access pasture or paddock with conspecifics, giving environmental enrichment, not isolating foals during weaning, not confining horses with stereotypies, because there is no proof of imitation by other horses (Sarrafchi and Blokhuis, 2013).

Crib-biting and weaving, together with box-walking, are the most prevalent stereotypical behaviours of horses (McBride and Hemmings, 2009). If we consider stereotypies as frustrated appetitive behaviours that, because of the restrictive nature of the environment, cannot reach the consummatory goal and the negative feedback on the motivation to perform them (Hughes and Duncan,1988), weaving can be easily explained. Indeed, weaving emerges every time that horses cannot reach the consummatory goal (grazing and interacting with conspecifics), while it ceases when the motivation can be fulfilled, allowing the horse to have social interaction and a more frequent turnout (Roberts et al., 2017). While it is difficult that weaving becomes a habit, crib-biting tends to persist, even if the consummatory goal is achieved (Fig. 10). Indeed, neural differences in crib-biting animals facilitate the acquisition of this behaviour as a habitual response and make it difficult to stop with environmental intervention, so it is important to prevent its development (Roberts et al., 2017).

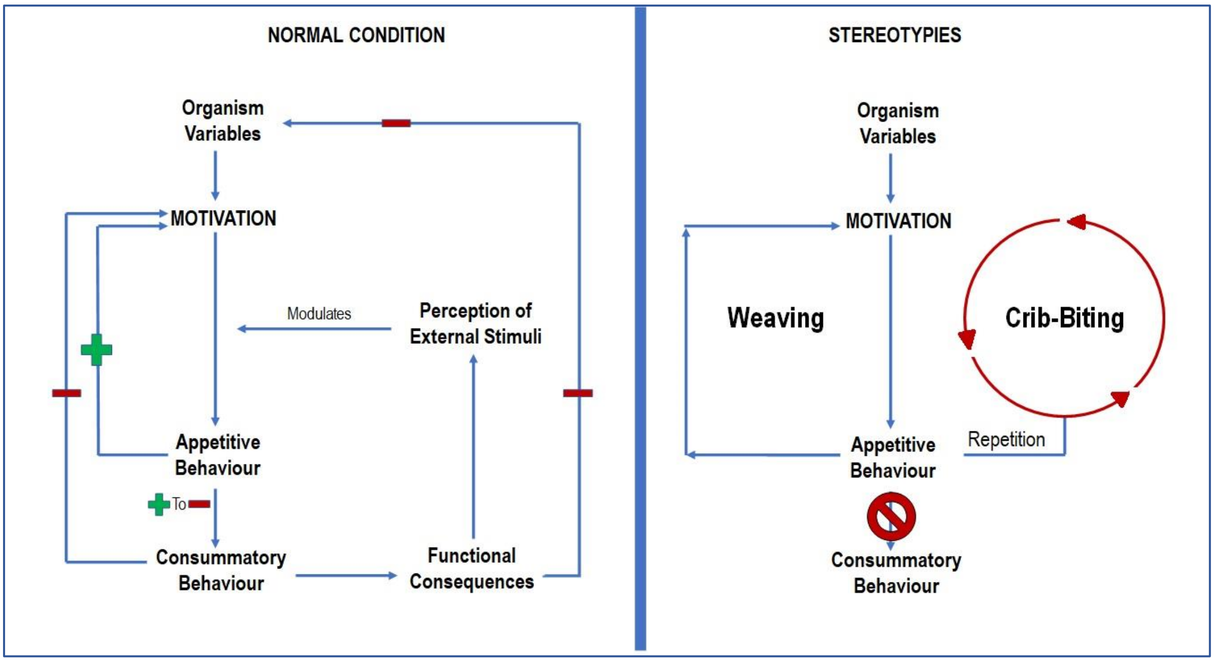

Figure 10. On the left, there is the schematic representation of the motivational model proposed by Hughes and Duncan (1988), according to which organism variables determine an animal's motivation to adopt appetitive strategies to reach the consummatory goal. Appetitive behaviour has a positive feedback effect (✚) on motivation, which increase until the goal has been fulfilled. The expression of consummatory behaviours has a negative feedback effect (▬) on motivation and leads to functional consequences that solve the organism's needs. When the animal cannot perform the consummatory behaviour (🚫) can remain in an appetitive state and can develop stereotypies (on the right). While waving can cease once the animal can reach its consummatory goal, Crib-Biting becomes a habitual response and does not stop even if the consummatory aim has been met (Modified from Roberts et al., 2017)

Once crib-biting has become part of the behavioural repertoire of the individual, it will be shown often during the postprandial of highly palatable food ratio, usually rich in cereals. Cereal-based concentrate feeds reduce the time of mastication and production of saliva, causing the reduction of pH of the digestive system, and crib-biting consents to produce more alkaline saliva to contrast the visceral discomfort caused by acidosis. This seems also the cause of the origin of crib-biting, which is often linked with the presence of gastric ulcers and gut inflammation (McBride and Hemmings, 2009; Roberts et al., 2017). However, it is difficult to establish if this behaviour is an attempt to ameliorate gastric discomfort, caused by the neural sensitization induced by visceral pain, or a combination of these conditions (Roberts et al., 2017).

McBride and Hemmings (2009) conclude that equine stereotypies are directly related to the impossibility of fulfilling their behavioural needs and that chronic stress conditions, especially during animals' development and probably together with genotypical characteristics, may predispose animals to develop stereotypies, causing the alteration or "sensitization" of the mesoaccumbens system (ventral striatum). Indeed, the central nervous system (CNS) and basal ganglia are strictly involved in the development and maintenance of stereotypical behaviours. In particular, the dopaminergic system and the striatum, divided into ventral (nucleus accumbens) and dorsal (caudate nucleus and putamen) striatum, have a key role. The ventral striatum and mesoaccumbens dopamine pathway regulate goal-direct behaviour, in terms of acquiring a reward or avoiding aversive stimuli, so it is directly linked to animal motivational states and the development of stereotypies. On the other hand, the dorsal striatum and nigrostriatal dopamine system are involved in associative learning and the formation of habits (Ikemoto and Panksepp, 1999; McBride and Hemmings, 2009). The important role of dopamine in the expression of stereotypic behaviours is proved by the fact that the administration of dopamine can induce the manifestation of stereotypic behaviours similar to those induced by environmental conditions, and in the meantime, the administration of dopamine antagonists seems to attenuate the expression of spontaneous stereotypies (McBride and Hemmings, 2009). Indeed, in the striatum can be found two types of dopaminergic receptors, D1 and D2. Comparing crib-biting and non-stereotypic animals, it was found that the density of D1 and D2 receptors in the nucleus accumbens was higher in animals with stereotypy, while the density of D1 and the affinity of D2 receptors into the caudate nucleus were lower. This last result may be caused by an extensive period of expression of this ritualised behaviour by the animals (McBride and Hemmings, 2005). Therefore, the increased neural transmission ("sensitization") in the striatal region seems to be the cause of the development of crib-biting. In particular, animals with an upregulated mesoaccumbens dopaminergic pathway, which is the primary neural centre of initiation and regulation for goal-directed behaviour, can present an enhanced motivation in any goal-direct situation. Thus, if environmental conditions prevent these animals to achieve their goal, they remain highly motivated toward the goal achievement, in a persistent appetitive phase. As previously stated, this condition is the basis of stereotypies development. This hypothesis is sustained by the fact that often stereotypies, also weaving, is shown in concomitance with cues that indicate food arrival or the possibility to eat, which are both highly motivational conditions (McBride and Hemmings, 2009).

The “sensitization” of mesoaccumbens system can be caused by prolonged chronic stress conditions together with a genotypical predisposition. However, it cannot be excluded that some situations, such as the high exercise level with the excessive release of endogenous opioids or the highly palatable food far from what horses would eat normally, can influence the development of a hypermotivated reward-seeking phenotype (McBride and Hemmings, 2009). While the neural modifications under crib-biting have been widely studied, those under weaving are still unknown. However, Kirsty and colleagues (2015) suggest that also weaving animals present an increased activity of the ventral striatum, causing a high motivational state, but the dorsal striatum function is normal.

Another consequence of chronic stress, pain, poor environmental conditions and welfare can be unresponsive inactivity, which could be considered a depression-like state (Fureix et al., 2012). Fureix and colleagues (2015, 2012) identified some similarities with human depression like less reactivity, more anxiety, apathy, and, finally, anhedonia. Indeed, horses in a depression like-state were characterized by: a “withdrawn” atypical position (flattened position of the neck, fixed gaze and absence of movement of head and ears, which point backwards); less reactivity to tactile stimulation and the sudden appearance of a human; anhedonia; lower baseline plasma cortisol level; and, in front of a novel-object, less exploration and more high emotional response (Fureix et al., 2015, 2012). However, those results are not sufficient to identify the aetiology of this condition and a true diagnosis of clinical depression in horses cannot be done because further studies are needed (Beaver, 2019).

1.2 ANIMAL WELFARE

1.2.1 History and Evolution of Animal Welfare

Animal welfare includes several aspects: scientific, ethical, economic, and political (Carenzi and Verga, 2009). Indeed, even if animal welfare is about a potential measurable quality of an animal, thus science, this concept implies several ethical consequences, such as what humans should do about it. Therefore, it is important to understand the development of ethical and moral positions under animal welfare, before applying science (Broom, 2011).

Welfare has always been part of animals, but humans' knowledge and consideration about it have evolved a lot over time. Although humans have always known how to behave morally, what is changed is the consideration of which living species deserve moral treatment (Broom, 2003). In some human cultures, such as in Buddhist and Jain religions, the range of living beings that should be respected has always been wide. However, in most human societies, the improvement of communication ways and knowledge about the functioning of humans and other animals have contributed to broadening the audience of individuals that should be subjected to moral actions (Broom, 2011). Indeed, it is more difficult for people to act harmfully towards others if the fact is known by society. The explosion of communication in the twentieth century has contributed to the rapid spread of information around the world and hiding harmful actions has become harder (Broom, 2003). In addition, the increasing knowledge about how the brain controls behaviour, neurobiology, and physiological similarities between humans and animals have elicited a revolutionary change in human attitude (Broom, 2011).

However, even if Greeks, Maya and other cultures already made physiological, behavioural and pathological descriptions of animals, and recognized similarities between humans and animals and the existence of emotional responses, the view of animals as "automata" proposed by Descartes was often employed during human history because it resulted convenient to justify animal exploitation (Broom, 2011; Duncan, 2006). On the contrary, during the early years of the 19th century common-sense and scientific community recognised sentience and suffering in animals, starting with Jeremy Bentham (1789), who stated: "The question is not, Can they reason? nor, Can they talk? but, Can they suffer?". In 1824, the Royal Society for the Prevention of Cruelty to Animals (RSPCA) was founded and it firstly focused on cruelty in the London meat market. Thanks also to RSPCA, during the entire 19th century the legislation about animal cruelty was enlarged to a wider group of species and activities (e.g, sports or experimentation) and the existing measures were strengthened (Harrison, 1973). In 1913, also in Italy law 611, concerning the provisions for the protection of animals, was promulgated and it expressly prohibited cruel acts, torture and unjustified torture against animals[1]. However, in the first 70 years of the 20th century, the Behaviourism of James, Skinner and Watson, spread especially among North American schools of psychology and posed some reluctance to accept the idea of feelings and consciousness, especially because of the difficulty to investigate and measure them. Indeed, Behaviourism focus on what was objectively observable (Duncan, 2006). Certainly, there were some exceptions, such as McDougall (1926) and Young (1959), which suggest that affective states regulate motivation and behaviour. However, the advances made by biological sciences, the recognition of ethology and neuroscience by the scientific community, the use by the ethologists of certain terminologies, such as pain or hunger, which recalls feelings, and the publication of Griffin about awareness and mental experiences of animals in 1981 shifted the focus again on animal sentience (Duncan, 2006; Griffin, 1976). Sentience is a key point in animal welfare science and scientists have demonstrated an increasing interest in animals' capability of experiencing positive and negative affective states (Duncan, 2006).

In 1964, Ruth Harrison published "Animal Machines", where she denounced the intensive farming system and the treatment of animals as machines, considered only for the production of goods. Here she was concerned about not only the stress animals are subjected to but also how animals can feel (Harrison, 1964). Following the publication of this book, in 1965 the Brambell Committee, headed by Professor F. Rogers Brambell, was set up by the British government to investigate this matter (Broom, 2011). This led to the draft of the Brambell Report (1965) that recognized the necessity of new legislation about animal protections that took into account the feelings (especially suffering) of animals, understanding the importance of their sentience, and led to the foundation of the adoption of a scientific approach to animal welfare as a new discipline (Carenzi and Verga, 2009; Duncan, 2006). Indeed, the Brambell Report reported: ''Welfare is a wide term that embraces both the physical and mental well-being of the animal. Any attempt to evaluate welfare, therefore, must take into account the scientific evidence available concerning the feelings of animals that can be derived from their structure and functions and also from their behaviour" (Command Paper 2836, 1965). In

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[1] Legge 12 giugno 1913, n.611 concernente provvedimenti per la protezione degli animali. GU n.153; 02-07-1913

addition, W.H. Thorpe, an ethologist from Cambridge and a member of the Committee, in Appendix III of the report underlined how animals have biological needs, including showing species-specific behaviour, whose frustration can lead to several problems (Thorpe, 1965). This view was exemplified in the Five Freedoms, which were implicit in the Brambell Report but were codified and published in a press statement on 5th December 1979 by the Farm Animal Welfare Council (FAWC) for the definition of minimum welfare standards: "Freedom from thirst, hunger or malnutrition; appropriate comfort and shelter; prevention, or rapid diagnosis and treatment, of injury and disease; freedom to display most normal patterns of behaviour; freedom from fear"[2]. However, we can find the Five Freedoms and the consequent provisions as known today in the "Report on Priorities for Animal Welfare Research and Development" of the FAWC published in 1993[3]:

I. "Freedom from thirst, hunger and malnutrition - by ready access to fresh water and a diet to maintain full health and vigour
II. Freedom from discomfort - by providing an appropriate environment including shelter and a comfortable resting area.
III. Freedom from pain, injury or disease - by prevention or rapid diagnosis and treatment.
IV. Freedom to express normal behaviour - by providing sufficient space, proper facilities and company of the animal's own kind.
V. Freedom from fear and distress - by ensuring conditions and treatment which avoid mental suffering."

Following the publication of the Brambell Report, scientists began to study animal welfare, proposing applicable measures and definitions. Although Brambell Report pointed out the importance of feelings, during the 1970s scientists widely accepted the idea that welfare was well reflected by the status of physiological stress, finding stress measures useful indicators for the assessment of animal welfare (Benson and Rollin, 2004). Indeed, a biological function orientation on animal welfare developed in the 1980s. Scientists evaluated welfare status

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[2] Farm Animal Welfare Council, Press Statement on 5th December 1979. https://webarchive.nationalarchives.gov.uk/ukgwa/20121010012428mp_/http://www.fawc.org.uk/pdf/fivefreedoms1979.pdf Accessed 28/6/2022, 16:39:36

[3] Farm Animal Welfare Council, 1993. Report on Priorities for Research and Development in Farm Animal welfare. MAFF Publ., London, UK. https://webarchive.nationalarchives.gov.uk/ukgwa/20110909181241mp_/http://www.fawc.org.uk/pdf/old/animal-welfare-priorities-report-may1993.pdf Accessed 28/6/2022, 17:05:36

through physiological and behavioural indexes, without inferring experience of mental subjective states or affective states by animals. According to the biological function school, good welfare is considered when the animals grow, reproduce, and are healthy and without stress (Fraser, 2008, 2003; Green and Mellor, 2011). During the same years, the affective state orientation emerged. This was focused on the theory that welfare was entirely defined by how animals feel, calling “suffering” the presence of negative emotional states, and “pleasure” the presence of positive ones (Benson and Rollin, 2004). This school found its foundation in the book *Animal Suffering* by Marian Dawkins (1980), where she pointed out the centrality of animals’ feelings in animal welfare definition, but she recognised also the importance of other aspects, such as health. During the years, there was a progression from the idea that feelings were a necessary component of welfare (Dawkins, 1980; Duncan and Petherick, 1991; Duncan, 1993) to that whereby feelings are the only things that matter (Benson and Rollin, 2004; Duncan, 1996). In this last case, welfare is established when the adaptation of the animal to its environment occurs with positive affective states and without negative ones (Green and Mellor, 2011). Finally, a third orientation, natural living, arose in parallel to the others in animal welfare science. According to the natural living orientation, better welfare conditions may be when the animal is closer to its natural or wild state, especially considering the possibility of expressing its species-specific behaviour (Fraser, 2003). Considering only this last orientation can be inadequate because animals in their natural environment can suffer starvation, and malnutrition and can be exposed to predation. However, this approach can be useful, together with measures of biological functioning and affective states, to underline the consequence of restrictive housing conditions (Green and Mellor, 2011). These different orientations emphasized different value-based views (basic health and functioning, natural living, and affective states) and have led to the development of different criteria, which have become the rationale for a different approach to animal welfare (Fraser, 2008).

Therefore, animal welfare is surely a concept that can be scientifically discussed, but it is also value-based. Indeed, it has multi-disciplinary scientific roots but in the meantime is influenced by ethical, cultural, political, societal, religious and economic views (Fraser, 2008; Green and Mellor, 2011). The complexity of animal welfare has led to several different methods for welfare assessment and a general lack of consensus about its definition and how to improve it (Dawkins, 2021). One of the first and generally accepted definitions of welfare was that of Huge (1982), which defined animal welfare as a condition in which an animal was in harmony with nature or its environment. However, harmony cannot be measured, so, in 1986, Broom proposed another definition: ‘‘the welfare of an individual is its state as regards its attempts to cope with its environment’’. He considered coping as the condition where all mental and physical systems have worked so that the environmental impact is offset. In this definition, coping strategy defines the welfare state of an animal. In addition, feelings are biological mechanisms, results of natural selection and part of animals’ coping strategies (Broom, 2011). In 2005, Webster defined good welfare as “feeling good”, focussing more on the affective state. A similar definition to that by Broom was made in May 2008 by OIE (Office International des Epizooties, today the World Organisation for Animal Health or WOAH), which stated: “animal welfare means how an animal is coping with the conditions in which it lives”. One year later, the Farm Animal Welfare Council specified that “welfare principally concerns both physical and mental health, which is largely determined by the skills of the stockman, the system of husbandry and the suitability of the genotype for the environment”[4]. Similarly, the updated (2019) version of the ©OIE-Terrestrial Animal Health Code reports: “Animal welfare means the physical and mental state of an animal in relation to the conditions in which it lives and dies”[5]. Finally, Marion Dawkins tried to give a definition of welfare that could be universally accepted and understandable by scientists and not-scientists and could condense all the definitions already present. She explained that the impossibility to define animal welfare was caused by the complexity of the problem, due also to the existence of several different measures, and the problem to define consciousness scientifically. Therefore, she defined welfare as considering both health status but also what the animal wants (Dawkins, 2021, 2008). “Health and what animal wants” is a simple definition that gives the possibility to solve both the problem of complexity, prioritizing and understanding what measures are useful, and the problem of consciousness, because this definition avoids the term subjective conscious feelings but does not necessarily deny them. “Welfare” literally means “going well”, and it can be measured in terms of survival and offspring production. An animal with good welfare is healthy and has the prospective to be healthy in the future. Health is a common measure and is

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[4] Farm Animal Welfare Council, 2009 Farm Animal Welfare in Great Britain: Past, Present and Future. FAWC, London, UK. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/319292/Farm_Animal_Welfare_in_Great_Britain_-_Past__Present_and_Future.pdf Accessed 30/6/2022, 00:13:56

[5] World Organisation for Animal Health, 2021. Introduction to the recommendations for animal welfare. In: Terrestrial Animal Health Code, 29th Ed. World Organisation for Animal Health (OIE), Paris, France, Article 7.1.1.

universally accepted as an indicator of good welfare. Furthermore, "what animals want" helps to validate a series of measures to find out if the animals consider a certain situation positive (to be approached and repeated) or negative (to be avoided), thus defining the valence of affective states, which possibly could be conscious (Dawkins, 2021).

"What animals want" is also in agreement with the relatively recent attempt to define welfare not only negatively as an "absence of suffering", but also positively, considering the possibility for animals of having positive experiences and a life worth living (Boissy et al., 2007; Dawkins, 2021; Green and Mellor, 2011; Webb et al., 2019). In the last decades, the scientific community increasingly interested in emotional assessment and positive animal welfare (PAW). As demonstrated by the five freedom and welfare codes, traditionally welfare is over-focussed on the absence and neutralization of negative experiences and suffering. However, this is not a sufficient condition for good welfare. Therefore, although PAW recognises the importance of minimisation of negative welfare aspects, it affirms that this is not enough for animals for having positive experiences and emotions, so it is fundamental to give them also these opportunities (Boissy et al., 2007; Lawrence et al., 2019; Mellor, 2015; Yeates and Main, 2008). In the review by Lawerence and colleagues (2019), it was evidenced how positive welfare is defined by 4 key aspects: positive emotions, positive affective engagement, Quality of life and Happiness. Different scientific areas (ethology, neuroscience, psychology and also animal welfare science) have demonstrated that animals can experience emotions, and positive emotions are the central theme of PAW. In PAW, terms like pleasure, comfort, contentment, curiosity and playfulness are used to refer to positive emotions and the same methods of assessment previously employed for the evaluation of negative emotions are proposed for the positive ones (preference testing, judgment bias test, qualitative behavioural assessment (Lawrence et al., 2019; Mellor, 2012a). The second key component of PAW is positive affective engagement, which refers to the fact of giving the possibility to the animals being engaged in rewarding goal-direct behaviour, so experiencing positive emotions (Mellor, 2015). This concept is strictly linked to motivational affective states (MASs) proposed by Fraser and Duncan (1998). These authors have distinguished: a) negative MASs that emerged by "need situations", are caused by changes in the environment and the body and, if prolonged, can be a threat to animal's survival (e.g., hunger or thirst); b) positive MASs, that are evolved in "opportunity situations" and drive actions (e.g., exploring, playing) that bring advantages to animals with reduced fitness costs. These motivational states are affectively experienced as negative or positive, lead the animal to adopt different behaviours, and are genetically programmed, because of their adaptive values (Fraser and Duncan, 1998). Even if it is not always simple to separate positive and negative MASs, it is important to consider that some behaviours are determined mostly by positive MASs, while others by negative ones (Fraser and Duncan, 1998). Considering this background, Mellor (2015) defines positive affective engagement as: "the experience an animal may have when it actively responds to motivations to engage in rewarding behaviours, and it includes all associated appetitive and consummatory affects that are positive. For example, it represents a state of engaged aliveness that might attend an animal's goal-directed, energised exploration of and interactions with a stimulus-rich environment". This definition suggests that giving opportunities to the animals to be involved in situation-related positive affects, with possibilities of companionship or environmental enrichments, enhances their welfare. On the contrary, if they have small possibilities to engage in behaviours accompanied by rewarding affects, their positive welfare state is incomplete (Mellor, 2015). A wider view of animal welfare is given by the third concept of "Quality of life" (QoL), which is a key part of the PAW but it is less studied than positive emotions and positive affective engagement (Vigors et al., 2021). Currently, QoL has not a universally recognized definition. The idea of QoL in animal welfare has shifted the focus of animal welfare from preventing mistreatment to improving animal's life (Bono and De Mori, 2005). The focus of QoL is the individual and its mental experiences derived from the satisfaction of needs and expectations in the long term. QoL depends on individual perspective, which is influenced by the experiences that the subject had until the moment of the evaluation. Therefore, QoL, like other factors, varies during the lifetime of an individual (Green and Mellor, 2011; Wathes, 2010). The FAWC[6] in the Report of 2009 adopted the QoL concept and a positive approach to animal welfare and gave equal importance to positive experiences and the consequent affective states. According to the FAWC[6], the quality of life of an animal should be guaranteed during all its lifetime (including for the manner of its death), should be defined by an independent body and should be classified as: "a life not worth living", "a life worth living" and "a good life". Later Green and Mellor (2011) proposed a different four-scale

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[6] Farm Animal Welfare Council, 2009. Farm Animal Welfare in Great Britain: Past, Present and Future. FAWC, London, UK. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/319292/Farm_Animal_Welfare_in_Great_Britain_-_Past__Present_and_Future.pdf Accessed 01/07/2022, 16:17:56

classification of QoL: “a life not worth living”, “a life worth avoiding”, “a life worth living” and “a good life”. Those classifications were based on the idea that QoL depends on the balance of positive and negative mental experiences of an individual over an extended period. An acceptable QoL is when positive experiences outweigh the negative ones. When the negative and positive experiences are equivalent, it is recognised as the “point of balance” (Green and Mellor, 2011). The difference between the two classifications is that the first has only one QoL level under the point of balance, and the second has two. Indeed, Green and Mellor (2011) differentiate “a life not worth living”, for the animal whose suffering could not be reduced except by euthanasia, and “a life worth avoiding”, for those states where remedial actions can ameliorate animals’ QoL (Fig. 11).

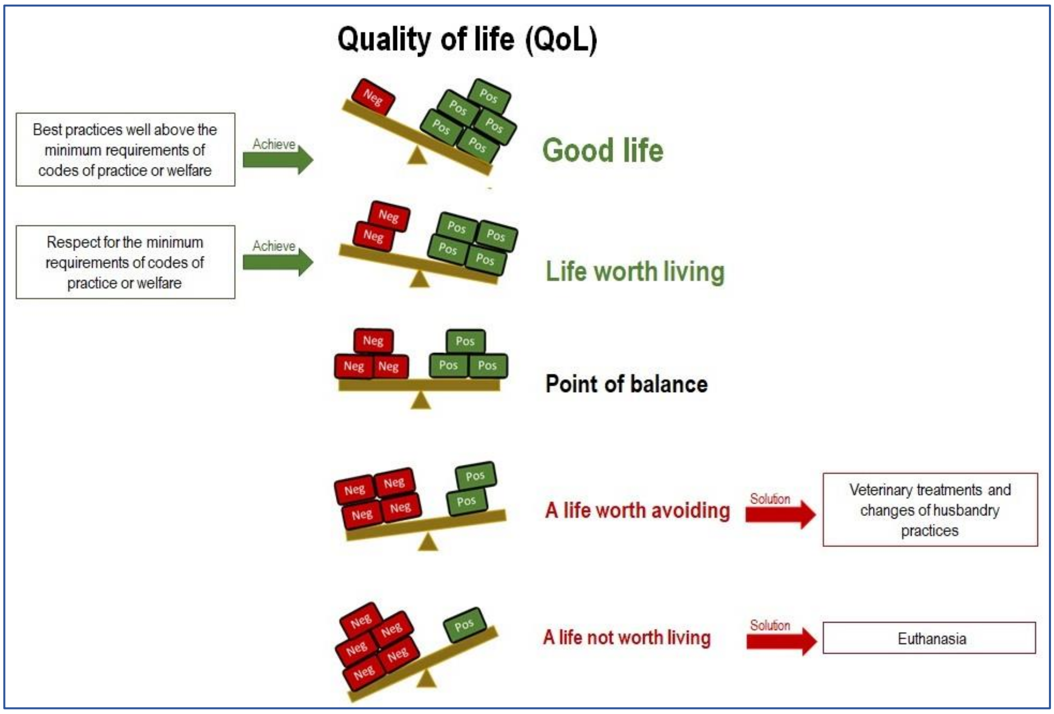

Figure 11 The balance between positive and negative salient experiences determines the different grades of Quality of life (derived from Green and Mellor, 2011)

The concept of QoL can be useful and should be taken into account when we have to make considerations or during the decision-making process, but its assessment over a long period is difficult. Indeed, one of the limitations of QoL is its assessment, which can be made for a short period of time and mostly in a qualitative manner, although standardised methods performed by experts and trained people (such as the Five Domains Model and the qualitative interpretation of animal behaviour) can be employed (Green and Mellor, 2011; Mellor and Reid, 1994; Wemelsfelder, 2007). Finally, the last core concept of PAW is “Happiness”. As for QoL, the literature about happiness in animals is scarce (Vigors et al., 2021). Animals’ happiness is identified as: “how an animal feels most of the time”, considering happiness as a long-term, relatively stable affective trait from a whole-life perspective (Webb et al., 2019). The notion of happiness focuses on the lived life of each individual, considering how all its experiences, also those of early life, can influence its ability to exploit opportunities, thus affecting its general PAW (Lawrence et al., 2019; Webb et al., 2019). Therefore, happiness differs from QoL because it considers the whole life of the animals and how their experiences in different life-stages influence their ability to achieve a positive life (Lawrence et al., 2019). However, this concept is understudied and future research could focalize on how different events across an animal’s life should interact to allow the achievement of a general level of happiness (Lawrence et al., 2019).

1.2.2 Animal Welfare Assessment

Most of the systems to assess animal welfare start from the Five Freedoms, which have been taken and developed also by the Ten General Principles of OIE (2012) (Fraser et al., 2013), the Five Domains Model (Mellor et al., 2020; Mellor and Reid, 1994) and the European Welfare Quality® assessment (Blokhuis et al., 2010).

The OIE 10 “General Principles for the Welfare of Animals in Livestock Production Systems” were adopted in 2012[7] and updated in 2019[8], when was added a new principle about the introduction of animals to a new environment. These principles were developed taking into account the scientific advancements in different disciplines that are relevant to animal welfare, and provide a scientific basis for animals’ husbandry. Indeed, they are a useful guide for the implementation of animal welfare standards for different species (Fraser et al., 2013). In Article 7.1.4 2021 © OIE - Terrestrial Animal Health Code, the World Organisation for Animal Health recommends that the standards should focus on favourable outcomes for animals. Those outcomes generally are evaluated considering the animals’ experience of the five freedoms and evaluating individuals, the entire group, or a sample of the group. The standards should include most of all animal-based measures, but also management-based and resource-based measures for each principle. All the measures should be defined according to scientific research and

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[7] OIE, 2012. Introduction to the recommendations for animal welfare. In: Terrestrial Animal Health Code, 21st Ed. World Organisation for Animal Health (OIE), Paris, France, Article 7.1.4.

[8] World Organisation for Animal Health, 2021. Introduction to the recommendations for animal welfare. In: Terrestrial Animal Health Code, 29th Ed. World Organisation for Animal Health (OIE), Paris, France, Article 7.1.5.

experts and should have a clear link with welfare outcomes, allowing the owners to identify what resources or management practices are necessary to improve welfare[9].

The principles listed in Article 7.1.5 of the 2021 © OIE - Terrestrial Animal Health Code[8] are:

I. "Genetic selection should always take into account the health and welfare of animals.
II. Animals chosen for introduction into new environments should be suited to the local climate and able to adapt to local diseases, parasites and nutrition.
III. The physical environment, including the substrate (walking surface, resting surface, etc.), should be suited to the species so as to minimise risk of injury and transmission of diseases or parasites to animals.
IV. The physical environment should allow comfortable resting, safe and comfortable movement including normal postural changes, and the opportunity to perform types of natural behaviour that animals are motivated to perform.
V. Social grouping of animals should be managed to allow positive social behaviour and minimise injury, distress and chronic fear.
VI. For housed animals, air quality, temperature and humidity should support good animal health and not be aversive. Where extreme conditions occur, animals should not be prevented from using their natural methods of thermo-regulation.
VII. Animals should have access to sufficient feed and water, suited to the animals' age and needs, to maintain normal health and productivity and to prevent prolonged hunger, thirst, malnutrition or dehydration.
VIII. Diseases and parasites should be prevented and controlled as much as possible through good management practices. Animals with serious health problems should be isolated and treated promptly or killed humanely if treatment is not feasible or recovery is unlikely.
IX. Where painful procedures cannot be avoided, the resulting pain should be managed to the extent that available methods allow.
X. The handling of animals should foster a positive relationship between humans and animals and should not cause injury, panic, lasting fear or avoidable stress.

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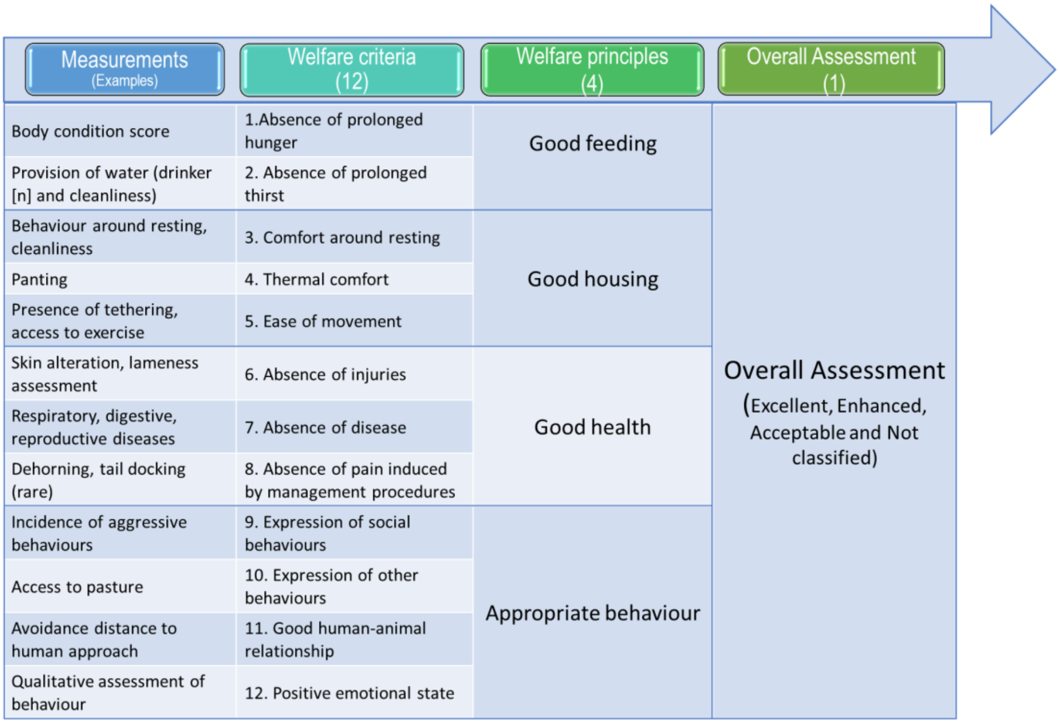
[9] World Organisation for Animal Health, 2021. Introduction to the recommendations for animal welfare. In: Terrestrial Animal Health Code, 29th Ed. World Organisation for Animal Health (OIE), Paris, France, Article 7.1.4.

XI. Owners and handlers should have sufficient skill and knowledge to ensure that animals are treated in accordance with these principles".

Before the adoption of general principles in 2012, the Welfare Qaulity® project was financed by the European 6th Framework Programme for Research and Technological Development (FP6). Its original title was "Integration of animal welfare in the food quality chain: from public concern to improved welfare and transparent quality". This project began in May 2004 and ended in December 2009. It was the largest European project on animal welfare with an integrated and multidisciplinary approach, which favoured the dialogue between science and society. Indeed, the project was developed to respond to the demand of consumers for high standards of animal welfare, health and food quality. Therefore, it had three principal drivers: (1) public concerns and demands; (2) product supply chains and markets; and (3) policy-making and regulatory bodies (Blokhuis et al., 2013; Botreau et al., 2007). The purpose of the Welfare Quality® project was to develop a scientific-rigours method of assessment but, in the meantime, it had to reflect broader public concern, be understandable by the public and useful for the stakeholders (Botreau et al., 2007; Keeling et al., 2013). Four criteria and 12 subcriteria were recognised as a base for the development of welfare assessment systems, which allow the classification of welfare in Excellent, Enhanced, Acceptable and Not classified, considering a score between 0-100 for each criterion (Botreau et al., 2009, 2007) (Fig. 12). This scheme takes into account: its applicability in different farm species; the fact that welfare is related to mental states so the measures should be animal-based to understand also animals' perception; the low compensation between criteria; the absence of hazards with a low probability (Botreau et al., 2007). In this project, animal welfare was defined by the quality of life experienced by an individual, and it is a dynamic state that is compromised by pain and suffering and enhanced by pleasure. In this view, animal-based measures gain more and more importance in the assessment of animal welfare. Resource and management measures are considered only if informative animal-based measures are not available. Indeed, the same environment and management practices can be perceived differently by different individuals because they can be influenced by their genetics, temperament and life experiences (Keeling et al., 2013; Veissier et al., 2013). The measures chosen for animal welfare assessment to verify all criteria and sub-criteria should be scientific-based, preferably animal-based, valid, feasible and reliable. All measures should be measurable in a farm environment and the overall assessment should be possibly made by one observer in a day (Veissier et al., 2013).



Measurements (Examples)	Welfare criteria (12)	Welfare principles (4)	Overall Assessment (1)
Body condition score	1.Absence of prolonged hunger	Good feeding	Overall Assessment (Excellent, Enhanced, Acceptable and Not classified)
Provision of water (drinker [n] and cleanliness)	2. Absence of prolonged thirst		
Behaviour around resting, cleanliness	3. Comfort around resting	Good housing	
Panting	4. Thermal comfort		
Presence of tethering, access to exercise	5. Ease of movement		
Skin alteration, lameness assessment	6. Absence of injuries	Good health	
Respiratory, digestive, reproductive diseases	7. Absence of disease		
Dehorning, tail docking (rare)	8. Absence of pain induced by management procedures		
Incidence of aggressive behaviours	9. Expression of social behaviours	Appropriate behaviour	
Access to pasture	10. Expression of other behaviours		
Avoidance distance to human approach	11. Good human-animal relationship		
Qualitative assessment of behaviour	12. Positive emotional state		

Figure 12 The Welfare Quality ® scheme of assessment. Most of the measurements are examples that derives from cattle assessment [9] *(Modified from Botreau et al., 2009)*

The Welfare Quality® project led to the development of welfare assessment protocols focalized on animal-based indicators for pigs[10], poultry[11], dairy and beef cattle[12]. During the 7th Framework Programme in 2011, the European Commission developed and funded a second project, called AWIN (Animal Welfare Indicators), to implement welfare assessment protocols for other commercially important species: sheep, goats, horses, donkeys and turkeys. AWIN project was based on the four criteria and the twelve sub-criteria of Welfare Quality® and developed welfare assessment protocols principally composed of valid, reliable and feasible animal-based indicators, with a particular focus on animals' pain and its recognition (Minero et al., 2015).

The four criteria of Welfare quality® ("good feeding", "good housing", "good health" and "appropriate behaviour") were anticipated by the four physical/functional domains, part of the

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[10] Welfare Quality® Protocol, 2009. Welfare Quality® Assessment Protocol for pig (sows and piglets, growing and finishing pigs). Welfare Quality® Consortium, Lelystad, The Netherlands, 122 pp.

[11] Welfare Quality® Protocol, 2009. Welfare Quality® Assessment Protocol for poultry (broilers, laying hens). Welfare Quality® Consortium, Lelystad, The Netherlands, 114 pp.

[12] Welfare Quality® Protocol, 2009. Welfare Quality® Assessment Protocol for cattle. Welfare Quality® Consortium, Lelystad, The Netherlands, 180 pp.

Five Domains Model proposed by Mellor and Reid in 1994. The Five Domains Model was developed starting from the Five Freedoms, considering that total freedom from the negative states, as stated in the Five Freedoms, is impossible to achieve. In addition, even if freedom is considered as animals being “as free as possible from” particular welfare problems, the concept of “degrees of impaired freedom” was difficult to interpret and coherently evaluate (Mellor, 2016; Mellor and Reid, 1994). In addition, the Five Freedoms paradigm does not differentiate between physical/functional (e.g., malnutrition) and mental components (e.g., hunger) of animal welfare. Therefore, the Five Domains Model was initially developed for the evaluation of animal welfare impairment and its minimisation. It comprises four “physical/functional” domains (“nutritional condition”-1; “physical environment”-2, “health conditions”-3, “behavioural interactions”-4) and one “mental” domain (5). In the context of welfare impairment, domains 1 to 3 refer to internal physiological and pathophysiological disturbances, and domain 4 is about external factors and social conditions in the environment that can compromise the expression of species-specific behaviour (Mellor et al., 2009) These internal and external factors can be assessed, and their anticipated affective consequences are assigned to the fifth “mental” domain. The net of affective outcomes in the mental domain represents the state of welfare (Fig. 13) (Mellor et al., 2009; Mellor, 2016; Mellor et al., 2020).

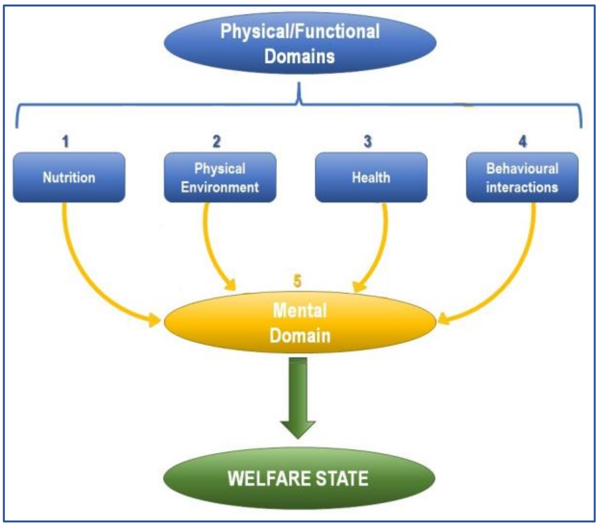

Figure 13. The Five Domains Model (Modified from Mellor and Reid, 1994; Mellor et al., 2020)

Initially, the Five Domain Model was formulated for animals used in research, teaching and testing, and it focuses on the assessment of negative welfare impacts (Mellor and Reid, 1994). However, over the years, the model was improved, both broadening the range of the considered negative affective states, and including positive affective states to evaluate not only the welfare impairment but also the welfare enhancement (Mellor, 2012b; Mellor and Beausoleil, 2015).

According to the model, the negative affective states can be divided into survival-related and situation-related. The survival-related negative affective states arise from conditions listed in domains 1, 2 and 3, and are motivational states that are part of genetically pre-programmed homeostatic mechanisms. Indeed, these states make animals adopt behaviours crucial for survival (Mellor and Beausoleil, 2015). These genetical pre-programmed states cannot be eliminated, thus it is important to maintain them within tolerable limits because they are important for the expression of life-sustaining behaviours (Mellor and Reid, 1994). The situation-related negative affective states arise from those situations of restricted environment that prevent animals to have opportunities to engage in rewarding behaviours, derived from their interaction with the environment or other animals. Therefore, in these impoverished environments animals cannot satisfy their genetic or learned motivations. These negative affective states are related to the cognitive responses of animals and to conditions that arise in domain 4 (Mellor and Beausoleil, 2015). Similarly, the enhanced positive affective states can be distinguished into positive affects elicited when some negative ones are minimised, and positive affects that replace negative ones. The first category of positive affective states can derive indirectly from the reduction of the intensity of survival-negative affective states within tolerable levels, or as a result of the expression of survival focused-behaviours motivated by negative affects. Therefore, these positive affective states are usually transient and linked to domains 1, 2 and 3. The second category includes those positive affective states that can replace situation-related negative ones (domain 4) and can improve animal welfare from neutral to positive. These affective states, arise from the improvement of environmental conditions, providing opportunities for animals to engage in rewarding behaviours. One example can be enrichment and environmental variability, which allow animals to actively engage in environment-focused and animal-to-animal interactive behaviours (Mellor and Beausoleil, 2015). The last update of the Five Domain Model in 2020 substituted the name of the fourth domain from “Behaviour” to “Behavioural Interactions”, considering that animals are sentient beings able to consciously choose goal-direct behaviours during their interactions with the environment, other animals and humans. As previously explained, when animals achieve the goal, they experience positive affects, enhancing their welfare state. On the contrary, when external circumstances prevent them to express those self-selected goal-direct rewarding behaviours, their welfare can be compromised. In particular, the last update of the Model focus on the wide range of human-animal interactions that is part of those external circumstances that can promote the enhancement or impairment of animals’ welfare state (Mellor et al., 2020).

1.2.3 Animal Welfare Legislation - focus on horse

The Western hemisphere law derived from Roman law, which was influenced by the thoughts of Greek philosophers, such as Plato, Socrates and Aristotele. Considering both Greek and Roman culture, nature was ordered hierarchically and slaves and animals were not part of the society, but they were "living tools" that can be used for the benefit of the society itself. Similarly, Roman law recognised three categories: person, things and actions. Women, children, slaves, mentally incompetent humans, and animals were all excluded from being persons but fell into the category of legal "things", meaning that, contrary to "persons", they did not have free will and legal rights, and, if owned, they were classified as "property" (Wise, 2014). The idea that animals were created for humans' purposes continued also in early Christianism (Kelch, 2012).

During the 18th and 19th centuries, in Britain, increasing attention to social justice for women, children and animals arose, and, in 1796, the philosopher John Lawrence argued: "the rights of beasts [animals] be formally acknowledged by the state, and that a law be framed upon that principle, to guard and protect them from acts of flagrant and wanton cruelty, whether committed by their owners or others". Despite this growing interest in animals' morality, the law remained conservative and the only formalised protections towards animals were those that safeguard the owner's interests (Robertson and Sparks, 2022). Indeed, only in 1822, the very first legislation was implemented with "The Cruel Treatment of Cattle Act (3 Geo. IV c. 71)", known as "Martin's Act", after the introduction by Colonel Richard Martin of the bill that made it an offence to "wantonly and cruelly beat or ill-treat [any] horse, mare, gelding, mule, ass, ox, cow, heifer, steer, sheep or other cattle". Even if this legislation referred only to a restricted group of species, it can be considered a legislative reform. Indeed, it poses boundaries to the traditional unlimited property of the owners and the bases for the Cruelty Animal Act, which was approved in 1849 and made illegal ill-treatment, abuse and torture of animals (Robertson and Sparks, 2022; Cardoso et al., 2017). This inspired many other European countries, and, thanks to the extension of the British empire, it spread all over the world, such as in the United States (US), where, in 1867, the first anti-cruelty legislation ("Act for the more effectual prevention of cruelty to animals") was implemented. Like the Britain act, the US law considered the common cruelties against animals, but, in addition, imposed some duties to avoid useless suffering for animals, and prosecute cock fighting and bull and bear baiting (Cardoso et al., 2017; Robertson and Sparks, 2022). In addition, this was an era of great scientific research, and the natural selection theory by Charles Darwin mined the idea of animals being created for human purposes (Wise, 2014).

This early legislation was followed by a series of regulations and laws in different countries, which focus on unnecessary cruelty towards animals and adopt the Five Freedoms paradigm as a benchmark. Animal protection laws worldwide prohibit owners and persons in charge of animals from causing suffering to animals and consider the nature, duration, severity and circumstance of animal suffering. However, animal law changes significantly between countries. Modern animal welfare legislation comprises primary legislation (established directly by the legislature) and secondary legislation (derived by the power of the legislature). The legislation is supported by guidelines for the husbandry of animals and codes, which do not have legal values but can be used as evidence for the defence or persecution of actions under animal welfare legislation (Robertson and Sparks, 2022). For example, the Welfare Act of 2006 give specific guidance for the interpretation of "necessity" and has both secondary legislation and codes and guidelines about good practices and scientific knowledge, to support the courts in the application of law (Robertson and Sparks, 2022).

The European (EU) policy on the protection and welfare of animals has a long story that starts in 1968 with the European Convention for the Protection of Animals during International Transport. This convention was followed by other four conventions: the European Convention for the Protection of Animals kept for Farming Purposes (1976); the European Convention for the Protection of Animals for Slaughter (1979); the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (1986); European Convention for the Protection of Pet Animals (1987). Conventions were signed by several countries and formed the basis for the development of the current EU animal welfare legislation (Villa et al., 2014). In addition, the reform of Common Agriculture Policy (2003)[13] established that farmers that do not fulfil animal welfare standards receive reductions of or exclusion from direct support. Furthermore, Regulations (EC) n.73/2009[14] established that the Member States

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[13] Council Regulation (EC) No 1782/2003 of 29 September 2003 establishing common rules for direct support schemes under the common agricultural policy and establishing certain support schemes for farmers and amending Regulations (EEC) No 2019/93, (EC) No 1452/2001, (EC) No 1453/2001, (EC) No 1454/2001, (EC) 1868/94, (EC) No 1251/1999, (EC) No 1254/1999, (EC) No 1673/2000, (EEC) No 2358/71 and (EC) No 2529/2001

[14] Council Regulation (EC) No 73/2009 of 19 January 2009 establishing common rules for direct support schemes for farmers under the common agricultural policy and establishing certain support schemes for farmers, amending Regulations (EC) No 1290/2005, (EC) No 247/2006, (EC) No 378/2007 and repealing Regulation (EC) No 1782/2003

can give specific economic support to those farmers who practice enhanced animal welfare standards. However, we have to remember that one of the important drivers of welfare policy is ethics. An example is Article 13 of the Treaty on the Functioning of the European Union, better known as the Treaty of Lisbon[15], which was enacted in 2009 and stated: "In formulating and implementing the Union's agriculture, fisheries, transport, internal market, research and technological development and space policies, the Union and the Member States shall since animals are sentient beings, pay full regard to the welfare requirements of animals while respecting the legislative or administrative provisions and customs of the Member States relating in particular to religious rites, cultural traditions and regional heritage". The identification of animals as sentient beings was already present in the preamble of the old 1997 Treaty of Amsterdam[16] (protocol 33), but in the Treaty of Lisbon, this concept was included in the body part (Cardoso et al., 2017; Villa et al., 2014). The Treaty of Lisbon was ratified by 27 countries and identified a list of EU policies where the EU has a legal base to act on animal welfare. Therefore, EU actions on animal welfare are restricted to some policies (e.g., agriculture for farm animals), and the Member States can have their regulations regarding religious rites, cultural traditions or regional heritage that may limit the EU actions. Some policies, like the environment, are not mentioned in Article 13, and this does not represent a limitation for the EU actions on animal welfare in these policies (Simonin and Gavinelli, 2019). Thus, European Union has developed animal welfare legislation for farm and laboratory animals and has employed audits, training, scientific expertise and advice to harmonise actions through the Member States, which have the responsibility to implement the EU rules. The EU rules can be distinguished in directives, which are transposed by the Member States to their national legal order and states can go beyond the minimum standard imposed by the EU, and regulations, which are directly applicable without any transformation. In this last case, Member States have limited actions and they have to adopt the standards of the EU regulations, even if they have to establish sanctions in their national legal system (Simonin and Gavinelli, 2019). Regarding EU legislation on animal welfare, there is one EU directive that is an umbrella directive about all farmed species (Council Directive 98/58/EC-animals kept for farming purposes), and four specific directives about laying hens (Council Directive 1999/74/EC),

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[15] Treaty of Lisbon amending the Treaty on European Union and the Treaty establishing the European Community, signed at Lisbon, December 13, 2007. *Official Journal of the European Union*, C 306, December 17, 2007.

[16] Treaty of Amsterdam amending the Treaty of the European Union, the Treaties establishing the European Communities and certain related acts, was signed on October 2, 1997, and entered into force on May 1, 1999.

broilers (Council Directive 2007/43/EC), calves (Council Directive 2008/119/EC), and pigs (Council Directive 2008/120/EC). In addition, the EU has ratified two regulations on animal welfare during transport (Council Regulation No 1/2005/EC) and killing (Council Regulation No 1099/2009/EC).

Considering specifically horses, there are no specific EU regulations or directives on their protection and welfare. Indeed, horse protection is under the general Council Directive 98/58/EC that regulates animal welfare of “any animal, including fish, reptiles or amphibians, bred or kept for the production of food, wool, skin or fur, or for other farming purposes”, but it does not concern “animals living in the wild, those intended for use in competitions, shows, cultural or sporting events or activities, experimental or laboratory animals and any invertebrate animal”[17]. One of the major problems with horses is their classification as farm or companion animals and the fact that they can be bred or kept not only for food production but also for sporting and cultural events, which are explicitly out of the field of application of the EU directive (Peli et al., 2012). Therefore, horses that came from professional farms and stables, not only for food production, are always subject to the general rules of Council Directive No. 98/58/EC as part of the economic activity of a farm enterprise. On the contrary, horses of a private owner or from non-professional farms for recreational activities and leisure purposes are not subjected to the EU directive. In the meantime, specific administrative regulations for the protection of horses used in competition are predisposed by technical and sporting boards, which identified the conditions to be respected in fields, tracks and competition areas (Peli et al., 2012). Similarly, animal welfare during transport is regulated by Council Regulation No. 1/2005/EC, but its application considers only transport for commercial purposes. Transport for commercial purposes is defined as: “transport for commercial purposes is not limited to transport where an immediate exchange of money, goods or services takes place. Transport for commercial purposes includes, in particular, transport which directly or indirectly involves or aims at a financial gain”[18]. Therefore, also for transport, equines are in a particular position, and a case-by-case evaluation should be done because horses’ transport for sports activities can

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[17] Council of the European Union 1998. Council Directive 98/58/EC of 20 July 1998 concerning the protection of animals kept for farming purposes Off J, L 221, 8.8.1998, 23-27.

[18] Council of the European Union 2005. Council Regulation (EC) No. 1/2005 of 22 December 2004 on the protection of animals during transport and related operations and amending Directives 64/432/EEC and 93/119/EC and Regulation (EC) No 1255/97. Off J, L 003, 05.01.2005, 1-37.

imply a financial gain. Finally, also the Council Regulation No. 1099/2009/EC[19] does not give specific indications for equine slaughtering and killing (Peli et al., 2012).

The lack of a harmonised and specific regulation for equine welfare and husbandry, especially for those not involved in commercial or economic activities, has led to the development of different conditions of management and different lairaging and training practices, which not always are respectful of ethological requirements of horses and can cause the development of behavioural and health problems and physical and psychic losses (e.g, single stall, insufficient daily exercise in paddock, constrictive training methods) (Peli et al., 2012). However, in 2017, the European Parliament, recognising also the importance of the economic impact of the equid sector, "calls on the Commission to develop European Guidelines on Good Practice in the Equid Sector for various users and specialists, drawn up in consultation with stakeholders and organisations from the equid sector and based on existing guides, including a focus on species-specific welfare and behavioural care, in addition to end-of-life care"[20]. In the same resolution, European Parliament underlines that owners should have a minimum level of knowledge about equids' husbandry and they are responsible for their welfare. Finally, it highlights the importance of the exchange of knowledge about scientific advancement, legislative developments and new learning methods between owners and State Members to enhance equine welfare[20]. Before the European Parliament, in May 2016, the World Organisation for Animal Health (WOAH) added a chapter in the OIE©-Terrestrial Code about working equids, which specifies guidelines about their welfare, observing the Five Freedoms[21].

Focussing on Italy's regulations on equids' welfare, the Council Directive 98/58/EC was enacted through Legislative Decree No. 146/2001[22]. However, as stated before, the Directive focus only on some categories of equines, thus the welfare of those animals that were excluded from the regulations on animal protection in farming was covered only by the general

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[19] Council of the European Union 2009. Council Regulation (EC) No. 1099/2009 of 24 September 2009 on the protection of animals at the time of killing. Off J, L 303, 18.11.2009, 1-30.

[20] European Parliament resolution of 14 March 2017 on responsible ownership and care of equidae (2016/2078(INI)) https://www.europarl.europa.eu/doceo/document/TA-8-2017-0065_EN.html Accessed 06/07/2022 20:22:47

[21] World Organisation for Animal Health, 2021. Welfare of Working Equids. In: Terrestrial Animal Health Code, 29th Ed. World Organisation for Animal Health (OIE), Paris, France, Chapter 7.12.

[22] Decreto Legislativo 26 marzo 2001, n. 146. Attuazione della Direttiva 98/58/CE relativa alla protezione degli animali negli allevamenti. Gazz Uff, Serie Generale del 24.04.2001, 195, 21-24.

legislation against animal mistreatment (articles 727, 544-bis, 544-ter; 544-quater of the Italian Criminal Code) (Peli et al., 2012). Furthermore, the Order issued by the Health Ministry on 10 August 2020[23], amending the Order issued on 21 July 2011, gives rules for public or private shows outside of the official authorised events that involve horses (e.g., authorised by FEI-International Equine Federation, or FISE-Italian federation for equestrian sports, or ASSI-the Italian agency for the horseshow activities). This introduces various measures, such as the Code of Conduct for events, the prohibition of doping for riders and horses, the exclusion from the competitions of those riders who have reported convictions for mistreatment or killing of animals, provisions on horse care, and the conditions of authorisation for show procedures, track, field grounds and holding walls where events take place[23]. To support the legislation the Italian Ministry for Labour, Health and Social Politics implemented a “Code for the protection and management of equids” in 2009[24], renewed in “Principles for the protection and management of equids” in 2017[25]. This document has no legal value, but it consists of guidelines about horse welfare standards and management practices. This document is a useful reference for both owners and people that have responsibilities towards equids (horses, ponies, donkeys, mules and hinnies) and authorities, which can find support in absence of specific legal parameters and regulations (Peli et al., 2012). The code provides criteria for the proper management of equids, considering good practices and ethical behaviour, to protect the health and well-being of these animals and promote a right human-animal relationship that respects the equines’ dignity [24, 25]. The latest version has included the “10 Training Principles” of the International Society for Equitation Science (ISES)[26], which promote training method that respects the learning abilities of the horse and its well-being. In addition, the Italian Ministry for Labour, Health and Social Politics has developed the Ethical Charter[27] for the protection of horses, which represents a commitment “for life”. The subscription of it by the main

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[23] Ministero della Salute (Italy), 2020.Proroga dell'ordinanza contingibile e urgente 21 luglio 2011, e successive modificazioni, in materia di disciplina delle manifestazioni popolari, pubbliche o private, nelle quali vengono impiegati equidi al di fuori degli impianti e dei percorsi ufficialmente autorizzati. G.U. Serie Generale, n. 222 del 07 settembre 2020 https://www.trovanorme.salute.gov.it/norme/dettaglioAtto?id=76079 Accessed 06/07/22 22:49:30

[24] Ministero della Salute (Italy), 2009. Codice per la Tutela e la Gestione degli Equidi. Ministero della Salute , Rome https://www.salute.gov.it/imgs/C_17_pagineAree_1911_listaFile_itemName_0_file.pdf Accessed 06/07/2022 21:43:23

[25] Ministero della Salute (Italy), 2017. Principi per la Tutela e la Gestione degli Equidi. Ministero della Salute , Rome https://www.salute.gov.it/imgs/C_17_opuscoliPoster_292_allegato.pdf Accessed 06/07/2022 21:32:23

[26] International Society for Equitation Science. ISES 10 training principles. https://www.equitationscience.com/ises-training-principles Accessed 06/07/2022 21:49:43

representatives of the equestrian sector means that they undertake to respect equids' ethological needs and protect their health and well-being in the practice of all the activities that involve equids. In addition, they will guarantee a dignified destination, other than that of slaughtering, to the animals at the end of their careers[27]. Recently, Legislative Decree No. 36/2001 has been enacted and officially recognises the status of a horse as an athlete. According to this regulation, a horse can be defined as an “athlete” when: a) it is “definable *registered equid*, according to Article of Commission Regulation (EU) No 262/2015 of 17 February 2015 as stated in the identification document, following the same European Regulation; b) is declared not intended for food production, as provided for by Regulation (EU) No. 262/2015 and as resulting from the Identification Document compliant with the same Regulation (EU) No. 262 of 2015, even after the cessation of sports activity; c) is registered with the Italian Equestrian Sports Federation or the Modern Pentathlon Federation or the FitetrecAnte, or a Sports Promotion Body as shown by the identification document or the document issued by the card system of the same sporting body”[28]. This decree gives particular rules about horses’ health care and eligibility for sports activities and highlights the general obligation of their owners to ensure the protection and welfare of their animals, avoiding training that could compromise their health. Furthermore, according to the decree, a veterinarian has always to be present during competitions, horses can take part in sports events only if their physiological status is compatible, competitions must take place where the safety and welfare of animals are guaranteed and all equipment used must meet safety criteria. Finally, the transport of animals should be made employed means that are cleaned, disinfected and suitable for ensuring the safety of animals and animals cannot be slaughtered or killed at the end of their sportive career, except for “humanitarian killing”[28].

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[27] Ministero della Salute (Italy), 2009. Carta Etica per la Tutela degli Equidi. Ministero della Salute , Rome https://www.salute.gov.it/imgs/C_17_pagineAree_1911_listaFile_itemName_1_file.pdf Accessed 06/07/2022 22:15:42

[28] Decreto Legislativo 28 febbraio 2021, n. 36 -Attuazione dell'articolo 5 della legge 8 agosto 2019, n. 86, recante riordino e riforma delle disposizioni in materia di enti sportivi professionistici e dilettantistici, nonche' di lavoro sportivo. Titolo IV. GU n.67 on 18-3-2021. https://www.gazzettaufficiale.it/atto/vediMenuHTML?atto.dataPubblicazioneGazzetta=2021-03-18&atto.codiceRedazionale=21G00043&tipoSerie=serie_generale&tipoVigenza=originario#:~:text=DECRETO%20LEGISLATIVO%2028%20febbraio%202021%2C%20n.%2036%20Attuazione,di%20lavoro%20sportivo.%20%2821G00043%29%20%28GU%20n.67%20del%2018-3-2021%29 Accessed 06/07/2022 23:20:48

1.3 EMOTION

1.3.1 Definition of emotions

A key problem in animal welfare science is the ability of animals of experiencing suffering. Therefore, an important question that still today is extremely difficult to answer is whether and how animals experience emotions. In addition, identifying similarities and differences in emotional processes between human and non-human animals is a useful tool for the development of comparative sciences and study of the emotional disorders in humans (Paul and Mendl, 2018). However, among authors agreement on the definition of emotion lacks (Kremer et al., 2020). Paul and Mendl in 2018 tried to find a definition of emotion valid for both human and non-human animals and they proposed the following descriptive definition: "An emotion is a multi-component response (subjective, physiological, neural, cognitive) to the presentation of a stimulus or event. The conscious, subjective component of an emotion is generally regarded as its central, key feature. It is always valenced (i.e., either positive or negative; occasionally both). It can be intense or mild; long-lasting or brief. The exact type of emotion experienced (e.g., sadness, grief, remorse) will depend on the precise nature of the emotive (emotion-producing) event. An emotive event can be external or internal (i.e., emotions can be generated by imagination and recollection as well as by events occurring in the environment). An emotive (i.e., emotion-producing) event is usually one that is in some way important to the goals or relevant to the well-being of the individual. If an emotive event is reliably predicted, that prediction will also often generate an emotional response." This definition underlines the many components of emotional experiences and points out the centrality of subjective experience, which in non-human animals is largely debated. Furthermore, this definition does not evidence the basic biological process of emotions and, as the authors themselves recognised, is too human-centred to be applied in animal science without ambiguity (Paul and Mendl, 2018). Therefore, for the study and understanding of emotional processes in non-human animals can be more useful to adopt perspective definitions that do not describe how the word emotion is used in everyday life (descriptive), but underline the events that scientific theories of emotion want to explain (Paul and Mendl, 2018). One of the perspective definitions is that of Anderson and Adolphs (2014) that argue: "an "emotion" constitutes an internal, central (as in Central Nervous System) state, which is triggered by specific stimuli (extrinsic or intrinsic to the organism). This state is encoded by the activity of particular neural circuits that give rise, in a causal sense, to externally observable behaviours, as well as to associated cognitive, somatic and physiological responses". In this definition, emotions are seen as central neural states that arise after sensory stimuli or memories and cause behavioural, cognitive and somatic changes. In this view, behaviours are seen as consequences of emotion central states, even if also behaviours, once expressed, can influence emotional states, in a sort of causal loop (emotion states→ behaviours→ emotion states). These central emotional states are characterized by four basic evolutionary "building blocks" or "emotion primitives," which are common to different emotions and different animal species:

1) scalability, which indicates how emotions are characterized by gradations of intensity (level of arousal) that can be associated with different intensity of a certain behaviour or with qualitative shifts in the behaviours associated with the emotions;
2) valence, indeed "antithetical pairs" of emotions (e.g., joy vs. anger; happiness vs. sadness) can be recognised and they are expressed by physically opposite and complementary behaviours;
3) persistence, which refers to the fact that certain emotions can outlast the trigger stimulus, and this influences both cognition and behaviour;
4) generalization, which is when the emotional state which persists after the stimulus can cause a generalisation to different contexts, influencing behavioural and cognitive responses to different stimuli (Anderson and Adolphs, 2014).

If we consider emotions as central neural states with evolutionary similarities in their expression (four primitives) between non-human and human animals, this implies that all animals (invertebrates as well as vertebrates) have central emotion states. In the meantime, this idea neither affirms nor *a priori* excludes the possibility that they are consciously aware of them. Indeed, feelings, as subjective experiences of emotion that can be only assessed in human beings by the verbal report, are not part of emotions but are consequences of that, like behaviours (Anderson and Adolphs, 2014).

The primal emotional neural networks, indeed, exist in some of the most ancient regions of the brain, the subcortical brain regions, which present evolutionary homologies among species (Panksepp, 2011). Seven basic affective circuits that interact with each other have been identified: SEEKING System; RAGE/Anger System; FEAR/Anxiety System; LUST Systems; Maternal Nurturance CARE System; PANIC/GRIEF/Separation Distress System; PLAY/Rough-and-Tumble, Physical Social-Engagement System. These circuits are large transverse networks that occupy and connect midbrain regions (Panksepp, 1998, 2011), and are characterised by descending and ascending components, which work together for the generation of various instinctual emotional behaviours and the associated raw feelings. These primary-process systems are the basis of raw affects that consist of ancestral memories (instincts), which promote survival and mediate the process of reinforcement. They are "building blocks" for higher emotions, modelled by cognition, such as guilt, hatred, and loneliness. Decorticated animals maintain the integrity of these systems but they lose connection to the higher brain regions and their control (Panksepp, 2011). Indeed, higher prefrontal neocortical regions rarely are responsible for affective changes, probably because higher brain areas lack the circuits involved in the generation of affective experiences. However, the neocortex has an important role in the regulation of emotional arousal, and neodecorticated animals show important learning impairments (Panksepp, 2010). In the layered stratification brain, we can find clear historical and evolutionary imprints. Indeed, the more ancient systems are located in the medial and caudal areas, while the more recent systems were lateral and rostral. The brain is organized in "nested hierarchies", where the lower levels are again presented in the higher systems, and there are two-way (circular) communications among all levels. The regulation of emotionality follows a hierarchical control where: (a) primary-process core affects are the results of ancient subcortical processes; (b) secondary-process elaborations or emotional learning derive from classical conditioning and operant learning principles; (c) tertiary process emotions that consist of cognitive-affective combinations, such as reflections and affectively charged thoughts, and emerge by the interactions between neocortex and paralimbic and limbic structures (Panksepp and Watt, 2011a).

The first emotional circuit that is part of the primary process is the SEEKING system. This system is a dopamine-energized network confluent with the Medial Forebrain Bundle, called the "brain reward system", which courses from the ventral midbrain to the nucleus accumbens and medial frontal cortex. It is an appetitive motivational system that stimulates the animals to acquire the resources they need for survival, provoking exploration and stimulating learning and, so, numerous acquired behavioural manifestations. It is diminished in depression states and bilateral lesions of this system can cause an amotivational state where all appetitive behaviours are reduced. It profoundly influences the function of other basic emotional circuits (Panksepp, 2011, 2010). In opposition to the SEEKING system, there is the RAGE system, which mediates anger and facilitates dopamine release. The RAGE circuit comprises an area that extends from the medial amygdala and hypothalamus to the dorsal Periaqueductal grey (PAG). This network is stimulated by Substance P and glutamate and inhibited by endogenous opioids and γ-aminobutyric acid (GABA). The activation of the RAGE system evokes aggressive behaviours when animals are irritated or restricted and when they have to defend themselves (Panksepp, 2011, 2010). This circuit is near the trans-diencephalic FEAR/Anxiety system and interacts with it, justifying the terminology "fight-flight" strategy. The FEAR network run from the amygdala to the PAG and it is regulated by the Neuropeptide Y and corticotrophin-releasing factor (CRF), and inhibited by GABA transmission. It has evolved to protect the animal from painful situations and to avoid dangers which can cause severe injury or death. To do that, this system elicits flying or freezing behavioural responses (Panksepp, 2011, 2010). The fourth system is the sexual LUST, mediated in females and males by different social neuropeptide transmissions regulated by the sex hormones: oxytocin transmission is promoted by estrogens, while testosterone stimulates vasopressin transmission. This system regulates sexual urges and gender-specific sexual tendencies. However, because bodily sex characteristics and brain circuits are independent, an animal that has certain sex characteristics may have sexual urges typical of the opposite sex. The dopamine-driven SEEKING system can take part in the search for sexual rewards. In the meantime, the LUST circuit shares some chemistries, such as oxytocin, with another emotional network, which is the Maternal Nurturance CARE System. This suggests the intimate evolutionary relationship between female sexual rewards and maternal motivation (Panksepp, 2011, 2010). CARE System assure the care of parents, in mammals especially mother, towards off-springs. Before the birth of the offspring, progesterone declines and estrogen, prolactin and oxytocin increase. These hormonal and neurochemical changes stimulate maternal urges and facilitate the establishment of maternal bonds with offspring (Panksepp, 2011, 2010). When young mammals and birds are separated from their caretakers, from which they are dependent, this condition activates the PANIC/GRIEF system or separation-distress system, inducing crying and calls to attract their attention and care. This system is inhibited by the principal chemicals involved in the social-attachment (oxytocin, opioids and prolactin). A decrease in these chemicals and an increase in corticotropin realising factor (CRF) and glutamatergic drive can exacerbate the feeling of distress. The PANIC/GRIEF circuitry starts in midbrain central grey regions (PAG), ascends through medial diencephalic structures, especially the dorsomedial thalamus, and terminates in various basal forebrain nuclei and subcallosal anterior cingulate forebrain regions (Panksepp, 2011; Panksepp and Watt, 2011b). Finally, the 7th emotional circuit is the PLAY system. This circuit is regulated by a complex neurochemical framework, where opioid, cannabinoid, dopaminergic, and cholinergic systems interact together in the regulation of play. Young animals need strongly physical play (running, chasing, wrestling), but play is also important to learn social rules and interactions. Therefore, PLAY can be an emotion that promotes the development of the higher social brain. The activation of the PLAY system is accompanied by a positive affect, and helps to develop social competencies, such as CARING for others and learning about the world, while they SEEK. The positive affect induced by the activation of the PLAY circuit in rats is signalled through high-frequency (~50 kHz) chirping sounds, that are like laughter (Panksepp, 2011, 2007; Siviy and Panksepp, 2011).

The activation of the brain networks mediates the emotional process that evolved in animals to develop the ability to respond to rewards (seek and approach valuable resources) and punishments (avoidance) (Panksepp, 2011). Therefore, in this view, all mammals have basic discrete emotional systems with an adaptive value that represents the “building blocks” of all emotional reactions. However, this “discrete emotions” approach may be limiting, because it does not consider the entire framework of all possible emotional states, their interactions and how they are manifested and possibly measured (Mendl et al., 2010). Thus, Mendl and colleagues (2010) proposed the integration of the discrete emotions approach with the dimensional theories, applied in the study of human emotions (Russell, 2003). According to the dimensional theory, the subjective conscious emotional experiences, that in humans are vocally reported, are characterized by valence and arousal and can be represented in a two-dimensional space, composed of four quadrants (Q1, Q2, Q3, Q4). All the emotional experiences that can be identified according to valence and arousal dimensions constitute the core affect (Russell, 2003). This concept has born from the need of understanding the subjective experience of emotions. However, this experience is accompanied by physiological, behavioural and cognitive responses that can be measured objectively. Those measures can be useful to identify the position of the animal in a certain location of the core affect space but it is not certain that it can experience consciously the emotion (Mendl et al., 2010). Indeed, applying a functional view of the core affect and maintaining an agnosticism about the possibility of a conscious experience of emotions, it is plausible that animals have states that can be equivalent to those of core affect. Considering the evolutionary development of affective responses in animals, the valence of emotional states is determined in terms of reward and punishment. Emotions arise from punishing and rewarding stimuli and the consequent affective responses coordinate the entire organism to achieve two survival goals: “maximizing acquisition of fitness-enhancing rewards and minimizing exposure to fitness-threatening punishers” (Mendl et al., 2010). Therefore, the position of animal emotion in the core affect is determined by their functions:

Q1, positive high-arousal affective states that are induced by appetitive motivations and promote seeking and obtaining rewards;
Q3, negative low-arousal affective states that follow the lack or loss of reward, and may facilitate the conservation of energy when resources are not available;
Q2, positive low-arousal affective states that are associated with the low presence of threats and facilitate the manifestation of maintenance and recovery activity;
Q4, negative high-arousal affective states that are provoked by the presence of danger and important threats and coordinate response to them (Fig. 14) (Mendl et al., 2010).

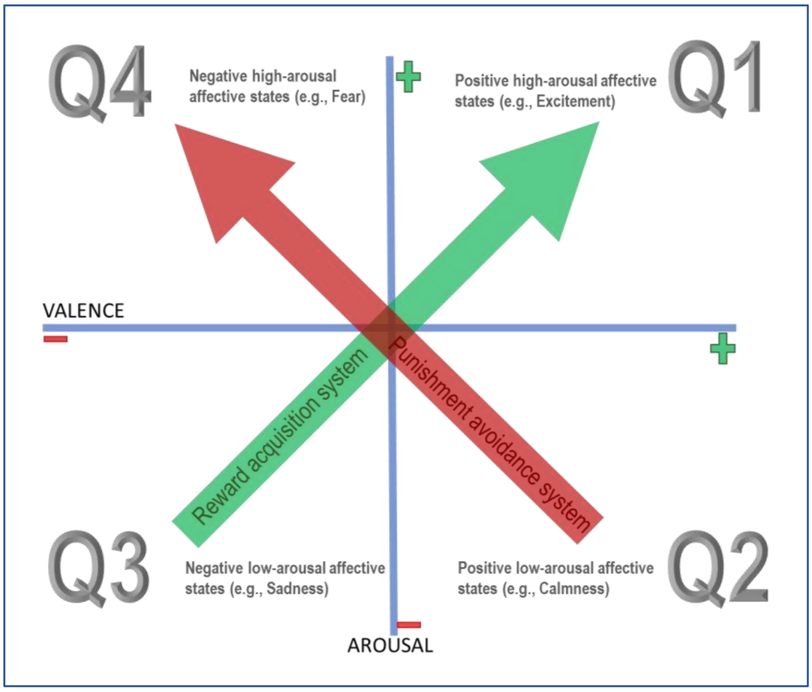

Figure 14. Representation of the two-dimensional core affect. In the upper quadrants there are high-arousal emotions, in the lower quadrants the low-arousal emotions, while in the left quadrants there are positive emotions and in the right quadrants the negative ones. The green and red arrows indicate respectively the reward acquisition biobehavioural systems and the punishment avoidance one (Modified from Mendl et al., 2010).

Considering this classification, two axes that lie at 45° to the core affect axes can be identified: the Q3-Q1 axes or Reward Acquisition System (RAS), and the Q2-Q4 axes or Punishment Avoidance System (PAS). The arrival of a reward or punishment enhances the activity of respectively RAS and PAS leading to a High-Reward or a High-Punishment state. On the other hand, the failure to obtain a reward or the successful avoidance of punishment causes a Low-Reward or Low-Punishment state, decreasing the activity of the two systems. Thus, arousal is determined by the activation of the systems and prepares the organism to act for the avoidance of punishment or the acquisition of reward. While, the activation or deactivation of one system instead of the other established the valence (high PAS activity and /or low RAS activity for negative valence, and the contrary for positive one) (Mendl et al., 2010; Mendl and Paul, 2020). PAS and RAS can be independent or can mutually influence, also inhibiting one another. They are regulated by the action of different neural substrates. RAS is associated, especially, with mesolimbic dopaminergic and opioidergic circuits, (medial) orbitofrontal cortex, nucleus accumbens and ventral pallidum, while PAS is regulated by serotonergic circuits, (lateral) orbitofrontal cortex, amygdala, anterior insula, and lateral habenul (Mendl and Paul, 2020).

The location of the animal in the core affect can rapidly change and it depends on success or failure in acquiring a reward and avoiding punishment. The short-term changes in the core affect are determined by discrete emotions, which are elicited by rewarding and punishing stimuli and favour physiological and behavioural responses to facilitate the immediate survival of the organism. Sensations also, mediated by the connection between sensory organs and the brain, and motivations, determined by physiological needs or external stimuli associated with rewards, have a valence, so can be located in the core affect, and cause, as discrete emotions, short-term changes in the animal's location in it (Mendl et al., 2010; Mendl and Paul, 2020). The cumulative function of short-term emotional experience determines the mood state, which is a long-lasting background state that represents a "running mean" of the positions occupied within the space defined by the arousal and valence axes (core affect) over a previous period and changes slowly with the acquisition of new experiences (Mendl et al., 2010; Mendl and Paul, 2020). The mood state is that background core affect state that remains in absence of emotion-inducing events and is informative of the environment where the animal leaves, in terms of rewards' opportunities and presence of threats, and how it is coping with it. Mood state influences cognitive processes, such as memory, attention, and decision-making, thus it affects also the evaluation of the situations and the respective emotional responses. Therefore, there is a bidirectional influence and a mechanism of positive feedback between short-term discrete emotions and longer-term core affect mood (Mendl et al., 2009, 2010). After making the distinction between emotion and mood, we should consider that in the literature we can find the term affect as synonymous with both emotion and mood. However, it could be better considered and used "affect" as an umbrella term that encompasses both emotion and mood (Kremer et al., 2020; Paul et al., 2005).

1.3.2 Measurement of animal affective states

Affect is a multi-component state that comprises feeling/subjective, motor/behavioural, somatic/physiological and cognitive components (Kremer et al., 2020). In addition, it should be considered that affect has both a functional role (promoting the acquisition of reward and avoidance of punishment, facilitating reproductive success and survival and influencing the judgment of unknown situations and attention to certain stimuli) and a social role, facilitating the group-living, the formation of social bonds and the communication. Indeed, affect can spread among individuals, as demonstrated by phenomena like social facilitation and emotional contagion, which have been studied in different species (Kremer et al., 2020; Špinka, 2012).

The feeling/subjective component of animals' affective states cannot be measured and it remains an assumption. On the contrary, the physiological and behavioural responses to different tests or situations that are supposed to induce particular emotional states are often employed and measured for the study of affective states, in particular discrete emotions (Kremer et al., 2020; Mendl et al., 2010). In addition, to physiological and behavioural measures, there is the investigation of the cognitive component of affective states. Affect-induced cognitive bias, such as memory, attention and judgement bias, can be useful in the objective evaluation and measure of mood-like states in animals. If animals are exposed to the same stimulus, according to their background mood state, they can have a different appraisal of the situation and hence different short-term emotional responses that can induce several behavioural and physiological measurable changes. Indeed, the negative cognitive biases induced by a particular background mood may cause a negative appraisal of a situation or a stimulus and, as consequence, also a negative short-term emotion (Mendl et al., 2010; Paul et al., 2005). Physiological, behavioural and cognitive parameters can be indirect measures of affective states but present several limitations and cannot prove the subjective emotional experience. Thus, coupling several parameters of different nature and the application of different techniques is probably the best method for interpreting affective states in animals (Kremer et al., 2020; Mendl et al., 2009; Paul et al., 2005).

Physiological measures

The study of the physiological component of affective states often overlaps with research about stress. The term "stress" refers to the state that arises following challenges to the homeostasis of the organism. Thus, even if stress is not necessarily negative (Ralph and Tilbrook, 2016), it is generally viewed as a response of the organism to an aversive situation, which induces negative affective states, such as “anxiety” or “frustration”. Therefore, also because a lot of research focuses on stress, the effort in the study of negative affective states has been greater than that of positive ones (Boissy et al., 2007; Paul et al., 2005). In addition, it should be considered that often the physiological changes employed for stress evaluation, such as the increase of cortisol level and heart rate in front of a stimulus, can be useful in the evaluation of the arousal of an affective state but not necessarily in the understanding of its valence. Physiological responses can be measured by neuroendocrine activity, immunological parameters and autonomic nervous system (ANS) activity, in particular considering the balance between sympathetic and parasympathetic systems (Boissy et al., 2007; Kremer et al., 2020; Paul et al., 2005).

The research about neuroendocrine activity in affective states considers the evaluation not only of glucocorticoids and catecholamines levels (indices of the hypothalamic-pituitary-adrenal (HPA) and sympathetic-adrenal-medullary (SAM) axis activation usually employed in stress research) but also of oxytocin, vasopressin, prolactin, opioid peptides, dopamine and serotonin levels (Kremer et al., 2020; Paul et al., 2005). For example, oxytocin (OT) and arginine-vasopressin (AVP) are two important nonapeptides involved in social behaviour, cognition and stress response (MacLean et al., 2017). The dynamic balance of these two peptides mediates a large range of emotional states. AVP is anxiogenic and increases sympathoadrenal activity, while OT has the opposite role because is anxiolytic and inhibits the sympathoadrenal axis (MacLean et al., 2017). AVP is usually associated with stress and anxious, fearful and aggressive behaviours (Neumann and Landgraf, 2012) and preliminary studies in dogs seem to confirm this (Hydbring-Sandberg et al., 2004; Jeong et al., 2020; Pirrone et al., 2019). On the other hand, OT inhibits fear-like states and facilitates affiliative behaviour in stable social contexts, such as during affiliative human-dog interactions (Nagasawa et al., 2015; Neumann and Landgraf, 2012; Odendaal and Meintjes, 2003; Rehn et al., 2014). The increase of OT in response to positive stimuli was largely studied, thus OT is often employed as an indicator of positive affective states (Kremer et al., 2020; Mitsui et al., 2011). However, it should be considered that most of the research that considers OT is especially focused on positive affective states and this creates a bias. Indeed, other studies have demonstrated that oxytocin can increase also during negative affect, as in socially challenging situations. Therefore, it could be better to consider OT as linked to social coping strategies, and its increase per se has no precise valence but it is an adaptive mechanism to maintain existing social bonds. Thus, the valence of oxytocin is modulated by the environment and social context and it is important to maintain this contextualization during the study design and interpretation of results (Kremer et al., 2020; Rault et al., 2017). Like OT, also the activation of the endogenous opioids system is associated with both negative and positive affective states and opioids modulate social bonds and affiliative behaviour (Nummenmaa and Tuominen, 2018). Indeed, endorphins are released during reward and danger signals (Spruijt et al., 2001) and the administration of the exogenous opioid stimulates both negative and positive affects (anger and, in humans, pleasure) (Nummenmaa and Tuominen, 2018). In addition, the opioid system regulates the mesoaccumbens dopamine system. Opioids act on the mesolimbic axis inducing the stimulation of dopaminergic neurons in the ventral tegmental area or the increase of dopamine in the nucleus accumbens. Usually, dopamine and opioids are associated, respectively, with liking and wanting motivational states, regulating appetitive/seeking and consummatory behaviours. Thus, both dopamine and opioids are involved in the reward system and are important players in positive affective states (Boissy et al., 2007; Spruijt et al., 2001). However, even if evidence suggests that central dopamine mediates positive affect and has a central role in affective process, further research in animals is needed to clarify its role as a marker of affective valence. Indeed, recent research suggests that only a part of dopamine central neurons are inhibited by aversive stimuli and activated by appetitive ones, while most of them are excited by both types of stimuli (Kremer et al., 2020; Matsumoto and Hikosaka, 2009). Interesting to notice that humans affected by depression have a lower level of dopamine in peripheral blood mononuclear cells. Similarly, depletion of serotonin seems to be associated with depression state in humans and a pessimistic bias in animals. This makes peripheral and central serotonin a promising indicator of negative affective states, but further studies are needed (Kremer et al., 2020).

Affective states can influence also immunity indirectly, through the interaction of hormones that bind to the white blood cells' receptors (e.g., glucocorticoids), or directly via sympathetic fibres that connect the brain and lymphoid tissues. Therefore, other physiological promising markers of affective states can be found in the immunological system, such as acute phase protein, cytokines, immunoglobulin and immune cellular response (Kremer et al., 2020; Pressman and Cohen, 2005). Most research has been made in human science. For example, positive affect has been associated with a decrease in C-reactive protein, and it is inversely proportional to fibrinogen levels (Ironson et al., 2018; Steptoe et al., 2007, 2005). Considering cytokines, it has been found that negative affective states can induce the decrease of interleukin

(IL)-2 and IL-3, and the increase of the secretion of TNFα (tumour necrosis factor-alpha) (Mittwoch-Jaffe et al., 1995; Pressman and Cohen, 2005). Human research has also reported an increase in plasma antibodies, immunoglobulins (Ig) A, G and M, induced by positive mood. In particular, secretory Ig-A, which is responsible for the defence of mucosal surface, is easier to measure in saliva. This and the other research have stimulated also animal science, and, for example, it has been found that salivary Ig-A increase during positive affective states not only in humans, but also in animals, and seems to be a promising tool for their assessment (Boissy et al., 2007; Lv et al., 2018; Pressman and Cohen, 2005). Another study in mice has evidenced that high anxiety and restraint stress can induce a decrease in the total number of T-lymphocytes (CD4+ and CD8), and in IgA, IgE, and IgG serum concentrations. In addition, restraint stress can induce an increase in the number of granulocytes and monocytes. These results suggest that anxious subjects may present an impaired immune system and are particularly predisposed to the development of infections and inflammation, especially during stressful situations (Rammal et al., 2010). However, it is important to notice that communication between the nervous and the immune systems is bidirectional. Therefore, not only affective states can influence immunity, but also activation of the immune system can influence brain structures, including those that regulate emotional processes. Indeed, the activation of the immune system can change the affective value, or valence, of a perceived stimulus. In particular, immune activation does not directly induce a negative affective state, but it enhances the reactivity of the subject to potentially negative aspects of a situation, facilitating defensive responses. Thus, inflammatory markers can be useful for the evaluation of the vulnerability to stress, and immune activation could be indicative of the plasticity of valence (from pleasant to aversive) of features in the environment of an animal. However, the immune system results are not so obvious to interpret, because various immune factors can influence each other and, in addition, they can be easily influenced by also non-obvious infectious or inflammatory conditions (Boissy et al., 2007).

Another useful index for animal research to investigate the animal response to affective stimuli can be the autonomic response. The most common parameters investigate are cardiovascular, respiratory and electrodermal responses (i.e., heart rate variability, respiratory rate and surface humidity) and peripheral temperature (Kremer et al., 2020; Mauss and Robinson, 2009). The autonomic nervous system (ANS) is efferent of the limbic system and receives inputs from the amygdala, which, via hypothalamic centres, influences autonomic brainstem nuclei. The excitatory sympathetic system (SNS) and inhibitory parasympathetic system (PNS) are part of the ANS and their often antagonistical activity, which is highly dependent on a great variety of emotions, determines the physiological arousal and state of the organism and regulates its homeostasis (Boissy et al., 2007; Duarte and Pinto-Gouveia, 2017). SNS is usually associated with high arousal negative states, while PNS seems to control the activities during resting and has been associated with positive affective states (Duarte and Pinto-Gouveia, 2017). These two systems regulate the beating activity of the heart, influencing the activity of the sinoatrial node (the principal pacemaker of the heart), the atrioventricular node and the atrium muscle. The SNS, through postganglionic sympathetic fibres and noradrenalin, and PNS, through the vagus nerve and acetylcholine, can act independently or simultaneously in the regulation of the heart rate. Indeed, an increase in the heart rate can be caused by either an increase in sympathetic activity or a decrease in vagal activity or changes in both systems. Therefore, it is challenging to distinguish what component is activated considering the heart rate alone. For this reason, researchers usually employed Heart Rate Variability (HRV) to distinguish between the activation of the two systems. The HRV indicates the variability in time intervals between consecutive heartbeats (IBI). This variability is determined by the independent action of the two components of the ANS, influenced by humoral, neural and physiological factors. When plotted consecutively on a time scale, IBIs produced an oscillatory curve called “tachogram”, (von Borell et al., 2007). The HRV is a useful non-invasive method to assess stress conditions and affective states in humans and animals and can be useful in the evaluation of sympathovagal balance, which can be altered by psychological stress also in absence of detectable variations in heart or respiratory activity (von Borell et al., 2007). HRV can be measured using frequency domain or time domain indices. One of the most important measures of the time domain is the rMSSD (Square root of the mean of the sum of the squares of differences between consecutive IBIs). An increase in HRV reflects the prevalent activity of PNS over SNS. Studies in humans and animals have associated a decrease in HRV, therefore a lower PNS activity, with negative affective states, such as fear, anger, depression and anxiety. On the other hand, putative positive affective states have been related to the PNS activation and higher HRV (e.g., rMMSD), even if this last relationship seems not to be linear (Duarte and Pinto-Gouveia, 2017; Kremer et al., 2020; Reefmann et al., 2009b; von Borell et al., 2007). The cardiovascular system is strictly linked with the respiratory system (as demonstrated by respiratory sinus arrhythmia). Respiratory function is regulated by ANS and it should be considered for a complete evaluation of ANS activation and a correct interpretation of heart parameters results (Kreibig, 2010; von Borell et al., 2007). Indeed, respiratory rate influences the location of the high-frequency (HF) band in the analysis of the spectrum of HRV. The spectrum analysis of HRV consents to analyse frequency domain parameters, which can be divided into high frequency (HF) power (in humans is 0.15–0.40 Hz, and in other species change according to respiratory rate) that represents the modulation of vagal tone, the low frequency (LF) power (0.04–0.15 Hz) indicative of both sympathetic and vagal influences, and the very low frequency (VLF) band (≤ 0.04 Hz) that reflects sympathetic activity (Kreibig, 2010; Li et al., 2019; von Borell et al., 2007) (Li et al., 2019; von Borell et al., 2007). In humans, it has been noticed that anger, happiness, fear and sadness are characterized by different combinations of heart rate and HF of heart rate variability coupled or not with respiratory variations (Rainville et al., 2006). However, even if some research reports a correlation between respiratory rate and the emotional valence of a certain stimulus, others find a relationship between respiratory rate and affective arousal but not with valence (Briefer, 2012; Reefmann et al., 2009a; Von Holst, 1998).

Another interesting parameter that should be coupled with respiratory and heart measures to obtain a comprehensive view of autonomic response and can help to distinguish different emotional valence, especially if considered with other parameters, is electrodermal responses (Collet et al., 1997; Kreibig, 2010). Electrodermal responses, measured through the activation of sweat glands, and skin blood flow, directly linked to peripheric temperatures, are both regulated by sympathetic activation. Skin innervation is composed of autonomic (predominantly sympathetic and only in little part parasympathetic) and sensory nerve fibres because has a double role in receiving sensory information and maintaining the body’s homeostasis. The sympathetic C-fibres provide two different types of innervations: one determines the vasoconstrictive adrenergic activity of the blood vessels and the activation of arrector pili muscles; the other is cholinergic and stimulates the eccrine sweat glands and vasodilatation of cutaneous blood vessels (Fig. 15) (Collet et al., 1997; Glatte et al., 2019). The electrodermal response can be a useful index of SNS activation, which, acting directly on the sweat gland, is reflected by the variance in body surface humidity. Indeed, positive emotional states can determine a deactivation of the SNS and a consequent decrease in variance in body surface humidity (Reefmann et al., 2009a, 2009b).

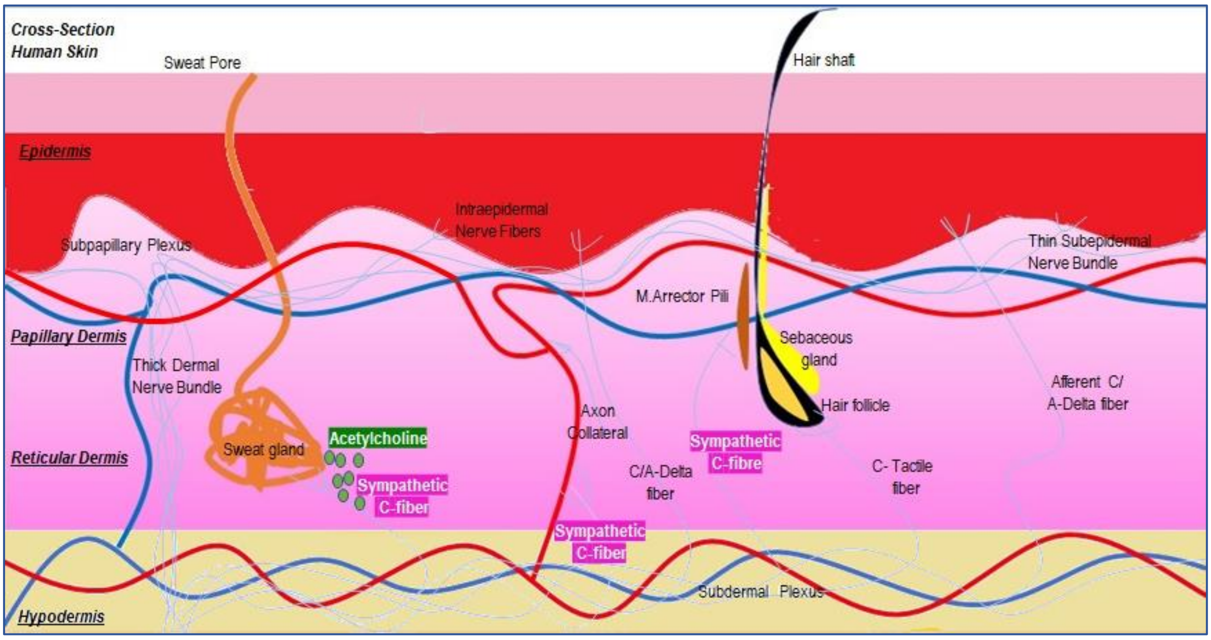

Figure 15. Schematic representation of skin anatomy and innervation. The sympathetic C-fibers innerve blood vessels and the arrector pili muscle. The cholinergic sympathetic C-fibers innervate sweat glands. In the epidermis, there are the free endings of afferent intraepidermal C and Aδ nerve fibers. The same fibers have collateral axons that innerve blood vessels with an efferent antidromic action. Small sensory fibers, which derive from thick nerve bundles, innervate the epidermis forming thin subepidermal nerve bundles (Modified from Glatte et al., 2019).

Also, body temperature, which depends on vasoconstriction and vasodilatation of blood vessels, is determined by the activation of SNS and PNS and can be a useful parameter. Indeed, psychological or physical acute or repeated stresses and unpleasant stimuli can induce the so-called phenomenon of “emotional fever” or “stress-induced hyperthermia” (SIH), during which there is a short-lived increase in core body temperature. The activation of SNS caused by stress stimulates HPA and glucocorticoid production, which leads to the activation of the SAM system and an increase in catecholamines from the adrenal medulla. This process regulates the “flight or fight” responses and induced SIH, which is regulated by the same neural pathway of fever that includes the activity of dorsomedial hypothalamic neurons. Thus, SIH is usually associated with peripheral vasoconstriction and a drop in peripheral temperature. Indeed, during the “flight or fight” responses, the SNS activation limits the blood loss from vulnerable areas and redirects the blood flow to important organs, such as the brain and muscles, which have high metabolic needs. On the other hand, the PNS regulates the “rest and digest” strategy, which causes heart rate reduction, bronchoconstriction, vasodilation, and miosis. Changes in SNS and PNS determine dilatation or constriction of blood vessels but it should be considered that these two systems are not rigidly in opposition, for example, PNS can be active during both pleasant and unpleasant events (Proctor and Carder, 2015; Travain and Valsecchi, 2021). Infrared thermography is a useful and non-invasive method for the detection of body temperature variations, even if can be expensive and need specific training. The eyes are highly irrorated with a rich capillary bed that rapidly reflects the changes in blood flow. Therefore, the temperature of the lacrimal caruncle can reflect the core temperature (Fig. 16). Peripheral temperature can be assessed using infrared thermography in regions like the nose (primates), tail (rat), ear (rabbit) and chicken comb. These areas are particularly rich in arteriovenous anastomoses, where stress-induced vasoconstriction is particularly evident. Drop in these regions has been demonstrated in front of both positive and negative stimuli. It seems that this drop is not indicative of the valence of an emotion per se but can be determined by changes in valence affective state (from positive to negative and vice versa) regardless of its arousal, while other research underline that arousal is quite important to determine temperature changes (Kremer et al., 2020; Proctor and Carder, 2015; Travain and Valsecchi, 2021).

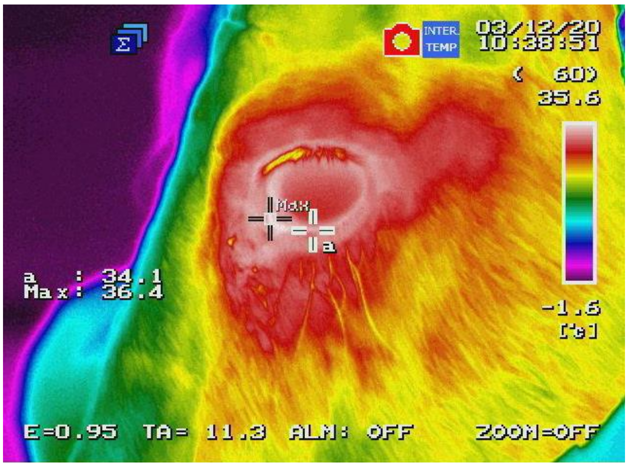

Figure 16. Example of a thermographic image of the lacrimal caruncle of a horse (Source- picture of Irini Kiumurgis).

When measuring physiological parameters, should be considered the time of activation, such as temperature changes in front of stress are quicker than those of heart, baseline level and methodology employed for the assessment. In addition, is more indicative to consider the combination of different parameters than to use only one index (Kremer et al., 2020; Travain and Valsecchi, 2021).

Behavioural measures

The behavioural component of the affective state can be investigated in the environment or during specific test situations. However, most behavioural tests, where the animal is exposed to a novel object, a new environment or an unfamiliar human being, are often designed to measure negatively valenced states, look mostly at the reactivity of the animals, and not always are useful in the evaluation and distinction of affective states (Désiré et al., 2002; Kremer et al., 2020).

In evaluating animal behaviour, we can observe the whole animal behaviour or we can focus only on specific parts. For example, the avoidance or approach to a certain stimulus can be useful indicators of the general valence that the animals attribute to this. Other examples can be specific behaviours, such as freezing and play, which are two behavioural indicators of negative and positive emotional experiences. Interesting to notice that play is suppressed during negative affective states but it is still unclear if this behaviour reflects positive affect, even if some studies consider this behaviour as a positive indicator because its expression occurs when other behavioural needs are satisfied (Ahloy-Dallaire et al., 2018; Boissy et al., 2007; Paul et al., 2005). Other potential indicators of positive affective states are explorative, affiliative and sexual behaviours and auto-grooming, which are all rewarding behaviours. To be self-rewarding the behaviours should be associated with the activation of the mesolimbic system, for example by the production of endorphins. However, stress can sensitize the dopaminergic system and self-rewarding behaviours can become uncontrolled. Indeed, depending on species and circumstances, animals may perform self-rewarding behaviour, such as excessive locomotion or auto-grooming, when an animal's wanting state for a reward is amplified by stressors and rewards are unavailable (Boissy et al., 2007). For example, in non-human primates, auto-grooming and scratching can become displacement behaviours indices of anxiety (Coleman and Pierre, 2014). Other useful behaviours to consider are vocalizations, which can be associated with particular affective states and can be indicative of both arousal and valence of the emotions of the sender. On the other side, vocalizations can also influence and modulate the emotional states of receivers (Briefer, 2012; Manteuffel et al., 2004). Finally, it should be taken into account behavioural lateralization. Indeed, negative and positive affect are processed respectively by the right and left-brain hemispheres, which control the contralateral part of the body. This determines behavioural lateralization, which indirectly reflects the activation of one of the two hemispheres. The preferential use of one side in the motor pattern and sensory investigation reflects cerebral lateralization, so can be employed in investigations of affective states. For example, in horses, the use of the left eye seems to be preferred for the investigation of negative valenced stimuli (De Boyer Des Roches et al., 2008; Leliveld et al., 2013; Siniscalchi et al., 2021).

Considering specific body parts of animals, facial expressions, head and tail posture, ear movement and the percentage of visible white of the eyes can be employed for the assessment of affective states. In particular, for the study of facial expression, an objective system (Facial Action Coding System or FACS) has been developed for several species, including horses, for the documentation of facial movement, described in terms of the underlying muscle contractions (Kremer et al., 2020; Waller and Micheletta, 2013; Wathan et al., 2015). Another system, the Grimace Scale, has been created for the objective investigation of pain through the use of facial expressions in different animals, including mice, horses and donkeys (Costa et al., 2014; Langford et al., 2010; Orth et al., 2020).

Using behaviours as indicators of affective states should be made with caution. Indeed, some behaviours can occur in different affective situations and low arousal affective states can be difficult to detect using behavioural indices alone. Furthermore, the evaluation of behaviour should be always made considering the entire context, all the environmental influences and subject-related factors (Kremer et al., 2020; Paul et al., 2005).

Cognitive measures

Physiological and behavioural measures offer a lot of information about affective states but more often are indicative of arousal, while are less informative about emotional valence (e.g., HPA and SAM activations). Furthermore, only a few measures are useful indicators for positive affective states, and there is a lack of *a priori* hypothesis indicative of how these measures change according to changes in affective states (especially emotional valence) (Boissy et al., 2007; Boissy and Erhard, 2014; Mendl et al., 2009; Yeates and Main, 2008). Therefore, in both human and animals there are growing interest in the study of brain areas that regulate the affective process and their role in influencing decisions. However, in animals, this evaluation can be difficult and usually involves the subjects' restraint. Thus, one promising approach can be to study the cognitive component of emotions (Mendl et al., 2009; Paul et al., 2005). Cognition has been defined as "the mechanisms by which animals acquire, process, store and act on information from the environment" (Shettleworth, 1998). This definition comprises many types of processing information, from sensory perception to associative learning, considering also the conscious and rational processes. From human sciences, we know that in people when short-termed emotions are triggered or the mood state change, contemporary also the way of thinking can modify. This is indicative of the existence of a strict relationship between affective states and cognition. However, there are controversial points of view about the interactions between affective and cognitive processes (Paul et al., 2005). Some authors argue that affect and cognition cannot be independent because the only identification and evaluation of a situation or a stimulus that triggers an emotional response should be made through a cognitive process (Forgas, 2000; Lazarus, 1999). On the other hand, other authors maintain a distinction between emotional and cognitive processes, both conceptually and neurobiologically. This view recognises the existence of a strict link between affective states and cognition, but, in the meantime, argues the anatomical, functional and phylogenetical independence between the two systems. Indeed, affective states are regulated by subcortical areas of the brain and are visceral, while the cognitive system is more evolutionary recent, is controlled by the cortical part of the brain and presents more variations across species (Panksepp, 2003). Another argument that supports this independence is that affective states can be induced without cognitive involvement (Russell, 2003; Zajonc, 2000). Even if subcortical areas are crucial for the generation and expression of affective states, it is also true that cognitive processes are highly involved in the both production and results of affective states (Paul et al., 2005). Indeed, the division between cognitive and affective brain regions is not clear-cut. For example, the amygdala, which is the core of emotional neural circuits, is involved also in cognitive processes, such as attention and memory. In the meantime, the brain areas involved in cognitive processes, such as the dorsomedial and ventrolateral prefrontal cortex, have a clear role in emotional experiences. Therefore, also at the brain level, cognition and emotion are integrated (Roelofs et al., 2016). The bidirectional causal relationship between affective states and cognition gives the possibility of employing the study of cognitive processes to obtain important theoretical and practical means for the study of affective states in both humans and non-human animals. Cognition influences and is influenced by affective states. Thus, different tasks that consider cognitive processes, in particular attention, perception, memory, expectation and risk assessment, have been developed and employed in the study of affective states in people that were not able to linguistically report their feelings. Therefore, human research already provides a series of useful tools that can be modulated for the study of affective states in non-verbal animals (Mendl et al., 2009; Paul et al., 2005). The study of the affect-cognition relationship in animals should focus on the interactions between these two systems, considering both the process of appraisal of stimuli, which can trigger certain emotional responses and cognitive outputs, which can be influenced by affective states (Paul et al., 2005).

The appraisal theories, employed also in human cognitive psychology, offer a possibility for understanding emotional experience also in animals (Boissy et al., 2007; Désiré et al., 2002). The appraisal process of a stimulus made in a certain moment allows an individual to assign an emotional value to the situation considering the available information, and to adequately respond (Paul et al., 2005). There are different appraisal theories, according to which affective states evolve from a series of cognitive evaluations in front of a situation or of an event by the individual. This cognitive appraisal influences other emotional components (neurophysiological, behavioural and subjective responses) and *vice versa* (Désiré et al., 2002). According to the appraisal theory developed by Scherer (2001) for humans, to make the evaluation of a certain situation that elicits a consequent affective state, "situation evaluation checks" or SECs are employed. SECs follow a precise organization and sequence and can be divided into four appraisal criteria from the most elementary to the most complex, according to the type of information that they bring for the evaluation of the situation: 1) RELEVANCE, the characteristics of the event and its relevance for the individual (sadness, pleasantness, predictability, familiarity); 2) IMPLICATIONS, how the consequences of the situation are consistent with individual's expectations; 3) COPING POTENTIAL, the possibility of the subject to cope with it, reacting, controlling or adapting to the situation; 4) NORMATIVE, how the response to the situation fits the social or internal standards (Scherer, 2001). Scherer postulates were adapted to animals by Désiré and colleagues (2002). The appraisal criteria that most animal species can employ to acquire information are relevance implication and coping potential, while normative criteria are used by some gregarious species to modulate their response according to social standards (Désiré et al., 2002). The SECs that an organism can employ depend on their ontogenetic and phylogenetic development, and the process of the appraisal can be classified according to three processing levels: sensorimotor, when the processing is automatic and carried out by motor expressive programs and innate mechanisms triggered by the stimulus; schematic, which implies learning patterns but the processing is still automatic; and conceptual, when the evaluation process is made through conscious thoughts supported by memorised information (Leventhal and Scherer, 1987). The existence of long-lasting memories and the fact that animals can assign an affective value to the knowledge that they acquire through their experiences are indicative facts that the SECs belonging to the relevance category can be elaborated at conceptual levels. Implications, potential coping and normative criteria need more investigation but the possibilities of elaborated appraisals cannot be excluded (Désiré et al., 2002). Therefore, in both non-humans and human animals, we can find similar appraisal processes and mutual neural substrates that regulate the expression and the experience of a wide range of emotions. Thus, animals not only express affective states but can feel a wide range of them. However, animals lack verbal reports and the subjective and motivational feelings can be only inferred by employing physiological, behavioural and cognitive measures, which can be objectively described or quantified (Boissy and Erhard, 2014; Désiré et al., 2002; Veissier et al., 2009).

It is also true that cognitive processes, such as memory, attention and decision-making, can be influenced by affective states, and the results of cognitive performance can be useful indicators to evaluate and measure affective states. In this case, also, researchers have made parallels between animals and humans (Mendl et al., 2009). For example, in human beings, a negative affective state, such as anxiety, can induce more attention to threats, because anxiety and fear have the primary aim of protecting the body, so anxious cognitive bias will be related to greater vigilance (Bradley et al., 1997; Paul et al., 2005). Furthermore, there is a strict link between memory and affective states, proven by the fact that emotionally positive or negative memories are easier to recall than neutral emotional ones and the amygdala has a central role in the storage of emotional memories in both human and non-human animals. In addition, the moderate activation of HPA and SAM favours the consolidation and storage of information in long-term memory, while too high or low activation of these axes is detrimental to the same process. Thus, the affective state influences the ability to store and retrieve memories, such as depression and unhappiness that facilitate an easier recall of bad memories than positive ones (Bradley and Mathews, 1988; Paul et al., 2005). Finally, negative affective states induce a negative judgment of ambiguous stimuli (pessimism), while positive states facilitate an optimistic evaluation (Nygren et al., 1996). Judgment in humans has been studied as interpretations of ambiguous stimuli, expectations about the future and risk-taking. Affective states can influence directly judgement or can induce bias in memory and attention processes that participate in judgment (Paul et al., 2005). Emotional modulation of the cognitive process has an adaptive value and has been subjected to the selective pressure of evolution. Therefore, it is realistically possible to think that this occurs in both human and non-human animals and that cognitive bias in memory, attention and judgement can be useful indicators in understanding animals' affective processes. However, it should be taken into account that cognitive bias could be influenced by personality traits rather than being associated with the affective state, and this can constitute a complication in their interpretation (Paul et al., 2005).

Attention bias congruent with affect is the tendency to increase awareness and attention towards negative and novel stimuli in animals that experience negative affective states. Attention bias has been studied in animals in association with negative affective states, while the influence of positive affective states is still understudied (Kremer et al., 2020). However, animals tend to increase vigilance and scanning behaviours during both threatening circumstances (presence of predators) and when there are positive stimuli (potential mates or food sources). Indeed, the amygdala seems to lower neural threshold activation of the sensory system, facilitating orientating behaviours to stimuli with an affective value. For example, this neural structure is directly involved in the enhancement of attention and vigilance during fearful situations. Therefore, the only increase in attentive behaviours is more indicative of the arousal of affective states rather than of their valence (Paul et al., 2005). However, attention bias tests have been recently proposed and developed in animals, considering those already present in humans, but methodologies are still under investigation and further research is needed (Kremer et al., 2020; Monk et al., 2018). The tests modulated on animals consider the attentive response of subjects to visual threatening stimuli (Lee et al., 2016; Monk et al., 2018; Paul et al., 2005) or novel auditory cues. For example, horses in depressive-like states show decreased attention to novel auditory stimuli (Rochais et al., 2016). Another potential way to assess attentive bias could be to consider how easily the animal can be distracted from a task that required attention, theorising that animals in an anxious or negative affective state could be more easily distracted (Paul et al., 2005). These tests required short or no training time but should be considered that attention bias can be influenced, as other cognitive biases, by personality and social rank (Kremer et al., 2020).

Memory bias congruent with affect corresponds with the tendency of animals to remember specific events according to their affective state (Kremer et al., 2020). The emotional modulation of memory has an adaptive value. Indeed, memories of emotionally arousing stimuli that can have positive (food) and negative valence are more advantageous for survival and reproduction than neutral emotional memory. Considering this, memory performance can be more indicative of emotional arousal than of valence but the findings in humans demonstrate that the valence of memories retrieved is congruent with mood state (Paul et al., 2005). Paul and colleagues (2005) proposed to test memory bias by exposing animals to positive and negative stimuli on separate occasions when they are in the same affective state and context, and then re-exposing them later. The prediction is that animals in a depressive-like and negative state would behave as they recall negative memories, while animals in a positive affective state would recall positive memories and behave consequently (Paul et al., 2005). In animals, studies about memory bias are few and are limited to rodents. Furthermore, memory bias tests need training and are time-consuming (Burman and Mendl, 2018; Takatsu-Coleman, 2013).

Judgment bias congruent with affect indicates the tendency of an individual to respond positively or negatively in front of ambiguous cues according to its affective state. Such tendencies can be defined as “optimism” or “pessimism” (Kremer et al., 2020; Mendl et al., 2009). Roelofs and colleagues (2016) combined the definition of Bateson and Nettle (2015), Boleij et al. (2012) and Douglas et al. (2012): “A judgment bias is a relative reaction to an ambiguous stimulus, expressing an ‘‘interpretation’’ of this stimulus and an ‘‘expectation’’ about the consequences of the reaction (Boleij et al., 2012). In Judgement bias tests (. . .) animals that respond to the ambiguous stimuli similarly to the positive stimulus are interpreted as displaying a high expectation of reward in the presence of ambiguous information, and hence an ‘‘optimistic’’ cognitive style indicative of a positive affective state. In contrast, animals that respond to the ambiguous stimuli similarly to the negative stimulus are interpreted as displaying a higher expectation of punishment or lower expectation of reward, and hence a more ‘‘pessimistic’’ cognitive style indicative of a more negative affective state’(Bateson and Nettle, 2015). Therefore, in judgement bias tests, optimism is operationally defined as a higher proportion of responses to an ambiguous cue as if it were the cue predicting the positive outcome, and pessimism as a higher proportion of responses to an ambiguous cue as if it were the cue predicting the negative outcome (Douglas et al., 2012)’’. The first published study in animal research on judgement bias and judgement bias test (JBT) is that of Harding and colleagues (2004), which has founded the basis for subsequent studies in other species (Mendl et al., 2009). The general structure of the JBT includes, before the real test, a training phase. The animal is trained to associate auditory, visual or spatial cues with positive or negative reinforcers and they will respond consequently. The response can be a go/no-go response or an active choice (or go/go task). Therefore, in the first case, for example, the animal in front of cues predictive of a positive reinforcer learns to respond in a certain way (e.g., approach the cue or touch a screen), while in front of negative cues the subject does not respond (e.g., not approach or approach slower the cue or not touch a screen). In the second case, the animal gives a certain response (e.g., peck green or red key) according to the positive or negative perception of the cue. Once trained, intermediate/ambiguous/novel cues (probe cues) are presented to the animal, which will probably respond according to its positive or negative affective state, as if it predicts respectively a positive or negative event (Harding et al., 2004; Kremer et al., 2020; Mendl et al., 2009). 70% of the studies employed a Go/No-Go task. A pre-determined cut-off gives the possibility to classify the response as Go or No-Go. However, the suppression of the response in front of the negative stimulus can influence emotion and can be considered an omission of reaction rather than a pessimistic bias. Active choice can be a possible solution because required a behavioural response of the animal in front of both the positive and negative stimulus. Nevertheless, if the trial in a Go/No-Go does not stop immediately after the reaching of the cut-off of the No-Go response but lasts more than the criterion duration, then the data can also be considered as an active choice response (Roelofs et al., 2016). Even if the JBT structure proposed by Harding and colleagues has constituted the basis for subsequent studies, for the successful advancement of animal research the task employed in JBT for different species should be validated and adapted to the abilities of the species considered (Roelofs et al., 2016). Therefore, in animal research, JBT structure differs in several aspects: the type of cue (visual, auditory or spatial), the number of ambiguous probe cues employed, if the ambiguous probe cue is rewarded or not and how (e.g., randomly), the type of negative reinforcer (absence of reward or a slight punishment such as a loud noise), the type of response (go/no go or active choice). A common point is that most JBTs use food as a positive reward associated with the positive cue (Roelofs et al., 2016). The validity of the hypothesis that judgement bias can reflect affective states is reinforced if affect manipulation confirms it. Therefore, different researchers on different species have used different types of long-term and short-term affect manipulations, confirming that judgement bias can be a potentially useful indicator of affective states. However, the diversity in affective manipulation and cue modalities employed and the broad range of species where the judgement bias test has been performed can cause difficulties in the systematic interpretation of results, especially if research disagrees with each other (Mendl et al., 2009). In addition, several confounding factors can affect the JBT results. First of all, we should consider that judgement bias can be influenced not only by affective state but also by traits. Emotional trait, which is part of the personality, is a quite constant individual feature that depends on the genome, environment and interactions between them. However, the environment can determine gradually changes over time because traits are not static. An emotional trait determines a stable and individual-specific propensity to behave and respond similarly in front of a variety of situations. Traits can be identified using tests whose results are highly repeatable. On the other hand, states are transient conditions that can be observed in different situations. Anxiety can be considered a trait when it is an intrinsic characteristic of the individual that non varies among situations, and a state when it is experienced by the subject in a particular moment (Roelofs et al., 2016; Strelau, 2001). Therefore, the JBT is a useful tool to assess animals' moods but it should be correlated with other tasks or tests that help to determine individuals' personalities, and so evaluate the influence of traits on JBT (Roelofs et al., 2016). In JBT, the choice of ambiguous cues is also important. Indeed, ambiguity is determined by the possibility of interpreting a stimulus in two or more different ways. In the case of JBT, an ambiguous stimulus should be perceived as positive or negative. Therefore, it is suggested to use cues on a single dimension and scale them in a way that can be differentiated by the studied species. Thus, ambiguous stimuli have not been totally different from trained reference cues, because the risk is to test a response to novelty and not a response to ambiguity (Roelofs et al., 2016). Once the characteristics of ambiguous cues are decided, it should be guaranteed that they maintain their ambiguity during repeated test trials. Most of the researchers decide to leave ambiguous stimuli unrewarded. This lack of reward will be recognized by the animal after extensive training during which reward is always present and this can facilitate the learning process. Therefore, the repetition of test trials can induce animals to learn to associate ambiguous stimuli with a determined outcome. In this case, probe cues can lose ambiguity, influencing the subsequent choices, which probably will be more pessimistic, and leading to false conclusions after the JBT. Similarly, rewarded ambiguous cues can facilitate associative learning. Therefore, during JBT should be taken, precautions against the loss of ambiguity and avoid the associative learning process during the test progression (Roelofs et al., 2016). Different methods exist to avoid this problem. One can be to associate a secondary reinforcer during training, such as a clicker. The secondary reinforcer was associated with a food reward during training, while, during the test, the ambiguous trials were only associated with the clicker reinforcer. This consents maintenance of responsiveness toward the ambiguous cue (Keen et al., 2014). Another method is to employ a partial reinforcement of positive trials during training and testing. In this case, positive cues were associated with a reward only 50% of the time, while the punishment of negative cues remained 100%. Thus, animals learned to have lower expectations of reward during ambiguous trials (Neave et al., 2013). Other authors proposed to minimize the exposure to ambiguous trials, also employing a between-subject design. However, lowering the number of ambiguous trials can make JBT results prone to chance findings (Brilot et al., 2010; Rygula et al., 2013; Vögeli et al., 2014). Another potential confounding factor is the isolation of social animals during training and testing. Indeed, in social animals that are separated from their group to be trained and tested individually the stress level can increase (Roelofs et al., 2016). Stress should generally be avoided because can diminish learning abilities because it increases distraction and induces acute or chronic detrimental effects on memory formation and retrieval, even if mild stress can promote memory consolidation (Mendl et al., 2009). Indeed, separation from the group can decrease the possibility of learning during the training or can influence JBT results. To avoid this problem can be necessary a prolonged time of habituation and pre-training (Roelofs et al., 2016). Furthermore, as previously stated, the validation and development of JBT should be made considering the studied species, implementing a discrimination task that animals of this species can learn easily and without stress and that needs a short pre-training period. This will lower the exclusion rate of animals that during training do not succeed in learning the task. Indeed, if the proportion of “learners” is low, it can constitute a bias in the studied population and it will be difficult to generalize the JBT results (Roelofs et al., 2016). In addition, it is important to determine the value of the reinforcement associated with positive and negative cues. Indeed, if the affective value of the reinforcement of negative training cues is mild (e.g., no food), this may induce animals to generally respond to intermediate probes because the risk associated with the negative outcome is low. In this condition, the affect-induced bias can be observed in the intermediate probe nearest to the negative cue, where the perceived probability of receiving a negative reinforcer is highest. When the negative reinforcer is more severe (noise or unpalatable food), subjects tend to be more cautious to respond in front of ambiguous cues, and the affect-induced bias can be detected in the intermediate position nearest to the positive training cue. Therefore, it would be better if the strength of the reward and that of the punisher are equal to the animal’s motivation (Mendl et al., 2009). The motivation of animals should not change during the entire procedure of JBT. Indeed, the problem of the employment of food as a reward is that motivation towards food could change during the training and the test because of increasing satiety, but this problem could be evidenced only *a posteriori* (Kremer et al., 2020). JBT has been widely employed in companion, farm and zoo animals to study welfare and the effect of common practice or handling procedures (e.g., enrichment or dehorning) on animals’ affective state. However, extensive training that is needed before performing the JBT can decrease the applicability of this cognitive test in welfare science. In addition, training can perceive by animals as enrichment and can hide the effect of the experimental manipulation. Therefore, the results of JBT for welfare studies should be cautiously interpreted, considering that animals in different affective states can be influenced differently by the handling and training that cognitive tests require (Roelofs et al., 2016).

The decision-making process that results in the judgement of ambiguous stimuli is composed of different components that can be influenced by the affective state. The different components are sensory registration, evaluation and probability of the decision outcomes, and selection of the response (Mendl et al., 2009). Indeed, initially, the sensory cortices receive the incoming sensory information and make a neural representation (percept) of it. Then, the percept is elaborated and the value and the probability of the outcome of certain behaviour in response to it (expected utility) are evaluated in other brain areas, such as the amygdala and orbitofrontal cortex, causing changes in the dopaminergic activity (DA) in the mesocorticolimbic system.

Finally, probably the basal ganglia are involved in the selection of behavioural responses after the evaluation of the expected utility of a decision. These components are interconnected by multiple circuits that work in parallel and are widely distributed in the brain (Fig. 17) (Mendl et al., 2009).

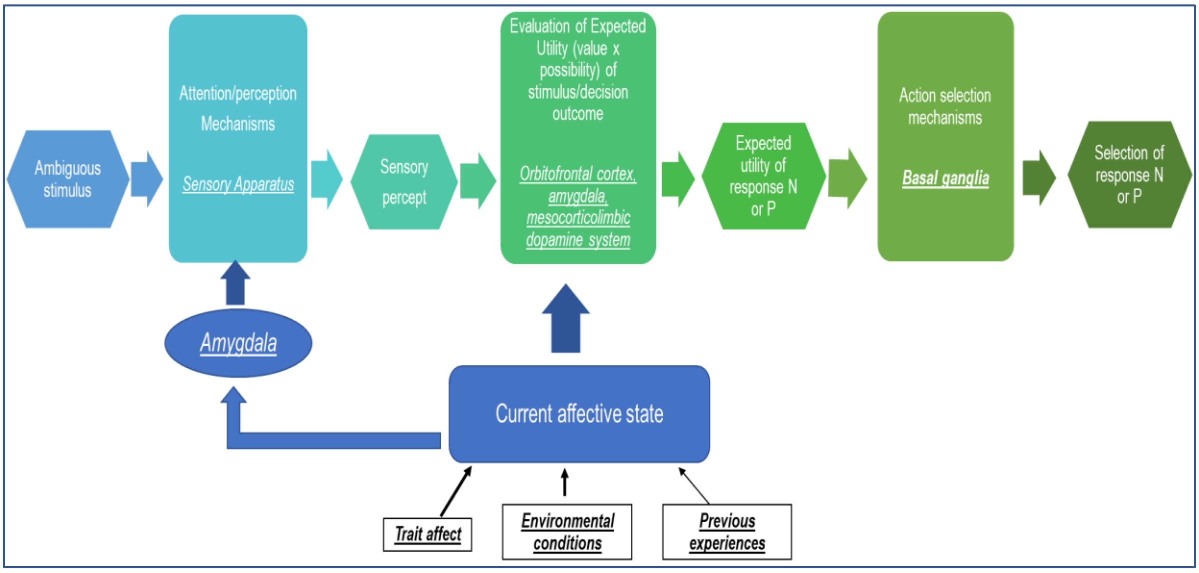

Figure 17. Scheme of the events under the decision-making process that guides the judgement of an ambiguous stimulus. This process depends on inter-connected and parallel circuits and can be influenced also by motivation. N=negative; P=positive (Modified from Mendl et al., 2009)

However, the first process of registration of ambiguous stimuli is not always "value-free". Indeed, perception is an active and selective process largely influenced by previous experiences, goals, motivation and expectations (Mendl et al., 2009). An emotional and motivational state can affect the active sensory perception of an ambiguous stimulus through a top-down feedback process, where the higher brain structures of the sensory processing pathway (e.g., prefrontal and parietal cortex) affect lower structures (e.g., thalamus), maybe giving "bias signal" to sensorimotor circuits (Miller, 2000). After the registration of the ambiguous stimulus, the anticipated value and the probability of different outcomes ("expected utility") are estimated involving different brain areas. In primates, the sensory stimuli representation acquires a possible value in the orbitofrontal cortex and amygdala that are directly connected to the mesolimbic dopaminergic system, which is involved in reward and motivation systems (Mendl et al., 2009). During the JBT, the unconditioned "liking" response to the food reward, mediated by opioids, activates the "wanting" response, mediated by dopamine. Therefore, this may cause the generation of reward prediction signals in the dopaminergic system. Long-term negative states, determined by chronic stressors or situations that animals cannot solve, cause a decrease in dopaminergic activity and a consequent decrease in the value of positive stimulus-response-outcome contingency. This condition decreases the chance that animals respond positively to ambiguous cues. Conversely, during short-term mild stress conditions that animals can easily solve, the dopaminergic system is stimulated and animals are more prone to show an adaptive optimistic-like response toward ambiguous cues (Mendl et al., 2009; Spruijt et al., 2001). The importance of positive and negative events also can be influenced by the modulation of the amygdala and orbitofrontal cortex. Mood states, which can be described as generalized changes of baseline neuronal activity in these brain areas due to the effect of individual emotional events and modulation of the dopaminergic system, can influence those neural valuation processes. In addition, mood influences memory retrieval, conditioning the value attributed to the ambiguous stimulus. The mood-congruent memory effect involves signals between brain areas that modulate emotion and areas that code for memory, such as the hippocampus (Mendl et al., 2009; Rolls, 2013). However, decision-making is influenced not only by the anticipated value of an outcome but also by the anticipated probability of it, which is affected by the similarities between the ambiguous cue and the two training cues. The anticipated probability of the outcome also seems to be modulated by the dopaminergic system, but the mechanism is uncertain and the neural substrates involved in probability are less studied. However, the pessimistic bias of depressed and anxious people, and probably also animals, can be influenced by the individual perception of the probabilities of good or bad consequences (Mendl et al., 2009). Valuation and probability of outcomes do not necessarily co-vary and affective state can influence them separately. Indeed, the valuation of the outcome can be more linked to the motivational state, while the probability of it may be influenced by more general states, such as mood. The separated modulation of value and probability can also influence the adaptive response that the affective state modulates. For example, anxiety can lead to an increase in individual perception of the probability of a negative outcome triggering alertness, while depression can cause a decrease in both value and perceived probability of a positive outcome, enhancing withdrawal strategy (Mendl et al., 2009). The integration of value and probability of outcome constitute the “expected utility” that is modulated by the affective states. “Expected utility” guides decisions and finds its neural substrate in the amygdala, orbitofrontal cortex and ventral striatum, which are all areas involved in coding the reward value, and the cingulate cortex and ventromedial prefrontal cortex. The affective states use these routes to influence the “expected utility”, as demonstrated by the activation of the cingulate cortex in people while imagining positive future events or, generally, in optimistic people (Mendl et al., 2009). Therefore, the influence of affective states on the attribution of value, probability and, in general, of “expected utility” of a stimulus and its outcome causes the emergence of judgement biases. “Expected utility” theory has led to several derivations, such as the prospect theory and the “risk-as-feelings” theory, which try to integrate emotional effects on decision-making and have been studied especially in people during gambling tasks. These theories consider the general people’s tendency to prefer to avoid losses and risky or ambiguous outcomes. Loss aversion can generate a bias in decision-making. Therefore, in front of JBT ambiguous cues, there will be a tendency to minimize losses. Considering that, if the discriminative training is made using food vs. no-food, it cannot be excluded that a subject will respond optimistically to reduce the risk of losing a potential reward (or prospect). The neural substrates and mechanisms involved in loss aversion are still unclear, but probably affect states use the same neural routes to influence this phenomenon (Mendl et al., 2009). For example, it has been evidenced that there is an activation of the amygdala, which is an emotional related structure, when losses are anticipated and experienced or in anxious people, where loss aversion is enhanced. Another possibility is an asymmetrical regulation of losses and gains in the brain areas, with the involvement of the ventromedial prefrontal cortex that receives signals from the midbrain dopaminergic system. Furthermore, it is interesting to notice that the noradrenergic system, which is activated during stress and anxiety, seems to have also a role in the enhancement of loss aversion (Mendl et al., 2009). Together with loss aversion, there are risk aversion and ambiguity aversion. The concept of risk can be applied when a subject knows the probability and variance of an outcome, while the concept of ambiguity involved those situations where there is no knowledge of the possibility of different outcomes. In animals, risk aversion seems to be enhanced in those situations where the size of the reward change, while is reduced in front of reward delay. However, how affect influences risk aversion and how this influences judgment biases is still to clarified (Mendl et al., 2009). On the contrary, ambiguity aversion seems to involve the amygdala and orbitofrontal cortex, which are emotional related brain areas, suggesting that an emotional process can occur in the perception of ambiguity and that the affective state can influence this process (Mendl et al., 2009). Indeed, the increase of ambiguity in choices corresponds to an increase in the activation of the amygdala and orbitofrontal cortex, and a decrease of activation in the striatal system, which, instead, correlates positively with expected reward. Ambiguity occurs when information miss and probabilities are uncertain, so the choice can lead to something unknown and potentially dangerous. In this condition, the brain is in a state of alertness, where cognitive and behavioural abilities are activated to find more information from the environment (Hsu et al., 2005). In JBT, the ambiguity of cues is determined by the fact that the subject does not know the reinforcement associated with them, but, as previously stated, if they are always followed by the same outcome over different trials, it is possible the verification of ambiguity loss. The ambiguity aversion can be modulated by affective states and can lead to different judgement biases but this phenomenon does not influence the response towards training unambiguous probes (Mendl et al., 2009). After outcome and stimuli evaluation, the information should be integrated leading to the final behavioural decision. Basal ganglia seem to be directly involved in the selection of how to act, considering the incoming information, even if other studies consider that selection of action happens in cortical structures. In the basal ganglia, there is the nucleus accumbens, which is an important brain structure that can be influenced by environmental factors that determine affective state changes (Mendl et al., 2009). Indeed, the nucleus accumbens plays an important role in the integration of valenced (positively or negatively) information and subsequent selection of the approach/avoidance behaviours (Hamel et al., 2017).

To conclude, cognitive biases are promising indicators for the evaluation of animals. However, they involve a series of neurobiological and physiological mechanisms that should be deepened. In particular, the judgment bias test has been implemented in a wide range of species and this has led to the development of several variations of JBT and its criteria. JBT have a series of potentially influential factors, such as traits, that should be considered during its implementation and interpretation of its results. In addition, should be taken into account that judgement bias tasks need to be always adapted to the species that we are studying. JBT has been used in animal welfare research, although its applicability in the field can be difficult. In particular, the prolonged training period makes this kind of test not appropriate for routine monitoring of animal welfare (Mendl et al., 2009; Roelofs et al., 2016).

2. OBJECTIVES

This PhD project aims to evaluate the welfare of the domestic horse (*Equus ferus caballus*) in different kinds of management.

The lack of legislation on precise standards for horse management has led to the development of different systems, which vary in terms of activities where the horse is involved, freedom of movement, the possibility of socialization, and feeding routine. In particular, this thesis has analysed and compared three realities that are in Italy: the traditional stable, the natural boarding system and the Ethological stable.

The first study considers the PNEI (Psychoneuroimmunoendocrinology) principles for the evaluation of horse welfare. Endocrine and immunological parameters have been used to make a comparison between two groups of horses kept in traditional and natural boarding systems. In this research, for the first time, the assay of the dehydroepiandrosterone (DHEA) in horsehair has been validated.

The second study investigates the management practices and housing characteristics that can favour the emergence of stereotypies and abnormal behaviours. Furthermore, cortisol and DHEA concentrations in plasma and horsehair were compared in healthy horses and horses that show behavioural problems, such as phobic, aggressive or anxious behaviours and stereotypies.

The third research aims to describe the daytime activity budget of horses housed in Ethological Stable, an alternative kind of management and housing system of horses in Italy that has been designed trying to fulfil the behavioural needs of this species.

Finally, the last two studies are focussed on the use of a no spatial go/no go Judgement bias test (JBT) as a useful test for the evaluation of affective state in horses. First of all, we have compared the results of this kind of test in three groups of horses kept in different management: traditional stable, ethological stable and natural boarding system. Successively, we analysed the limitations and possible confounding factors in the application of JBT in horses. In both these last studies, we have employed a multidisciplinary approach, considering also endocrinological parameters, personality traits, and, in the last article, behavioural data.

All the studies are reported in the *Paper compendium*, respectively in Papers 1, 2, 3, 4 and 5, which are already published:

Paper 1. Placci, M., Marliani, G., Sabioni, S., Gabai, G., Mondo, E., Borghetti, P., De Angelis, E., Accorsi, P.A., 2020. Natural Horse Boarding Vs Traditional Stable: A Comparison of Hormonal, Hematological and Immunological Parameters. Journal of Applied Animal Welfare Science, 23, 366–377. https://doi.org/10.1080/10888705.2019.1663737

Paper 2. Arena, I., Marliani, G., Sabioni, S., Gabai, G., Bucci, D., Accorsi, P.A., 2021. Assessment of horses' welfare: Behavioral, hormonal, and husbandry aspects. Journal of Veterinary Behavior, 41, 82–90. https://doi.org/10.1016/j.jveb.2021.01.006

Paper 3. Marliani, G., Sprocatti, I., Schiavoni, G., Bellodi, A., Accorsi, P.A., 2021. Evaluation of Horses' Daytime Activity Budget in a Model of Ethological Stable: A Case Study in Italy. Journal of Applied Animal Welfare Science, 24, 200–213. https://doi.org/10.1080/10888705.2020.1857252

Paper 4. Marliani, G., Balboni, A.., Tiberi, C., Melavasi, R., Gardini, A., Accorsi, P.A., 2022. Is the judgment bias test a good tool to assess the quality of horse management?. Journal of Veterinary Behavior, 58, 62-69. https://doi.org/10.1016/j.jveb.2022.11.002

Paper 5. Marliani, G., Vannucchi, I., Kiumugis, I.., Accorsi, P.A., 2022. Limitations of spatial judgment bias test application in horses (*Equus ferus caballus*). Animals, 12(21), 3014. https://doi.org/10.3390/ani12213014

3. PAPER COMPENDIUM

PAPER 1

Natural Horse Boarding Vs Traditional Stable: A Comparison of Hormonal, Hematological and Immunological Parameters

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Natural Horse Boarding Vs Traditional Stable: A Comparison of Hormonal, Hematological and Immunological Parameters

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ABSTRACT

In the equestrian world, two different types of management can be distinguished: traditional management and natural boarding. The aim of this research was to compare hormonal, hematological, and immunological parameters of 47 horses kept in these two different managements. Blood and horsehair of the horses were sampled to determine DHEA (dehydroepiandrosterone) and cortisol concentration through RIA. Moreover, blood count was conducted, and flow cytometry was employed to phenotype lymphocyte subpopulations. Results showed that, in horsehair, DHEA concentration was significantly higher in natural horses, whereas cortisol concentration and cortisol/DHEA ratio significantly lower. These hormonal parameters are used to assess the stress condition and the welfare of animals. The most favorable endocrine framework found in horses kept in natural boarding suggests that this management conveys most with ethological and physiological needs of the species. The research underlines the need of a modification of horses' husbandry systems. For the first time, this study validates the assay of DHEA in horsehair.

KEYWORDS: Horses; DHEA; cortisol; lymphocytes; management

INTRODUCTION

The concepts of “welfare” take into account animal body health, feelings, natural life conditions, and behaviors. A condition of good welfare depends on environment, husbandry and human-animal care (Ewing, Lay, & von Borell, 1999; Fraser, 2001; Moberg, 1987). Even if stress and welfare are two different concepts, they both depend on animals’ perception of environment and describe their physiological and behavioral responses (Moberg, 2000; Veissier & Boissy, 2007). Stress responses, such as cortisol release, are commonly used to assess animal welfare. Researches on stress have led to an increasing interest on psychoneuroimmunology (PNI), which studies the interactions among the central nervous system, the endocrine system, and the immune system, in particular under stress conditions (Padgett & Glaser, 2003).

Nowadays, the interest in animal welfare and the knowledge of equine physical and ethological needs have led to the development of natural horse boarding, which distinguish itself from traditional stables (Bekoff & Byers, 1988; Mills & Nankervis, 2001). Horses are grazing herbivores, social animals and preys, who, during their evolution, have developed the escape and group living as principal survival strategies. Current management practices of horses are driven by human requirements and costs limitations but often ignore basic equine needs. In developed countries, most performance and leisure horses are intensively managed. They are predominantly confined and socially isolated in a stable. The traditional management adopts individual stalls and maintains their horses in enclosed environments most of the time, because it is easier to control them, their diet, and to catch them and to maintain them clean. From humans’ point of view, stalls give protection from weather and a comfortable place where to leave, but horses have a different perception of it. In this condition, they are vulnerable and isolated in a closed place that prevents them to escape and restricts their sensory input to control the presence of predators. Moreover, the animals have few opportunities of socialization with other horses. In this kind of management, horses are usually fed twice or three times per day, using a different feeding from that natural (Strasser, 2005; Van Dierendonck & Goodwin, 2005). However, the isolation and the restriction of foraging time can lead equines to develop stereotypies and aggressive behaviors (Goodwin, 1999). In a natural environment, horses pass up to 18 h per day eating, searching and choosing different foodstuffs. For the characteristics of their digestive tract, they should eat small amount of food but continuously. In traditional management, food is provided twice or three times per day and this leads animals to boredom and to the development of pathological conditions, such as colic and gastric ulcers. Another deal is to guarantee to horses the chance of an adequate movement. Horses can travel more than 20 km per day and the free movement in stimulant open spaces gives them the possibility of developing and strengthening muscles and joints and it helps digestion process, metabolism, circulation, and respiration (Mills & Nankervis, 2001; Saba, Montagnani, & Ascione, 2014). The natural boarding of horses tries to fulfill the ethological needs of these animals, keeping them in wide-open spaces and paddocks, letting them to live in herds and giving them the possibility of socializing and of a natural and various feeding. In the traditional management, the equines are shod, and they are ridden using mouthpiece. In addition, during the winter usually, they are clipped and wear blankets. On the contrary, the natural boarding employed the barefoot and, most of the time, horses are ridden without the mouthpieces. In this management, horses are not clipped to favorite the natural mechanisms of thermoregulation (Strasser, 2005).

The aim of this research was to compare hormonal and immunological parameters of sporting horses kept in traditional and natural management. The study has focused on the determination of cortisol and dehydroepiandrosterone (DHEA) levels, and the assay of B-lymphocytes and lymphocyte subpopulations CD4+ and CD8 +. The hypothalamus-pituitary-adrenal (HPA) axis activation is involved in stress and inflammation (Silverman & Sternberg, 2012), and cortisol and dehydroepiandrosterone (DHEA) are the main players of this neuroendocrine system. Cortisol is the main glucocorticoid hormone, and its secretion is related with various diseases and stress conditions (Ayala et al., 2012). Most information on DHEA derives from human and laboratory rodent studies, while few information is available about the biological role of DHEA in ungulates, in particular, the horse. DHEA is an androgen of adrenal origin, but it is also produced in the gonads and in the brain. In humans, DHEA and its sulfated metabolite (DHEA-S) are the most abundant steroids in the circulation (Clark, Prough, & Klinge, 2018) and serve as precursors in androgen and estrogens biosynthesis in peripheral tissues (Hill, Dušková, M. & Stárka, 2015). DHEA has putative anti-glucocorticoid effects (Prall & Muehlenbein, 2018), and measuring cortisol and DHEA and analyzing the cortisol/DHEA ratio allow a more accurate evaluation of the HPA axis activation in stressful or inflammatory situations.

The research has not focalized on ethological observations that other studies apply but has wanted to highlight the influence of different management on physiological parameters, important also to investigate the state of health of animals.

MATERIALS AND METHODS

Ethical considerations

The entire study was previously evaluated and approved by Scientific Ethic Committee for Animal Experimentation of Bologna University. The trial was monitored by the responsible of DIMEVET (Department of Veterinary Medical Sciences) for Animal Welfare.

Subjects and environment

The subjects of the study were 47 horses employed in sporting activity at different levels: 21 bred through natural boarding (NM) and 26 kept in conventional management (CM) (Table 1). The horses came from 10 teams located in the north of Italy. Each stable was classified as conventional or natural depending on their management and taking into account the parameters specified in Table 2.

Sample collection

Sample period lasted from May to July. Blood and horsehair of each animal were sampled once and at the same moment. Blood sampling from jugular vein was made using one vacutest tube (Kina srl, Arzergrande – ITA) containing lithium-heparin and two vacutest tubes with sodium-EDTA.

Lithium-heparin vacutest tube was kept at room temperature and used for the determination of B-lymphocytes and lymphocyte subpopulations CD4+ and CD8+ through flow-cytometry. One sodium-EDTA tube, maintained at room temperature, was used for blood count. Flow-cytometry and blood count were performed within 24 h from blood collection.

The second sodium-EDTA vacutest tube was centrifuged at 4000 g for 10 minutes. Plasma was divided into aliquots and immediately frozen at −20°C, until radio-immuno-assay (RIA) for the determination of cortisol and DHEA concentration.

Samples of horsehair were taken from the mane, using scissors and they were put in airtight plastic bags. The mane was cut 1–2 mm from the base, without taking the roots; then the first 3 cm from the base were used for the analysis. Each sample was identified and stored at −20°C until RIA for the determination of cortisol and DHEA concentration.

Methods of analysis

Cortisol and DHEA determination

Extraction from plasma and horsehair.

For the extraction of steroid hormones from plasma, 5 ml of diethyl-ether (BDH Italia, Milan, Italy) were added to 100 µl of plasma (Tamanini, Giordano, Chiesa, & Seren, 1983). Extraction methodology from horsehair was conducted putting 60 mg of trimmed horsehair (1–3 mm) of each sample in a glass vial with 5 ml methanol (Accorsi et al., 2008). All samples were dried under an air-stream suction hood at 37°C, and the dry-residue was dissolved into phosphate-buffered saline (PBS) 0.05 M, pH 7.5.

Cortisol assay

Cortisol assay in both horsehair and plasma was performed in duplicate, following the method described by Tamanini et al. (1983). The cortisol RIA was performed using an antiserum to cortisol-21-hemisuccinate-BSA, raised in a rabbit, at a working dilution of 1:20 000 and [3H]-cortisol (amount 30 pg/tube vial) as tracer.

Validation parameters of the analysis were: sensitivity 0.26 pg/mg; intra-assay variability 6.8%; inter-assay variability 9.3%.

DHEA assay

DHEA was analyzed in horsehair and plasma extracts using a 96-well microtitre plate RIA and a commercial anti-DHEA-7-carboxymethyloxime-BSA (Biogenesis, Poole, UK) that showed the following cross-reactions: DHEA 100%, 5α-androstane-3α, 17β-diol 6.3%, androstenedione 1.3%, testosterone 0.1%, and other related compounds <0.05% (Gabai, Marinelli, Simontacchi, & Bono, 2004). The detection limit of the assay, as calculated by the software Riasmart (Perkin-Elmer Life Sciences), was 1.56 pg/well. The results of the intra- and inter-assay precision test, expressed as coefficients of variation (CV), were 7.4% and 7.2%, respectively.

Flow cytometry analysis and lymphocytes determination

In order to phenotype lymphocyte subpopulations CD4 +, CD8 + and B cells, 50µl of heparinized blood were mixed with 5µl of the specific antibodies for each surface antigen, in a plastic tube. After 15 min of incubation in the dark at room temperature, the cells were washed with Phosphate Buffer Solution (PBS)/1% Fetal Calf Serum (FCS) and centrifuged for 5 min at 400xg. For the CD4+ and CD8+, the cells were then mixed with a secondary antibody and incubated 15 min in the dark at room temperature, the cells were washed with Phosphate Buffer Solution (PBS)/1% Fetal Calf Serum (FCS) and centrifuged for 5 min at 400 x g. The contaminating red cells were lysed by treatment with NH4Cl solution, pH 7.2, for 15 min at room temperature in the dark. The cell suspension was washed twice with PBS/1% FCS, centrifuged for 5 min at 400 x g and re-suspended in 0.5 ml of PBS/1%FCS for the flow cytometry analysis (Epics XL-MCL,Colter).

Flow cytometry analysis was performed using mouse anti-horse CD4 (clone CVS4 IgG1 – Serotec) and mouse anti-horse CD8 (clone CVS8 IgG1-Serotec) primary antibodies followed by goat anti-mouse IgG1 FITC-labeled secondary antibody (DAKO) and a cross-reacting mouse anti-dog B cells-RPE (clone CA2.1D6 IgG1-Serotec). For each animal unstained cells and cells stained with an irrelevant mAb were used as negative and isotype controls. Additionally, secondary antibody was checked for unspecific staining by direct secondary antibody incubation for 15 min. Cell gates were set using size (forward scatter, FSC) and complexity (side scatter, SSC) characteristics, and data from at least 10,000 events within the lymphocyte gate were acquired. Dead cells were excluded using 1/1000 Sytox® Advanced Dead Cell Stain Kit (Life Technologies), according to the manufacturer’s instructions. Blood count with leukocyte formula was performed with the hematology analyzer for multi CELL DYN® 3500 (Abbott USA Rome, Italy), and B-lymphocytes and lymphocyte subpopulations CD4+ and CD8 + were quantified (Borghetti et al., 2006; Ferrari et al., 2016).

Statistical analysis of data

The distribution of each variable was evaluated with normality tests of Kolmogorov–Smirnow and Lillefors and W-test of Shapiro-Wilk. In order to highlight significant differences in blood and hormonal parameters between horses bred NM or CM, the ANOVA was performed. Spearman correlation test was used to find a significant correlation between cortisol and DHEA concentrations in plasma and horsehair, and between the other hematological parameters.

To assess the parallelism between standard DHEA and the endogenous hormone, a non-linear regression test was made.

Differences were considered statistically significant for p-value <0.05.

Plasmatic and horsehair cortisol/DEHA ratio was calculated.

RESULTS

Hormonal assay

It has been found a significant ($p < 0.01$) parallelism between standard DHEA curve and the endogenous DHEA one, measured using horsehair samples of subjects of the research (Figure

1). Mean value of DHEA in plasma and horsehair was higher in NM horses rather than in those under CM. Statistical analysis showed that only the difference found in horsehair DHEA concentration between the two groups was significant ($p < 0.001$). NM subjects registered also a not-significantly higher concentration of plasma cortisol, but they showed a significantly lower ($p = 0.003$) level of horsehair cortisol compared to that of CM horses. All results of hormonal assay are showed in Table 3.

CM horses' plasmatic cortisol/DEHA ratio was 56.92, and a similar value was found in horses kept with a natural management (56.27). However, horsehair cortisol/DEHA ratio was significantly ($p < 0.001$) higher in traditional (86.46) than natural subjects (23.93) (Figure 2).

Finally, correlations between cortisol and DEHA concentrations found in the two different matrices were evaluated. Negative significant correlations between horsehair cortisol and plasmatic DHEA ($r = -0.344$, $p = 0.018$; Figure 3) and between horsehair cortisol and horsehair DHEA were identified ($r = -0.578$, $p < 0.001$; Figure 4).

Blood parameters

Considering all the parameters of blood count (Table 4), no statistically significant differences were found between the two groups of horses. However, the mean counts of lymphocytes, neutrophils, basophiles, and eosinophils were higher in NM horses than in CM ones, even if not statistically significant.

Flow cytometry analysis did not show statistically significant differences in lymphocyte subpopulations. The average counts of B, CD4+ and CD8+ lymphocytes were higher in NM subjects; the percentage of B-lymphocytes was higher in NM horses; the percentage of CD8+ lymphocytes were lower in NM horses (Table 5).

DISCUSSION

This research can be considered innovative for the longitudinal and multidisciplinary approach employed. Hormonal, hematological and immunological parameters have been used to establish sporting horses' condition in two different kind of managements. Moreover, for the first time in this study, DHEA assay method from horsehair has been used and validated.

A standard approach to assess animal stress and welfare is evaluating hypothalamic–pituitary–adrenal (HPA) axis function, measuring levels of glucocorticoids (Mormède et al., 2007). During an acute stress, HPA axis activation and the increasing of plasmatic cortisol level immediately occur. Therefore, plasma is a useful biological matrix to assess acute stress, but often results are altered by blood sampling procedure, which is invasive and stressful for animals (Mormède et al., 2007). In order to evaluate chronic stress, hair sampling can be used. Indeed, cortisol deposits in growing hair, so this kind of sample integrates HPA axis activity over weeks and months and cortisol level is not influenced by acute stress during sampling procedure (Meyer & Novak, 2012). Cortisol and DHEA have the same precursor: pregnenolone. In human beings, during stressful situation, ACTH (Adrenocorticotropic Hormone) stimulates adrenal cortex to produce cortisol. Therefore, “pregnenolone steal” occurs and cortisol level increase, while DHEA production decrease. As a consequence, cortisol/DHEA ratio increases (Guilliams & Edwards, 2010).

In this research, horses kept in a natural management showed significantly lower horsehair cortisol level and a significantly higher horsehair DHEA concentration; as a consequence, a significantly lower cortisol/DHEA ratio was observed in NM subjects. Moreover, plasmatic DEHA concentration was higher in NM subjects, even if not significantly. Cortisol concentration in plasma was similar between the two groups, but probably it depended on stress inducted by sampling procedure. Considering hormonal concentrations as stress index, it may be supposed that natural management allows horses live in less stressful conditions, especially considering long time intervals. Higher levels of cortisol and cortisol/DHEA ratio found in horsehair of CM horses are probably due to a frequent or prolonged activation of the HPA axis.

High DHEA concentration in NM horses could be a positive finding, as this steroid seems to contribute to animal physical health condition and wellbeing. Indeed, DHEA and its sulfated metabolite (DHEAS) would be important as neuroactive steroids in the regulation of neural function, such as neuroprotection, neurogenesis, neuronal growth, and differentiation. Moreover, these hormones would influence catecholamine synthesis and secretion, would have anti-oxidant and anti-inflammatory action, and antagonize glucocorticoid effects (Pluchino et al., 2015). Several studies in humans and animals have demonstrated that neurosteroids could have psychotropic actions. In particular, studies in humans demonstrate that DHEA could have a potential role in the mitigation of stress and could have positive effects on mood symptoms in depressive disorders as antidepressant and on human well-being (Bloch, Ish-Shalom, Greenman, Klein, & Latzer, 2012; Dor, Marx, Shampine, Rubinow, & Schmidt, 2015; Russo, Murrough, Han, Charney, & Nestler, 2012; Maninger, Wolkowitz, Reus, Epel, & Mellon, 2009). On the contrary, researches demonstrate that high-prolonged cortisol concentration, detectable by hair analysis, can damage the organism (Stalder & Kirshbaum, 2012). Cortisol is the principal glucocorticoid hormone; it activates lipolysis and gluconeogenesis, insulin-resistance, increases blood pressure, and modulates the immune system responses (Gow, Thomson, Rieder, Van Uum, & Koren et al., 2010). Glucocorticoids have both pro-inflammatory and anti-inflammatory action. Initially, in response to an acute stress, glucocorticoids prepare the organism to a quick response and they have a pro-inflammatory action. However, during a chronic or repeated stress, the prolonged high level of glucocorticoids has anti-inflammatory effects and causes immunosuppression of the organism. The tight connection between the endocrine and immune systems makes that a prolonged exposure to stress conditions may lead to the development of inflammatory, autoimmune and allergic diseases (Cruz-Topete & Cidlowski, 2015; Stalder & Kirshbaum, 2012). As previously specified, DHEA also influences inflammatory response, and Hilderbrand et al. (2004) demonstrated that in rats this hormone causes the reduction of percentage of CD4+ and CD8+ populations.

The differences in the endocrine milieu between the two groups of horses involved in this research may lead to hypothesize differences also in the blood counts and especially in white cells populations and lymphocytes. Few studies were performed to analyze the effect of stress on immune parameters in horses; some researches showed that horses exposed to stress can have altered blood parameters, such as Neutrophil/Lymphocyte ratio and macrophage activity, indicating a possible reduction of immune response efficiency (Passatino et al., 2005; Popescu & Diugan, 2017). However, we did not find any significant differences between the two groups of horses. Many other factors can influence white cells populations and blood counts (Satuè, Hernandez, & Muñoz2012), and probably our results depend on the fact that the traditional stables enrolled in this work presented a good standard of management and did not have major deficits that compromise the immune system of animals.

To conclude, results of this research highlight that horses kept under two different kinds of management present different endocrine milieu. Considering the hormonal levels of horses, it can be supposed that CM probably put animals in a chronic stress condition, while the NM seems to comply more with the physiological needs of horses. Conversely, the immune system of animals seemed not to be influenced by the environmental and management conditions. However, the evaluation of cytokine and other inflammatory parameters can help to deepen this topic. Further studies are necessary to complete the assessment of equine welfare in different management conditions, such as behavior evaluation and incidence of diseases. This study wants to underline the importance of a multidisciplinary approach on the definition of animal condition and the need of scientific research for the improvement of animal management.

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TABLES

Table 1. Subjects of the study including in NM (Natural Management) and CM (Conventional Management) groups. G = Gelding; F = Female; S = Stallion.

Breed	Gender	Age (ys)	Type of equestrian activities	Group
Italian Saddle Horse	G	18	school and trekking	NM
Bardigiano	F	16	school and trekking	NM
Paint Horse	F	6	school and trekking	NM
Quarter Horse X Bardigiano	F	8	school and trekking	NM
Quarter Horse	G	9	trekking	NM
Irish Sport Horse	G	18	show jumping – dressage elementary level	NM
Thoroughbred	F	18	trekking – dressage elementary level	NM
Belgian Warmblood Horse	G	15	trekking – dressage elementary level	NM
Holsteiner	F	7	show jumping – dressage elementary level	NM
Holsteiner	F	6	show jumping – dressage elementary level	NM
Westfallien	G	11	show jumping – dressage elementary level	NM
Italian Saddle Horse	G	4	show jumping – dressage elementary level	NM
Italian Saddle Horse	F	9	show jumping – dressage elementary level	NM
Trakehner	F	11	show jumping – dressage low level – trekking	NM
Hannover	F	10	show jumping – dressage medium level – trekking	NM
Italian Saddle Horse	G	17	show jumping – dressage elementary level	NM

Arabian Horse	G	11	show jumping – dressage elementary level – endurance (60-90 km)	NM
Belgian Warmblood Horse	F	16	hippotherapy - show jumping – dressage elementary level	NM
Italian Saddle Horse	F	16	hippotherapy - show jumping – dressage elementary level	NM
Italian Saddle Horse	F	26	hippotherapy – show jumping – dressage elementary level	NM
Italian Saddle Horse	F	10	hippotherapy – show jumping – dressage elementary level	NM
Italian Saddle Horse	G	18	school	CM
Italian Saddle Horse	F	6	show jumping – dressage elementary level	CM
Belgian Warmblood	G	8	show jumping – dressage elementary level	CM
Italian Saddle Horse	G	4	show jumping – dressage elementary level	CM
Italian Saddle Horse	F	4	show jumping – dressage elementary level	CM
Belgian Warmblood	G	17	show jumping – dressage elementary level	CM
Italian Saddle Horse	G	14	show jumping – dressage elementary level	CM
Italian Saddle Horse	G	14	show jumping – dressage elementary level	CM
Kwpn	G	18	show jumping – dressage elementary level	CM
Anglo Arabo Sardo	F	18	School	CM
Ungarian Saddle Horse	G	11	show jumping – dressage elementary level – school - trekking	CM
Kwpn	G	30	show jumping – dressage elementary level – school	CM
Anglo Arabo Sardo	G	14	show jumping – dressage elementary level – school	CM
Italian Saddle Horse	G	9	show jumping – dressage elementary level	CM
Italian Saddle Horse	G	5	show jumping – dressage elementary level	CM
Italian Saddle Horse	G	6	show jumping – dressage elementary level	CM
Italian Saddle Horse	G	5	show jumping – dressage elementary level	CM
Italian Saddle Horse	G	4	show jumping – dressage elementary level	CM
Italian Saddle Horse	S	6	show jumping – dressage elementary level	CM
Italian Saddle Horse	F	8	show jumping – dressage elementary level	CM
Italian Saddle Horse	G	5	show jumping – dressage elementary level	CM
Italian Saddle Horse	F	7	show jumping – dressage elementary level	CM
Italian Saddle Horse	G	4	show jumping	CM
Arabian Horse	G	9	dressage elementary level	CM
Criollo Horse	G	22	dressage elementary level	CM
Italian Saddle Horse	F	6	dressage elementary level	CM

Table 2. Criteria used for the definition of conventional management or natural management.

	Conventional Management	Natural Management
Type of stable buildings and chance of movement	At least 18 h per day closed in stalls inside the barn. Limited daytime dedicated for free movement and / or equestrian activities (1-6 hours per day).	At least 12 h per day outside the barn, in paddocks (minimum criteria: 20mt*15mt). Most of the daytime dedicated for free movement and equestrian activities.
Socializing	Socially isolated in stalls with limited or no possibilities for social contact with other horses.	High motivation for free socializing with other horses when in paddocks.
Feeding	Feeding 2 times a day with large volume of hay-alfalfa hay and high amounts of concentrates.	Feeding ab libitum, or more than 4 times a day with grass hay or pasture.
Thermoregulation	Coat clipped and use of blankets	Free thermoregulation with no blankets or clipped coat
Shoeing	All horses shod	Barefoot trimmed
Type of bridle	Horses ridden with mouthpieces bridles	Horses ridden without mothpieces.

Table 3. Mean and standard error (SE) of DEHA and cortisol concentration found in horsehair and plasma of natural horses in two different management conditions. Statistical results are shown (NS = Not Significant).

	Natural Boarding	**Traditional Management**	
	Mean ± SE	**Mean ± SE**	**p-value**
Plasmatic DHEA (ng/ml)	0.46 ± 0.03	0.40 ± 0.03	NS
Horsehair DHEA (pg/g)	30.80 ± 2.25	16.23 ± 1.76	< 0.001
Plasmatic CORTISOL (ng/ml)	25.71 ± 1.06	22.07 ± 1.14	NS
Horsehair CORTISOL (pg/g)	0.16 ± 0.03	0.76 ± 0.12	0.003

Table 4. Mean and Standard Error (SE) of blood count parameters in the two groups of horses. NS = Not Significant. Hb = hemoglobin; PCV = Packed Cells Volume; RBC = Red Blood Cells; PLTS = Platelets; WBC = White Blood Cells; MCV = Mean Corpuscular Volume; MPV = Mean Platelet Volume; MCHC = Mean Corpuscular Hemoglobin Concentration; MCH = Mean Corpuscular Hemoglobin; RDW = Red cells Dispersion Width.

Parameter	**Natural boarding (n=21)**	**Traditional management (n=26)**	**p-value**
	Mean ± SE	**Mean ± SE**	
Hb %	13.46 ± 0.47	13.36 ± 0.69	NS
PCV %	37.32 ±1.21	37.01 ± 1.75	NS
RBC /mm³	7.79×10^6 ± 235,231.69	7.87×10^6± 423,723.38	NS
PLTS /mm³	112.23 $\times 10^3$ ± 7,234.88	115.068 $\times 10^3$ ± 21,213.72	NS
WBC /mm³	6.2 $\times 10^3$ ± 534.69	6.244 $\times 10^3$± 393.36	NS
MCV fL	47.96 ± 0.65	47.43 ± 0.55	NS
MPV %	6.53 ± 0.37	6.33 ± 0.36	NS
MCHC gr%	36.00 ± 0.13	35.96 ± 0.18	NS
MCH pgr	17.26 ± 0.23	17.05 ± 0.19	NS
RDW %	24.44 ± 0.28	24.42 ± 0.48	NS
Lymphocytes /mm³	2.516 $\times 10^3$± 184.81	2.299 $\times 10^3$± 226.73	NS
Monocytes /mm³	0.24 $\times 10^3$ ± 39.59	0.24×10^3 ± 79.50	NS
Neutrophils /mm³	4.02 $\times 10^3$ ± 248.18	3.67 $\times 10^3$ ± 213.99	NS
Eosinophils/mm³	0.237 $\times 10^3$± 41.33	0.153 $\times 10^3$± 24.81	NS
Basophiles /mm³	0.118 $\times 10^3$± 25.63	0.057 $\times 10^3$± 4.98	NS

Table 5. Absolute value, percentage, and ratio of lymphocytes subpopulations in the two horses' populations. NS = Not Significant.

Value	**Natural Boarding (n=21)**	**Traditional Management (n=26)**	
	Mean ± SE	**Mean ± SE**	**p-value**
CD4+ (%)	55.57±1.55	55.64±1.36	NS
CD8+ (%)	16.72±0.70	17.58±0.77	NS
B (%)	9.12±0.63	8.33±0.44	NS
CD4 / CD8	3.45±0.18	3.32±0.17	NS
CD4+ / B	6.92±0.65	7.26±0.50	NS
CD8+ / B	2.09±0.22	2.23±0.14	NS
CD4+ (n)	1,386.49±101.94	1,239.92±105.22	NS
CD8+ (n)	427.84±40.16	402.91±40.96	NS
B (n)	233.68±29.22	197.64±24.93	NS

FIGURES

Figure 1. Parallelism between standard DHEA concentration and endogenous DHEA dilutions, using horsehair of research subjects.

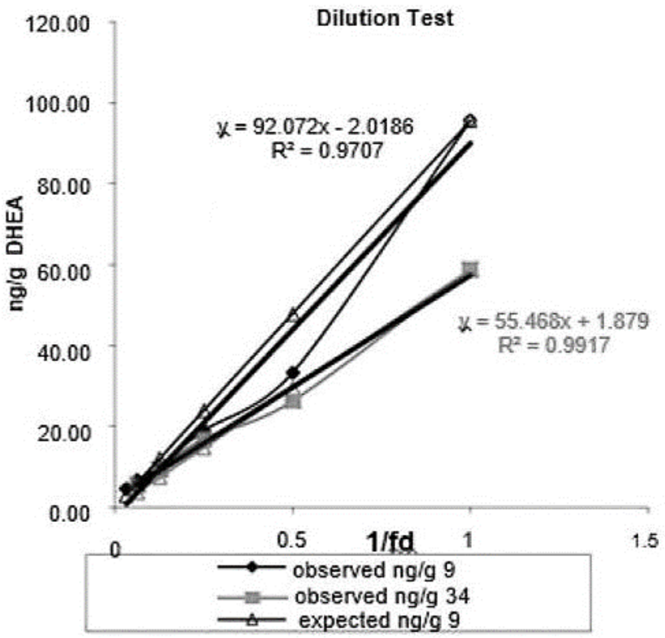


Figure 2. Horsehair cortisol/DHEA ratio in horses with different management. * = statistical significant result.

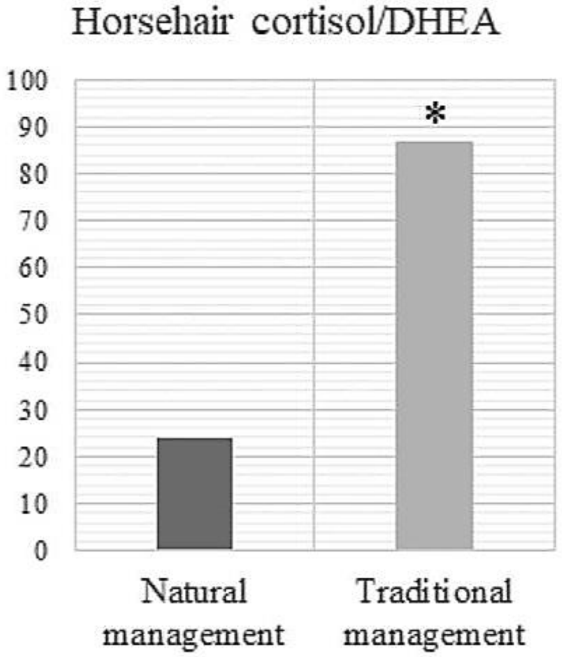


Figure 3. Negative significant correlation between horsehair cortisol concentration (pg/mg) and plasmatic DHEA levels (ng/ml).

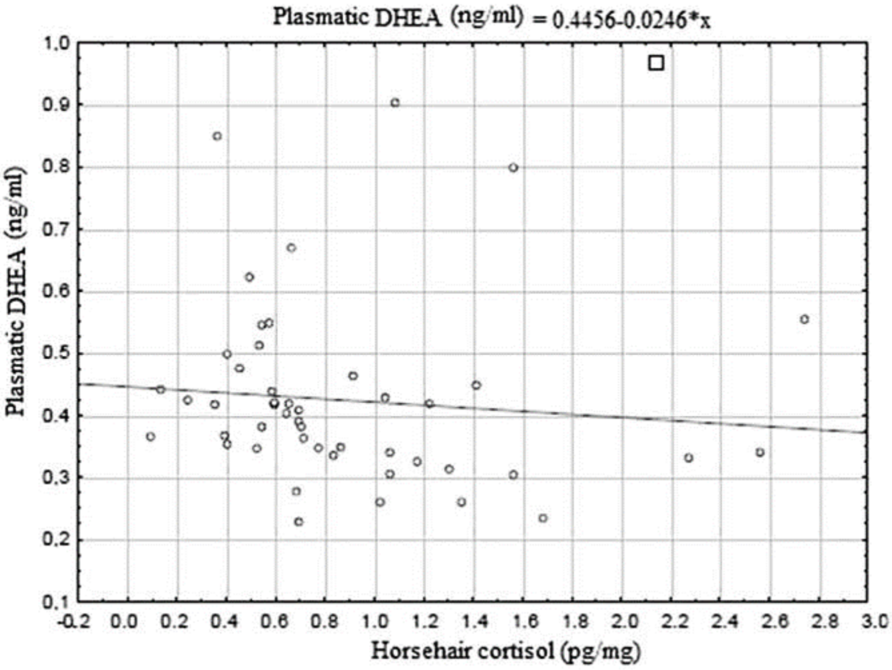
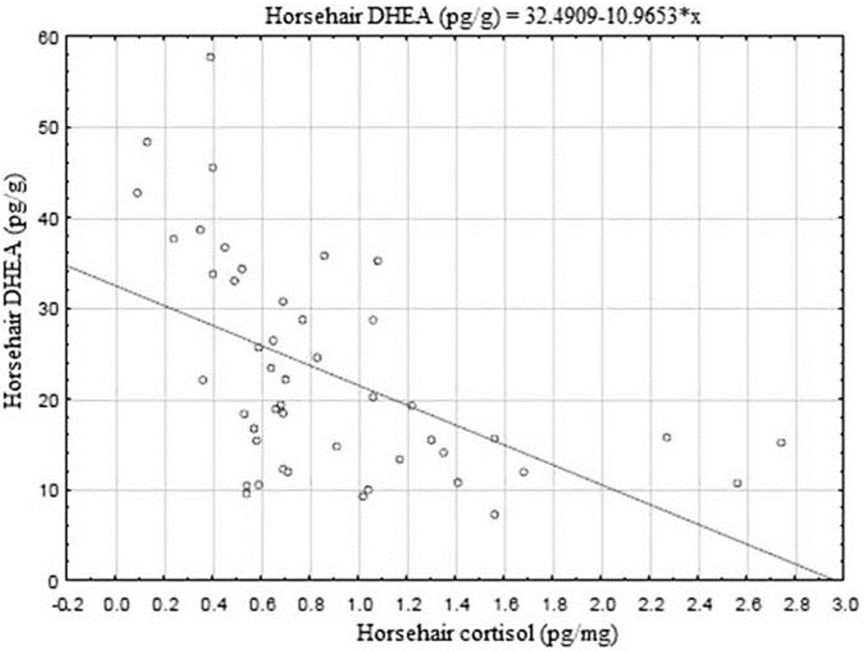


Figure 4. Negative significant correlation between horsehair cortisol concentration (pg/mg) and horsehair DHEA levels (pg/g).



PAPER 2

Assessment of horses' welfare: Behavioral, hormonal, and husbandry aspects

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Assessment of horses' welfare: Behavioral, hormonal, and husbandry aspects

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ABSTRACT

Horse practitioners have difficulties with undesirable behaviors exhibited by their horses (*Equus caballus*), such as cribbing, weaving, or head shaking. It has been suggested that most of behavioral disorders originate from the animals' attempt to cope with a suboptimal environment. To evaluate animal welfare, it is important to consider both behavioral and physiological parameters. Studies on animal welfare underline the importance of the monitoring of hormonal concentrations, particularly hormones that indicate stress, such as cortisol. The aims of this research were to compare cortisol and dehydroepiandrosterone (DHEA) concentrations of 14 healthy horses (HH) and 40 horses displaying behavioral pathologies (BPHs), to calculate the cortisol-DHEA ratio as marker of chronic stress and to find a relationship between aspects of horses management and the presence of BPHs. The methodology of radio-immuno-assay was used, to determine cortisol and DHEA levels in blood and hair. A questionnaire was completed by horse owners or barn managers and used to investigate the different characteristics of boarding barns, where the horses were housed. BPH horses registered a significantly ($P < 0.05$) higher plasma cortisol/DHEA ratio value than HH horses. It is possible that BPH horses suffer chronic stress, which might cause a dysfunction of hypothalamic-pituitary-adrenal. A positive correlation was found ($P < 0.05$) between the presence of BPH, time spent in stall and the frequency of work. Moreover, a low fiber, high-energy diet appeared to contribute to the development of BPHs. This study underlines the importance of a multidisciplinary approach to the evaluation of horse welfare. The improvement of welfare is closely correlated to a decreased risk of the development of stereotypic or undesirable behaviors, and a better understanding of equine ethological needs is fundamental to advances in this area.

Introduction

A stereotypy has been defined as the excessive repetition of the same motor activity, which remains unchanged over time without any apparent goal or short-term effect (Mason, 1991). Specific veterinary conditions such as brain damage, stress or frustration, environmental conditions that lack sufficient stimulation or do not accommodate the ethological and physiological needs of the animal, can lead to the development of stereotypic behaviors. For example, horses can develop these abnormal behaviors because of management and handling factors, such as feed restriction (lack of grazing), social isolation, or lack of exercise (Sarrafchi and Blokhuis, 2013). Many researchers have used the presence of stereotypies as an index of poor welfare (Mason, 1991; Mason, 2006; Sarrafchi and Blokhuis, 2013; Roberts et al., 2017). However, the display of stereotypical behaviors may not depend on current husbandry conditions, as it may have developed during a previous experience of poor welfare. Indeed, during suboptimal conditions, stereotypies can become habits and not linked from the original stressors. In such case, these behaviors are difficult to eradicate even af-

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ter the improvement of conditions of husbandry and management (Swaisgood and Shepherdson, 2005; Henderson, 2007; Hothersall and Casey, 2011). Stereotypies are characterized by regular, periodic, and predictable behaviors, which are always repeated in the same manner (Mason, 2006). Stereotypies do not appear to have a specific function or purpose, in contrast to the variability and relative unpredictability of functional and adaptive behaviors (Cooper and Albentosa, 2005; Hothersall and Casey, 2011). Stereotypies and other undesirable behaviors, such as redirected and avoidance behaviors, are often complained about by horse practitioners, who consider these behaviors dangerous and economically unfavorable, causing a loss of value and a depreciation of the animal (s.e. in case of redhibitory defects; Hothersall and Casey, 2011). Owners often define stereotypies “vices” and consider them as detrimental for the animals, although scientific evidences about the consequences of these behaviors on health are scarce (Cooper and Albentosa, 2005; Hothersall and Casey, 2011). Behavioral problems in horses can be categorized into 3 groups (McDonnell, 2003; Zeitler-Feicht, 2004):

- oral stereotypies, such as crib-biting, wind-sucking, wood-chewing, teeth grinding, self-mutilation, coprophagy, lip smacking;
- locomotor stereotypies, such as weaving, stall-circling, head-nodding, wall-kicking;
- social problems involving human beings or conspecifics, training problems (such as reluctance to move forward or leave the paddock or horse stall and defensive or aggressive behaviors when saddled)and phobic responses (such as separation anxiety, aggressive behaviors, overreaction to environmental stimuli, problems loading into a trailer).

On the farm, animals are in a confined environment and they are prevented from completely expressing their natural behaviors, so they may develop stereotypies (Lewis et al., 2006). Isolation and confined environment are imposed on the animals by humans and they can cause a state of anxiety, frustration, and boredom and a negative affective states included in the fifth domain (“Mental state”) of the Five Domains Welfare Model proposed by Mellor and Reid in 1994 (Mellor, 2017). This model considers how physical/functional states and external circumstances (“Nutrition”, “Environment”, “Health,” and “Behavior”) influence the mental experiences of the animals (“Mental state”), defining their state of welfare (Mellor and Reid, 1994; Mellor, 2017). Behavior is an important indicator to assess animal welfare, but it is not the only one. Indeed, a suboptimal environment and management practice can influence physiological parameters and the animals’ health (Marashi et al., 2003; Cooper and Albentosa, 2005; Carenzi and Verga, 2009). Stressful conditions may affect endocrinologic mechanisms and immune system, which both should be considered in the assessment of animal welfare (Marashi et al., 2003; Carenzi and Verga, 2009; Brown and Vosloo, 2017; Popescu and Diugan, 2017). The physiological indicators of welfare can be challenging to measure, but noninvasive methods, such as infrared thermography, heart rate evaluation, and quantification of salivary immunoglobulin-A, have become established in animal welfare science (Mmanus et al., 2016; Whitham and Miller, 2016; Zupan, 2016; Staley et al., 2018). In particular, the investigation of the activation of the neuroendocrine system (hypothalamic-pituitary-adrenal [HPA] axis) through the measuring of cortisol levels is useful to determine acute and chronic stress (Mormede et al., 2007; Palme, 2012). High blood cortisol level reflects acute stress. However, the collection procedure of blood can disturb the animal and cause the rise of cortisol concentrations, so the use of other matrices (s.e. saliva, urine, feces, hair/horsehair) has been proved to be a useful noninvasive alternative method (Mormede et al., 2007; Bennet and Hayssenn, 2010; Palme, 2012, 2019; Heimbürge et al. 2019). In particular, the analysis of cortisol in hair can be used to assess long-term or chronic stress. During its growth, hair incorporates cortisol, so the concentration of the hormone in hair reflects its secretion over a prolonged period (Stalder and Kirschbaum, 2012; Heimbürge et al., 2019). Another marker of adrenocortical activity and rise in response to stressful events is dehydroepiandrosterone (DHEA). DHEA is produced in the zona reticualris of the adrenal glands and has opposing functions to cortisol (Kamin and Kertes, 2017). It has also been reported that horses show a rise in DHEA following acute stress (such as castration, acute abdominal syndrome, or acute disease) and the release of Adrenocorticotropic Hormone (ACTH) (Ayala and Martos, 2013). Calculating the cortisol/DHEA ratio can be considered as the correct approach to study the relative abundance of the 2 hormones and determine which is preferentially produced (Kamin and Kertes, 2017). Depending on the nature and duration of stress, a prolonged or a repetitive stress can be followed by both an upregulation or a downregulation of the HPA activity (Miller et al, 2007; Lupien et al., 2009; Kamin and Kertes, 2017).

The aim of this study was to test the differences in behavioral and endocrinologic indicators (plasmatic and horsehair DHEA and cortisol) between horses under varying management conditions, identifying which management factors might have compromised the welfare of the animals and led them to develop behavioral problems. Most of the studies about DHEA refer to human beings, and there has been little research about animals and particularly horses. Therefore, this study can be considered as innovative.

Materials and methods

Horses

Fifty-six horses were analyzed from 9 different barns. The horses analyzed were 42 horses that displayed either stereotypes or behavioral pathologies (BPHs) and 14 healthy horses (HHs), without any behavioral problem (HH; Table 1). The BPH showed: oral stereotypies, locomotor stereotypies, social problems, and training behaviors. The BPHs were identified by a veterinarian, according to their clinical presentation and the clinical history of the animals and their completed questionnaire (Appendix 1). All animals were involved in competitive activities. Two horses of BPH group were excluded from the hormonal determination, because of the lack of plasma samples.

Study design

The research was made into different barns and was divided into 2 parts. During the first part, cortisol and DHEA concentrations were measured in BPH and HH from plasma and horsehair samples. In the second part, the main aspects of horses’ management were assessed by a questionnaire and the prevalence of BPHs were studied accordingly. Hormonal analysis was conducted in all horses. Samples were collected from June to July. Blood and horse-

Table 1
Number of horses considering sex and group, and the mean age.

	HH group	BPH group
Mare	6	12
Gelding	7	29
Stallion	1	1
Mean age ± SD	12.21 ± 5.91	10.02 ± 4.95

SD, standard deviation; HH, healthy horses; BPH, horses displaying stereotypies or other behavioral pathologies.

Table 2
The information about the management and the features of the different barns were obtained from the questionnaires and categorized into different classes.

	Class 0	Class 1	Class 2	Class 3	Class 4
Type of horse stall	Horse stall with outside area	Single stalls arranged in 2 lines separated by a central corridor. On the front, each stall has a metal grate and the horse cannot look out on the corridor. On the back, the stalls can have a window, from which the horse can look out	Single stalls arranged in 2 lines separated by a central corridor. On the front and on the back, each stall has a window that allows the horse to look out	N/A	N/A
Size of horsebox	From 8 m^2 to 12.5 m^2	16 m^2	24 m^2	N/A	N/A
Size of paddock	0 m^2	From 60 to 100 m^2	From 120 to 160 m^2	5000 m^2	N/A
Time spent in horse stall or in paddock	All the time in the horsebox	In the horse stall in case of bad weather, during the central hours of the day in summer and during the night. In paddock the rest of time	All the time in paddock	N/A	N/A
Bedding	N/A	Shaving	Straw	N/A	N/A
Forage	N/A	Hay from mixed-grass meadows 3 times per day	Hay from mixed-grass meadows twice a day	Hay from mixed-grass meadows once a day	N/A
Number of meals per day	N/A	N/A	2	3	N/A
Type of alfalfa	No alfalfa	Fresh alfalfa	Dried/pelleted/ alfalfa with molasses	N/A	N/A
Type of mash	None	Mixed pellet feed	Mixed flaked feed	Pellet feed + flaked barley or crushed oats	N/A
Amount of Alfalfa per week	0 kg	From 10 kg to 12 kg	30 kg	N/A	N/A
Forage/mash ratio	1	From 3 to 9	From 12 to 32	N/A	N/A
Competitions	Never	In the past	Sometimes	Often	N/A
Type of work	None	Trekking	Activities in riding school and horse trekking	Riding and/or competitive activities with beginner-intermediate riders	Riding and/or competitive activities with intermediate-advanced riders
Duration of work sessions	0 min	60 min	90 min	120 min	N/A
Weekly frequency of work	0-3 times per week	≥4 times per week	N/A	N/A	N/A
Lunging	No	Yes	N/A	N/A	N/A
Use of horsewalker	No	Yes	N/A	N/A	N/A
Behavior during lunging or in round horsewalker	None	Association (the horse shows calming signals and avoiding, stress and/or displacement behaviors)	Defense (the horse threats, kicks, and/or bites)	N/A	N/A
Weekly frequency lunging or in round horsewalker	Never/seldom	Sometimes	Often	N/A	N/A
Contact with conspecifics	Absent or only visual	Tactile-olfactory, low	Tactile-olfactory, abundant	N/A	N/A
Relation with conspecifics	Neutral or friendly	Little aggressive (the horse threats a conspecific but it does not attack, s.e. ears lowering back, bite/kick-threats)	Very aggressive (the horse attacks biting and/or kicking and fights with conspecifics)	Phobic	N/A

(*continued on next page*)

Table 2 (*continued*)

	Class 0	Class 1	Class 2	Class 3	Class 4
Employment of clipping	No	Yes	N/A	N/A	N/A
Employment of shoeing	No	Yes	N/A	N/A	N/A
Use of horse blankets	No	Yes	N/A	N/A	N/A
Behavior during the activities of grooming (with body brush, hoof pick, etc.) and harnessing	Neutral (the horse stands and lets it be groomed and harnessed)	Low degree of cooperation (the horse refuses to give the feet and/or to wear the harnesses. It moves, and it is difficult to groom and/or harness it)	Defensive/phobic/aggressive reactions (the horse threats and try to bite and/or kick the person during the grooming and/or harnessing)	N/A	N/A
Behavior with heterospecifics	Neutral or friendly	Little aggressive (the horse threats but it does not attack, s.e. ears lowering back, bite/kick-threats)	Very aggressive (the horse attacks biting and/or kicking)	Phobic or avoidance	N/A
Behavior close to mealtime	Normal	Little anxious (the horse stands alert and sometimes whinnies or paws the ground)	Very anxious (the horse shows anticipatory behaviors, repetitively whinnies, pacing in front of the door, repetitively paws the ground, and/or kicks the door)	N/A	N/A
Presence of abnormal behaviors	None	Oral stereotypy	Locomotor stereotypy	Undesirable and abnormal behaviors during the training or during social interactions, phobic responses	Presence of behavioral problems of class 1, 2, and 3 at the same time (s.e. an horse that show both oral and locomotor stereotypy)
Frequency of pathology	Never	Occasionally or in particular situations	Always	N/A	N/A

N/A, not applicable.

hair samples were taken from the horse at the same time and they were identified using horse's name, date of sampling, owner of the animal and the barn.

Questionnaire

As the barns had different facilities and spaces and the management approach varied between them, a questionnaire (Appendix 1 in Supplementary materials) was made to know the horses' daily barn routine. Information from feeding programs, to training, thermal regulation, and stall size were registered during an interview with the horse's owner or barn manager. Thirty-two questionnaires were analyzed and used for data analysis (HH, N = 14; pathological horses, BPH, N = 18). The information obtained through the questionnaires allowed the identification of 29 parameters regarding: stall, paddock, nutrition, and work. Then, different classes were created and ordered by number (Table 2).

Blood samples

Blood samples (20 mL) were taken from the jugular vein through venojet (Terumo Italia Srl, Roma, Italy) with Na-heparin as anticoagulant. Using a cooler (+4°C), all blood samples were transferred to the laboratory within 4 hours. Samples were centrifuged at 4000g for 10 minutes, in order to separate the cellular component and the plasma. The plasma was divided into aliquots and stored at −20°C until the determination of cortisol and DHEA concentrations.

Horsehair samples

Horsehair was cut at the base of the hair shafts using scissors and put in plastic bags with hermetic seals, transferred to the laboratory within 4 hours and stored at −20°C until the analysis for the determination of cortisol and DHEA.

Cortisol and DHEA determination

Hormone extraction from plasma

For the extraction of steroid hormones from plasma, 5 mL of diethyl-ether (BDH Italia, Milan, Italy) were added to100 μL of plasma. All samples were agitated with multivortex for 30 minutes and then centrifuged at 2000*g* for 4 minutes at 4°C. The ethereal extracts were transferred into glass tubes and evaporated under airflow at 37°C. The dry-residues containing steroid hormones were dissolved in 0.5 mL of 0.05 M phosphate buffer with bovine serum albumin (BSA), pH 7.5 (Tamanini et al., 1983).

Hormone extraction from horsehair

Horsehair samples were trimmed into 1-3 mm length fragments. Sixty milligrams of trimmed horsehair were put in a glass vial with 5 mL methanol and incubated at +50°C with gentle shaking for 18 hours. The vial content was filtered and the methanol extracts were dried under an under airflow at 37°C. The dry-residues were dissolved in 0.6 mL of phosphate-buffered saline (0.05 M with BSA), pH 7.5 (Accorsi et al., 2008).

Cortisol assay

Cortisol assay in both horsehair and plasma was performed in duplicate, following the method described by Tamanini et al. (1983). One-hundred microliters of ^{3}H-cortisol (specific activity 100 Ci/mmol, amount 30 pg/tube vial ≈ 12,771 dpm/100 μL) and 100 μL of an anticortisol antibody (dilution 1:20,000) were added to 100 μL of the solution obtained from glucocorticoid extraction. After incubation at +4°C for 18 hours, free steroid hormone was separated from bound with the addition of 1 mL of a 1% charcoal and 0.025% dextran solution (Sigma Chemical Co.), and the incubation at +4°C for 15 minutes, followed by centrifugation (4000*g*, for 4 minutes at +4°C). The supernatant containing the hormone bound to its antibody was decanted into scintillation vials and measured in a liquid scintillation β-counter (Perkin-Elmer Life Science Inc.). Validation parameters of the analysis were: sensitivity 0.26 pg/mg; intra-assay variability 6.8%; interassay variability 9.3%.

DHEA assay

Horsehair and plasma DHEA extracts were quantified using a microtiter radio-immuno-assay (RIA; Gabai et al., 2004). Microplates (96-well, Optiplate, Perkin-Elmer Life Science) were coated with goat antirabbit γ-globulin serum (1:1000 in 0.15 mM sodium acetate buffer, pH 9, by incubating overnight at 4°C. The plates were washed twice with phosphate-buffered saline, 0.1% BSA, pH 7.4 (RIA buffer and incubated overnight at 4°C with 200 µL of a commercial anti-DHEA-7-carboxymethyloxime-BSA (Biogenesis, Poole, UK; dilute 1:20,000). The antiserum showed the following crossreactions: DHEA 100%, 5α-androstane-3α, 17β-diol 6.3%, androstenedione 1.3%, testosterone 0.1%, and other related compounds <0.05%. Afterward, the antiserum solution was decanted, and plates were carefully washed with RIA buffer. Finally, standards, quality control, and unknown extracts were added, and plates were incubated overnight at 4°C (incubation volume: 200 µL). The tracer was [1,3,6,7 ^{3}H] DHEA (Perkin-Elmer Life Sciences; specific activity: 71 Ci/mmol; 30 pg/well). The standard curve was made by serially diluting (1.56-200 pg/well) a solution of DHEA (Sigma, Milan, Italy). The separation of bound from free hormone was performed by decanting the reaction mixture. Bound radioactivity was β-counted (Top-Count, Perkin-Elmer Life Sciences), after the addition of scintillation cocktail (200 µL/well; Microscint 20, Perkin-Elmer Life Sciences). Samples were analyzed in triplicate. The sensitivity of the assay was 1.56 pg/well. The results of the intra- and interassay precision test, expressed as coefficients of variation, were 7.4% and 7.2%, respectively.

Statistical analysis

Statistical analysis was conducted using R Software (R version 5.3.1.).

In order to investigate the relationship between the 29 dependent variables investigated with the questionnaire (Table 2) and the dichotomous variables HH and BPH (horse's group), the chi-square test was performed. However, when the frequencies were low (under 5 cases), the Fisher's test was employed.

The statistical comparisons between the hormonal data of the 2 groups were conducted using nonparametric (Wilcoxon) or parametric (Student *t* test) tests, depending on the distribution of the data, investigated through the Shapiro-Wilk normality test.

Differences and correlations were considered statistically significant at *P* value <0.05.

Results

Hormonal levels

The concentrations of horsehair and plasma cortisol were not significantly different between groups ($P=0.26$ and $P=0.44$) between HH and BPH (Figures 1 and 2).

Horsehair and plasmatic DHEA concentration did not show any significant differences ($P=0.84$ and $P=0.21$; Figures 3 and 4).

The horsehair cortisol/DHEA ratio was not different between groups (Figure 5), while the plasma cortisol/DHEA ratio was significantly lower in HH than BPH ($P < 0.05$; Figure 6).

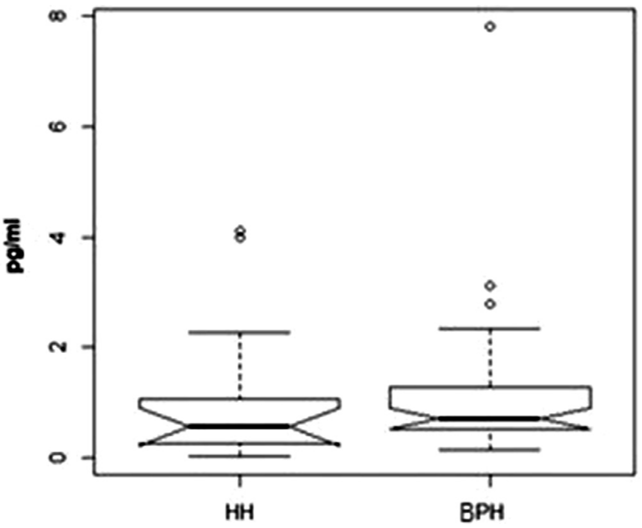

Figure 1. Boxplot of horsehair cortisol levels in healthy (HH) and pathological horses (BPH).

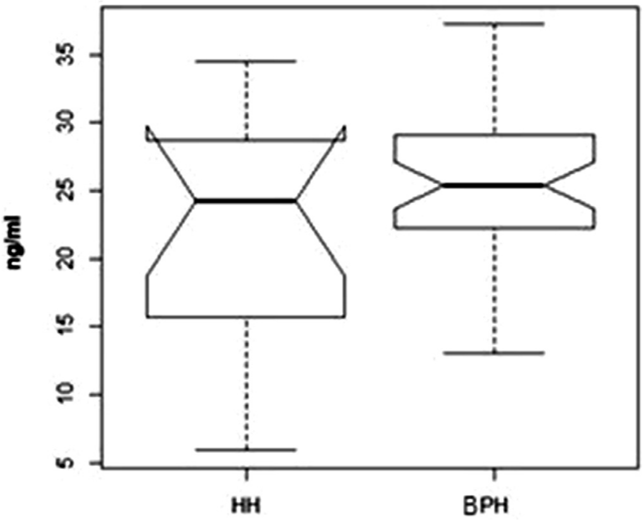

Figure 2. Boxplot of plasma cortisol levels in HH and BPH.

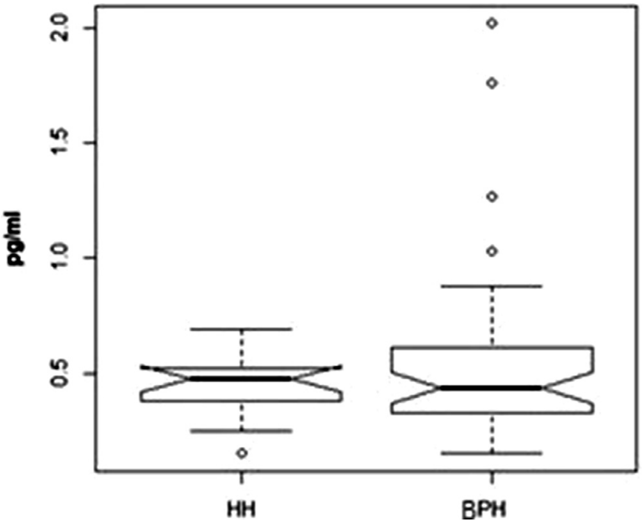

Figure 3. Boxplot of horsehair DHEA levels in HH and BPH.

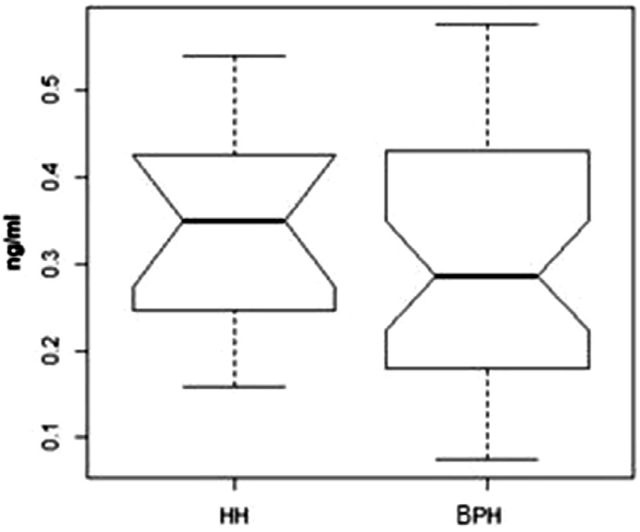

Figure 4. Boxplot of plasma DHEA levels in HH and BPH.

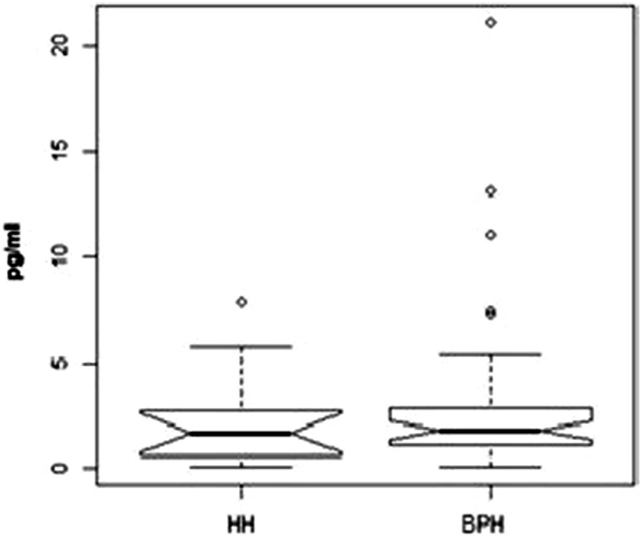

Figure 5. Boxplot of horsehair cortisol/DHEA ratio in HH and BPH.

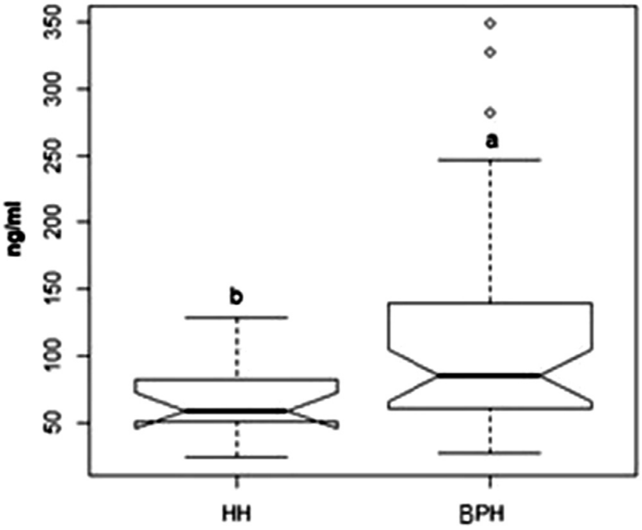

Figure 6. Boxplot of plasma cortisol/DHEA ratio in HH and BPH (a, b = $P < 0.05$).

Analysis of different models of stabling

The statistical analysis revealed significant differences between the 2 groups only for a few of the variables considered in the questionnaires: time spent in horsebox or in paddock; forage; type of alfalfa; weekly frequency of work; behavior close to mealtime (Table 3).

The time spent in a stall or in a paddock was one of the variables found to be significant ($P < 0.05$); indeed, the horses that display pathological behavior spent less time or no time at all in paddock compared to the control animals. The other 2 variables were the type of forage, which approached significance ($P = 0.053$), and the type of alfalfa, which was significant ($P = 0.047$). The HHs were fed with hay from mixed-grass meadows 3 times per day and ate only fresh alfalfa. On the contrary, the BPHs ate hay from mixed-grass meadows once or twice a day and their feed rations were completed by dried or pelleted alfalfa or alfalfa with molasses, but not fresh alfalfa.

The frequency of work resulted significantly different ($P < 0.05$) among groups. Indeed, the 79% of HH had a work frequency lower than 3 times per week, whereas most of the horses of BPH worked more than 4 times per week. Only the 33% of BPH had a work frequency lower than 3 times per week.

Finally, the horses' behavior close to the time significantly differ between the 2 groups ($P < 0.01$) and an higher percentage of BPH than HH shows very anxious behaviors close to the mealtime.

The distribution of the horses in the classes is represented in Figure 7.

Discussion

The study considers both behavioral and hormonal parameters, and it investigated which aspects of management can influence the presence of abnormal behaviors.

Stress is an important factor to consider in the assessment of animal welfare. Plasma cortisol levels usually reflect acute stress and the activation of the HPA axis occurs after a few minutes from the presentation of a stressor. For example, blood sampling procedures themselves can be considered as a stressor, which can alter cortisol results (Bennet and Hayssenn, 2010; Palme, 2012). The results of this study show that HH and BPH did not present any significant difference in cortisol levels. This result agrees with other previous studies, which have underlined the lack of a correlation between horses affected by crib biting and blood cortisol levels (Bachmann et al., 2003; Hemmann et al., 2012). It can be assumed that the activation of the HPA axis during acute stress is similar between the 2 groups.

In this research, the plasma cortisol/DHEA ratio is significantly higher in BPH than in HH. This may suggest that the BPH horses are suffering from the consequences of long-term stress. This hypothesis is supported by the presence of stereotypies and the behavioral disorders that they have developed. Unfortunately, the clinical history of the BPH is not available, and we do not know when the BPHs began. This limitation could be overcome by collecting the history of each subject in greater depth and interviewing any previous owners. During long-term stress, HPA axis try to maintain high level of cortisol, so it changes the metabolism of pregnenolone and it causes an increase of cortisol at the expense of androgens. This condition leads to a decrease in the activity of the enzymes responsible to produce DHEA in the cells of the zona reticularis (Lennartsson et al., 2013; Kamin and Kertes, 2017). Therefore, subjects chronically stressed produce less DHEA in response to an acute stressor and it cause an increase of the plasma cortisol/DHEA ratio (Lennartsson et al., 2012, 2013; Kamin and Kertes, 2017). The continuous activation of HPA axis can cause

Table 3
Management variables significantly affected by the presence of behavioral pathologies.

Variables	Groups	Classes				Sig.
		0	1	2	3	
Time spent in horse stall or in paddock*	HH	0.00	34.38	9.38		$P = 0.0036$
	BPH	21.88	34.38	0.00		
Forage	HH		9.38	34.38	0.00	$P = 0.053$
	BPH		9.38	28.13	18.75	
Type of alfalfa*	HH	9.38	34.38	0.00		$P = 0.048$
	BPH	6.25	31.25	18.75		
Weekly frequency of work*	HH	34.38	9.38			$P = 0.015$
	BPH	18.75	37.50			
Behavior close to mealtime*	HH	3.13	40.63	0.00		$P < 0.001$
	BPH	25.00	3.13	28.13		

The percentage frequency of association between the group (HH vs. BPH) and the class of each variable is reported. *, significant correlation. The classification criteria are described in Table 2.

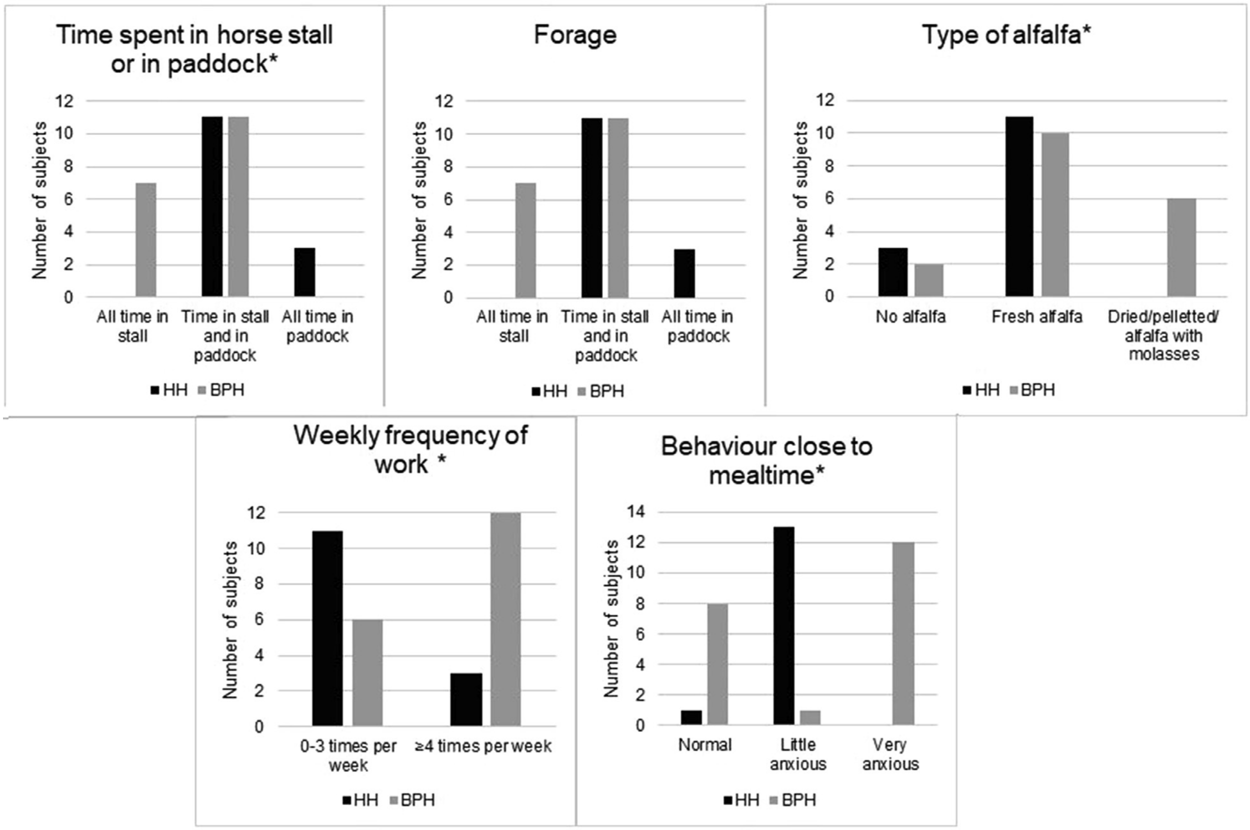

Figure 7. Histograms show the number of the horses of the 2 groups (HH and BPH) distributed for the management variables of Table 3. The * indicates the variables significantly affected by the presence of behavioral pathologies.

adrenal fatigue and a decrease of DHEA, followed by a decrease of cortisol (Kamin and Kertes, 2017).

The cortisol/DHEA ratio measured in horsehair extracts was not significantly different between BPH and HH. This finding is in contrast with the cortisol/DHEA ratio measured in plasma of our horses, as in the long-term cortisol and DHEA accumulation in the horsehair was not affected by the presence of stereotypies. This aspect, however, may be explained by the fact that all animals were employed in sport activity, which is known to stimulate cortisol release (Hill et al., 2008), and this may have masked the accumulation of both hormones in horsehair. In addition, the determination of DHEA concentration in horsehair is not a common practice and it can have some limitations. First, DHEA immobilized in horsehair can be lost over the time, leading to an underestimation of hormonal content (Cone, 1996; Anielski, 2008). Additionally, studies performed in humans have shown that the DHEA concentration in hair can be affected by age, sex, and pigmentation (Boyer et al., 2007). Finally, the efficiency of the method of hormone extraction could be lower in horsehair than in human hair (Boyer et al., 2007). It is therefore plausible that in the long-term, stressful situations leading to the development of stereotypies can affect the HPA reactivity but not the hormone accumulation, and testing HPA reactivity may be a better indicator of the horse wellbeing.

The second aim of the study was to investigate what characteristics of the management can be related to the presence of behavioral problems. In our work, BPH spent most of the time in a stall, and our results confirmed that most of the undesirable behaviors emerged when the ethological needs and motivation of horses are not catered for (Cooper and Albentosa, 2005; Hothersall and Casey, 2011). The isolation in the stall causes a lack of stimulation, an environmental challenge and limited space which can be a source of frustration (Meehan and Mench, 2007). Horses are prey animals that use, as a survival strategy, flight more than fight. Moreover, they evolved to travel over long distances (Cooper and Albentosa, 2005; Henderson, 2007). The high motivation for movement and the lack of possibility of escape in front of a potential risk can cause the observed undesirable behaviors, such as locomotor stereotypes or aggressive behaviors (Cooper and Albentosa, 2005; Henderson, 2007; Camargo, 2014).

An association was found between the presence of stereotypies or pathological behaviors and the use of dried or pelleted food or alfalfa with molasses and the feeding of mixed grass hay only once or twice a day. Horses are grazing animals and, in nature, spend most of the day eating a forage poorer in carbohydrates and protein compared to the ratio usually provided to sporting horses (Cooper and Albentosa, 2005). The high-energy and low-fiber ration often administered to stabled horses has been associated with undesired behaviors, especially oral behaviors, and stereotypies (Nicol, 1999; Cooper and Albentosa, 2005; Henderson, 2007; Camargo, 2014). For example, the eating of bedding can be described as a redirected grazing behavior by horses. Crib biting seems to be related to gastric ulcers. Indeed, the intake of too fermentable-based cereal concentrates reduces the mastication and saliva production, causing an increase of the acidity of the stomach and the formation of gastric ulcers (Cooper and Albentosa, 2005; Hemmings et al., 2007; Camargo, 2014).

A higher percentage of BPH than HH shows anxious behaviors close to the mealtime, such as repetitive pawing, pacing backward and forward, or repetitive vocalizations. This variable cannot be considered a cause of the development of the BPH, but a consequence. Horses displaying behavioral disorders showed anxiety and they accentuated their pathological behaviors, while they were waiting for food. We can assume that these are anticipatory behaviors, which occur during the appetitive phase, when the animal is waiting for a reward (Sprujit et al., 2001; Mendl et al., 2010). Captive animals often display stereotypic behaviors in anticipation of food. The research has demonstrated that the expression of the anticipatory behaviors for food can be related to the social isolation (Watters, 2014). In animals with a suboptimal welfare, anticipatory behaviors can be the expression of an increased sensitivity for a reward, because a positive experience can equate a negative one. Indeed, stressful conditions increase the sensitivity to a reward (Sprujit et al., 2001; Peters et al., 2012).

In this research, most of BPH worked more than 4 times per week. In other studies, the amount of exercise was found positively correlated to weaving and compulsive behaviors (Luescher et al., 1998; Christie et al., 2006). During the exercises, the riders have the control of horses' environment. The lack of control over the environment can be a prolonged or repetitive distress for the animals, which may develop abnormal behaviors (Christie et al., 2006). It can be also hypothesized that the horses that work more need more energy and are usually fed with high-energy concentrates. As explained above, the employment of low-fiber diet and high-energy concentrates does not respect the natural feeding system of the horse, and it may be the cause of abnormal behaviors' development (Cooper and Albentosa, 2005; Hemmings et al., 2007; Camargo, 2014). However, further studies about the correlation between abnormal behaviors and amount of forced exercise are needed.

This research underlines the importance of a multidisciplinary approach in the evaluation of horses' welfare, considering both physiological and ethological parameters, and the presence of abnormal behaviors can be considered one valuable index of a suboptimal management or environment.

Conclusion

Suboptimal welfare and stress conditions, caused by some management practice, may lead to the development of undesirable behaviors in horses. The prolonged confinement in a individual horse stall, the use of low-fiber and high-energy feeding and a high number of working hours may be factors positively correlated with the development of abnormal behaviors. The improvement of management practices should result in an improvement of horses' welfare.

In sport horses, the HPA axis can be highly stimulated in the long term, and hormone measurements in horsehair may fail to detect stressed animals. For this reason, testing the HPA reactivity may be a better tool for monitoring animal wellbeing. In this context, we suggest measuring the cortisol/DHEA ratio, although further studies are needed to characterize the DHEA release and its potential use in the assessment of animal welfare.

Data statement

Due to the sensitive nature of the questions asked in this study, survey respondents were assured raw data would remain confidential and would not be shared.

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Ethical considerations

The study was evaluated and approved by Scientific Ethic Committee for Animal Experimentation of the University of Bologna. The trial was monitored by the responsible of DIMEVET (Department of Veterinary Medical Sciences) for Animal Welfare.

Conflict of interest

The authors declare no conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jveb.2021.01.006.

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Appendix 1:

Questionnaire employed in the research

1. Name of the subject: ____

2. Breed and color:____

3. Sex and age: ____

4. Date of the orchiectomy (if applicable):____

5. Aptitude: ____

6. Stable:____

7. Name of the owner:____

Collective and Environmental history

8. Total number of horses in the stable: ____

9. Type of housing; order and measure of the stall; type of bedding:

   ____

10. Can horses go in a paddock? If they can, what is the arrangement and dimension of the paddocks? Do horses occupy always the same paddock? Are they alone or in group?
    ____

11. Are there some kind of enrichment put into the stall or in the paddock?
    ____

12. Method and schedule of the meals; type of food and amount; type of forage and way of presentation (s.e. on the ground, in the net):
    ____

13. Attendance of the stable:____

14. Lighting of the stable:______ ____

Past history of the subject

15. Was the weaning of the horse regular? How old the subject was when it was divided from its mother?: ____

16. If is a mare, has she never been pregnant? ____

17. How many owners the horse has had and how long it is with the present owner? ____

18. At what age was the horse tamed? ____

19. Its sporting career: ____

20. Has the subject never suffered physical or psychological trauma, or has it never been beaten? ____

Present history of the subject

21. Who ride the horse and what kind of training it does? ____

22. Type of mouthpiece used: ____

23. Number of hours of work in a week: ____

24. Does the subject work in horse walker or lunging? ____

25. In the horse walker, is the horse saddled? What kind of behaviour does it show towards the rider (defensive behaviour, association, fear)? Is it necessary some incitements? ____

26. Does the subject compete? How frequently? What kind of competition? ____

27. In stall or in paddock, does the horse allow to be approached and put the horse-collar easily? Or is it aggressive when someone approach it in box or when another horse or person walk nearby? ____

28. When the horse is released, does it try to escape before the horse-collar is removed?

____

29. Is the subject cooperative when it is cleaned-up (it stands still, it does not try to bite or kick). Does it stand peacefully bond?

____

30. When there are other horses, what behaviour does the horse show while it is free, ridden or bond?

____

31. Is the subject linked to another horse, with whom it shares the paddock or a horse that is in the near stall? Does it show anxiety when they are separated?

____

32. What is its behaviour during the administration of the meals?

____

33. Does the horse show stereotypies (s.e. Waving, crib-biting)? When did the stereotypy begin? How frequently and when does the horse show the stereotypy? Does the stereotypy intensify in special situation?

____

34. Have some measures adopted to reduce or abolish the stereotypy? If the answer is affirmative, what results these measures have brought?

____

35. Does the horse show phobias or excessive reactions?

____

36. Is the horse employed in hiking? How does it behave with the other horses? Does it want to stay ahead or to move through the pack? Can it go for a hiking alone or is it afraid to move away from the stable?

____

37. Is the subject timorous? If it is, what scares it most and how it reacts?

____

38. Is the horse gratified with food or prizes? When?

____

39. Has the subject recently suffered from inflammatory pathologies? Has it been treated with anti-inflammatory medications? Does it suffered from some chronic pathologies?

____

PAPER 3

Evaluation of Horses’ Daytime Activity Budget in a Model of Ethological Stable: A Case Study in Italy

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Evaluation of Horses' Daytime Activity Budget in a Model of Ethological Stable: A Case Study in Italy

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ABSTRACT

The increasing interest in animal welfare and the knowledge of equine physiological and ethological needs have led to the development of different types of horses' management and housing systems. The research presented here aimed to assess the daytime activity budget of horses. Focal animal sampling was used as an observational sampling method, and the five animals were observed for a total of 9920 minutes in the paddock and inside the stall. The results showed that horses spent most of the daytime in foraging behaviors, followed by resting behaviors, and locomotion. Social behaviors (s.e. allogrooming, olfactory investigation) were rare, and the stereotypic behaviors (s.e. oral and locomotor stereotypies) occupied 2.74%±2.74% of the total time. The percentage of time spent in foraging, resting, and locomotion, reflects the activity budget observed in free-roaming feral horses. However, the rare occurrence of positive social interactions and the presence of some stereotypies could be aspects to ameliorate. This kind of housing facility could be considered a good alternative traditional management; indeed, it might offer a better trade-off between the needs of the horse and the management goals from humans.

KEYWORDS: *Equus caballus*; welfare; management; behavior.

INTRODUCTION

The horse (*Equus caballus*) was domesticated during the late-Neolithic, and the behavioral and morphological characteristics of the modern horse are the result of an environmental adaptation (Davies, 2017). Horses are prey animals, their sensory abilities enable them to explore the environment and rapidly identify the presence of predators, and their locomotor apparatus enables them to reach high speeds (Mills & Nankervis, 1999; Pageat, 2007). They are animals characterized by a high level of sociality and they live in herds, where they find protection (Galli, 2006; Mills & Nankervis, 1999). Nowadays, horses have an important role as sport animals but also as companion animals. In response to economic and practical demands, different management and housing facilities have been developed (Baragli & Pagliai, 2016). However, there is still a long way to go to create a housing facility that can safeguard animal welfare, especially concerning the possibility of allowing horses to express their species-specific behavior (Paoletti et al., 2009).

Broom (1986) defined welfare as the condition of an animal in its attempts to cope with their environment. When an animal lives in suboptimal conditions, one of the first indicators of poor welfare can be the modification of its behavior, such as the development of anomalous behaviors (s.e. stereotypies). The modification of the behavior can be accompanied by a change in the physiological mechanisms (s.e. an excessive or prolonged activation of Hypothalamic–pituitary–adrenal axis and cortisol production), which can negatively influence the health of the animal (Broom & Johnson, 1993; Dawkins, 1990). Animal welfare is not only the evaluation of the health state of the animals, but it takes into account also the environment, the impact of human-animals' interactions, and animals' management. Indeed, human beings are responsible for the management and keeping of the animals and they must ensure that the five freedoms are respected (Biagi, Nannipieri, Signorini, & Bagliacca, 1998; Brambell Committee, 1965): freedom from thirst, hunger and malnutrition, freedom to have an adequate environment, freedom from pain, injury and disease, freedom to manifest species-specific behavioral characteristics, freedom from fear and discomfort. These freedoms are reported in the Brambell Committee (1965) and are the basis of the international animal welfare laws.

In Italy, most of the time the horses are maintained in individual stalls for much of the day, spatially isolated from their conspecifics, with whom they have only visual and auditory contact (Baragli & Pagliai, 2016). Sometimes, they are led out to an outdoor paddock for a period of time during the day, but without contact with other horses (Panzera, 2016). It has been observed that the behavior of horses in this kind of traditional management system diverges from the behavior observed in their wild conspecifics, which have the same biological and behavioral needs of domestic horses (Flannigan & Stookey, 2002; Franchini & Worthington, 2013). In traditional management, the failure to acknowledge horses' needs often leads to the development of anomalous behaviors, such as stereotypies, because the animals have difficulty in adapting to this kind of environment (Flannigan & Stookey, 2002; Hockenhull & Creighton, 2014). It has been shown that 40% of horses in traditional stables show anomalous behaviors, 10% of that was represented by stereotypies, such as cribbing, weaving, or pacing (Baragli & Pagliai, 2016). Epidemiological studies conducted by McGreevy, Cripps, French, Green, and Nicol (1995), Nicol, Davidson, Harris, Waters, and Wilson (2002), Henderson and Waran (2001) and Thorne, Goodwin, Kennedy, Davidson, and Harris (2005) have investigated the developmental causes of equine stereotypies and have linked the incidence of the stereotypies to some management factors, such as the food rich in concentrates, poor access to fiber and social isolation. A deficit in environmental stimulation can cause deprivation and an emotional state of frustration, boredom, and the horses can develop stereotypies (Cooper & McGreevy, 2007). For example, a diet rich in fiber, which requires a large percentage of a horse's time to consume in order to meet its dietary needs and helps to buffer the acidity of the stomach, can prevent or reduce the intensity of oral stereotypy, such as cribbing, compared to a diet rich in concentrates (Cooper & McGreevy, 2007). Indeed, horses are grazing animals, and, when it is possible, they spend most of the day eating (Cooper & McGreevy, 2007; McGreevy & Nicol, 1998). Another study has demonstrated that the visual and tactile contact between horses through the grates of the boxes can reduce significantly the incidence of locomotor stereotypies, such as nodding and waving (Cooper, McDonald, & Mills, 2000). Also, the physical exercise and the time that a horse spends outside its box can influence the development of stereotypies. Horses that spend most of the day inside the boxes can express locomotor stereotypies, such as box-walking and weaving (McGreevy, 1996). Usually, less stereotypic behavior is observed in the paddock, where the horse has more stimuli and the possibility of satisfying its motivations. However, horses in paddocks can also show stereotypies: especially older subjects that have spent most of their time isolated in individual boxes and have already developed stereotypies can show these behaviors also in other environments (Cooper & McGreevy, 2007). The traditional management systems do not take into account three main behavioral needs of the horse, sociability, locomotion, and foraging behavior. In traditional management systems, sociability is compromised by isolation and few opportunities for interaction with conspecifics. Confinement in boxes lasting for most of the day makes horses assume unnatural postures of the body. Finally, in the traditional management system, foraging behavior of the horse, which is a monogastric grazing animal, is affected by the amount and quality of the food. Indeed, often the ration is low in fiber and high in concentrate and the food is administered 2–3 times per day. Those are the aspects that are necessary to change. Housing facilities should favor the locomotion, a feeding system that respect horses' natural needs, and the socialization between conspecifics as well as between horses and people (Baragli & Pagliai, 2016).

This study aimed to evaluate the activity budget of a group of horses housed in a new stable, named Ethological Stable that has been designed taking into account the needs of both humans and horses. The main hypothesis was that horses in the ethological stable should show an activity budget more similar to the one reported in the literature for feral horses than the one of the animals who live in boxes, with a very low percentage of stereotypic and anomalous behaviors. This hypothesis was developed considering the layout and the management practices of the analyzed stable, which try to take into the biological and behavioral needs of horses more than other structures that are present in Italy.

MATERIALS AND METHOD

The Ethological Stable

The Ethological Stable considered in this study is about one thousand square meters wide, composed of 12 indoor Big Boxes© in addition to an area where the horse can be shod and treated, a saddlery, and a clubhouse.

The Big Box© is divided into two halves of about $20m^2$ each, for a total of $40m^2$ (8m x5m). In one half the floor is covered by sand, the other half of the bedding is made of high-density shock-resistant rubber that offers rigid support. It allows the horse to choose the surface on which to stand or lie on, between the soft sand and the shock-absorber rubber mat. The sand portion has an underlying system that suspends it from the ground without dispersing it. This system lets the urine drain away. The pavement underneath is washed by an automatic water system that transports the liquids outside into a specific tank. The sand is of fluvial origin with a large granulometry, and is washed and deprived of fillers, which constitutes the finest powdered part dangerous for breathing. The stalls are cleaned daily by collecting manure and later cleaning with automatic sweeping machines on the rubber mat. The sand can be collected only where dirty directly in daily management and it is replaced weekly replacing the sand removed during cleaning. When horses are inside the stable, the hay is placed outside the door of the box, easily reachable from the main openings, while cereals or dry food is fed in a bucket hooked to the grates in the lower front part of the Big Box© (Figure 1).

Outside the stall, there is an 18m round pen, an outdoor arena, a ‘Paddock Paradise’ of 1.5ha, and individual paddocks, divided by electrified fences, which extends to 5.0 ha. The “Paddock Paradise” was conceived for the first time with the idea of providing horses an environment more like their natural habitat, to satisfy the behavioral needs of the species as much as possible. It consists of a ring route, normally divided into two parts by an electrified mobile fence. Along its entire path, it has a variation in vegetation, varying from low shrubs to hedges and trees, areas where to roll, linear areas and plains are spaced by areas with a raised surface, and wood logs and a pool of water are also present. To increase the space available to the horses, there is a rectangular paddock that can be joined to the “Paddock Paradise”. Along the whole path, you find drinking and feeding stations, with the hay given on the ground (Figure 2).

During most of the daytime period, horses are in paddocks, however, if they are inside, hay is provided *ad libitum* allowing them to eat constantly and with their head lowered, as it happens when grazing. The same concept applies to water, which remains outside and placed close to the floor. The boxes are divided by grates and horses can see each other inside the stable and to touch and groom through the window between the boxes.

Subjects and Behavioral Assessment

The subjects observed were five horses, which were raised in traditional stables and were now moved into the Ethological stables. They were four mares (two Sella Italiano, 11 and 16 years old, one Trotter, 14 years old, and one Hungarian horse, 16 years old), and one gelding (Sella Italiano, 14 years old).

The observation period lasted from April to October 2019. Focal animal sampling was used for all of the five horses (Altmann, 1974). The observations were conducted in four daily bands: bands B (8.00 am −12.00 pm) and C (12.00 am – 6.30 pm), during which the horses remained outside in the paddocks (PP = Paddock Period), and the bands A (7.00 am – 8.00 am) and D (8.00 pm − 9.00 pm), when the horses were inside the stalls (BBP = Big Box© period). During the observation outside the stable, an observer recorded the animals using a Sony HDR-CX240E camera, whereas during the bands A and D webcams (Each ® Italy 7548AHD) were installed above the stall of each horse. A single observation lasts 60 minutes and a continuous recording method was employed. The ethogram employed was that reported by McDonnell (2003), and three other behaviors were added (Backwards, Licking/chewing, Lip-smacking).

The entire ethogram consisted of 110 behaviors divided into 12 categories (see Appendix 1). When it was not possible to observe the entire animal during the analysis of the video, it was recorded as “not visible.”

The analysis of video was made with the software BORIS (Behavioral Observation Research Interactive Software) (Friard & Gamba, 2016), employing a continuous recording method for all focal animals (Altmann, 1974). A total of 5320 minutes in the paddocks (PP) and 4200 minutes in the Big Box© (BBP) were analyzed.

Video Analysis

The behaviors were categorized into states and events. States have a measurable duration, while the events are instantaneous and only frequencies can be recorded (Altmann, 1974). Therefore, the mean frequency and standard error of the main events observed in PP and BBP were calculated and expressed in acts/hour, whereas the percentage duration of state behaviors was calculated, to analyze the daily activity budget of the group. The observed behaviors were divided into 12 behavioral categories: foraging behavior, elimination behavior (defecation and urination), locomotion, standing alert, resting, grooming and insect control, investigation, aggressive behavior, affiliative behavior, reproductive behavior, comfort behavior, anomalous behavior. The observer that recorded and analyzed the video did two weeks of training before the beginning of the study.

Initially, the daily activity budget of each subject was calculated including the observations from both the PP and BBP, and then separately in each period. The mean percentage and the standard error of each behavioral category was calculated, to obtain the activity budget of the group. Due to the small sample size, only descriptive statistics have been used to illustrate the time budgets of the horses and the differences between the two periods were not compared with statistical tests. Any power analysis was performed because the five animals considered was the only resident animals in the structure when the research was conducted.

RESULTS

Horses spent most of the daytime (7.00am-9.00pm) in foraging behavior (67.76%±2.51%), particularly in grazing, which occupied 98.99% of the total time spent in foraging. The second behavioral category most represented was resting (18.88%±2.12%), followed by locomotion (5.20%±0.51%), stand alert (4.03%±0.78%), and anomalous behaviors (2.74%±2.74%). On the other hand, the lowest percentages were recorded for grooming and insect control

(0.62%±0.04%), investigation (0.45% ±0.07%), elimination (0.23%±0.02%), affiliative behavior (0.04%±0.02%), reproductive behavior (0.03%±0.03%), comfort behavior (0.01%±0.004%), and aggressive behavior (<0.01%) (Table 1 and Figure 3).

Considering the single behavioral category of resting, we distinguished between resting recumbent (10.25%) and resting while standing (88.09%), sleeping recumbent (1.02%), and sleeping while standing (0.65%). Sleeping behaviors were observed only inside the boxes. The percentage duration of standing alert in BBP was almost twice that in the PP (Table 1 and Figure 4a). The observed anomalous behavior occupied only 2.74% of the total observational time, and, within this category, we observed miscellaneous abnormal behaviors (1.13%), locomotor stereotypies (4.80%), and oral stereotypies (94.07%).

Considering the activity budget recorded in PP and in BBP, the percentages of time spent in foraging behavior (PP = 81.31%±4.16%; BBP = 49.17%±1.81%), grooming and insect control (PP = 0.89%±0.08%; BBP = 0.24%±0.05%), social behaviors (PP = 0.10%±0.07; BBP = 0.04% ±0.03%), and elimination (PP = 0.32%±0.05; BBP = 0.11%±0.04%) were higher in PP than when the horses were in Big Boxes©. When the horses were inside the boxes, the percent of time of resting (35.97%±0.85%), standing alert (5.91%±1.24%) and investigating (0.64%±0.19%) were higher rather than for the horses in paddocks (respectively 6.47%±0.41%; 2.62%±0.60%; 0.31%±0.03%). The percentage duration of locomotion and anomalous behavior, recorded was similar in PP (5.27%±0.85%; 2.82%±2.82%) and BBP (5.13%±0.41%; 2.63%±2.63%) (Figure 4a, Figure 4b).

Regarding the events recorded during the observation, the most frequent ones were the stamp (Figure 5a) and the swish/swat insects (Figure 5b), two behaviors that belong to the grooming and insect control category. The mean frequency of both of these events was higher in the PP compared to in the BBP. Events that were observed more frequently in BBP were licking and chewing, yawning, and stretching (Figure 6a). General social communication behaviors were observed more frequently while the horses were in paddock, especially vocalization, and flehmen, whereas, the aggressive behavior “ears laid back” had a higher frequency in the BBP (Figure 6b).

DISCUSSION

The aim of this research was to observe the behavior of a group of horses stabled in a new facility (Ethological Stable) designed to promote natural behavior, recording them both inside the Big Box© and outside in paddocks. The layout and the management practices of the analyzed stable tries to take into account both the biological and behavioral needs of horses and the human's practical requests.

Our results showed that for most of the daytime, horses are occupied in foraging behaviors (67.76%±2.51%), mainly grazing. The second most common activity was resting (18.88%±2.12%), predominantly expressed when the subjects were inside the Big Boxes©. The third most common activity was locomotion (5.20%±0.51%). These data are very similar to the results of Ransom and Cade (2009), who monitored the daily activity budget of free-roaming horses. Previously, Kiley- Worthington (1990) underlined how feral horses spent 60% of their time in foraging. On the contrary, in a condition of social isolation and with a planned feeding schemes, horses spent 65% of their time in resting while standing, which is three times more than free-roaming horses, and only 15% of their time in foraging activity (Table 1) (Kiley-Worthington, 1990). In traditional management, horses mainly receive diets rich in concentrate and low in fiber (Marsden, 1995). This kind of diet can be consumed in less than 2 hours and alters the normal feeding behavior of the horse (McGreevy, 2004). In nature, horses are non-ruminant grazer, the food moves quickly through the digestive apparatus and it is low in energy and high in fiber. Therefore, these animals need to spend most of their time eating, to achieve their caloric and nutrient needs (Burla, Ostertag, Patt, Bachmann, & Hilmann, 2016). The main feeding behavior observed in feral and pastured horses, like in our study, is grazing (Mills & McDonnel, 2005).

The second most common behavior observed in our study is resting (18.88%±2.12%), which is usually divided into stand-resting and lying. Lying occupies a small part of the daily time (approx. one hour per day, about 4% of the activity budget), but this time usually does not vary during the day (Mills & McDonnel, 2005). On the contrary, the time spent standing while resting changes during the day and is inversely related to the time spent foraging, with the more time they spend in resting while standing, the less time they dedicate to foraging and vice versa (Duncan, 1985; Mills & McDonnel, 2005). Our results show that, while they were in the paddock (PP), horses spent more time in foraging behaviors (81.31%±4.16%) and less time resting (6.47%±0.41%) compared to in BBP, respectively, 49.17%±1.81% and 35.97%±0.85%. The lower duration of foraging recorded in BBP may be explained with the fact that, during the night, the horses were in the box and they did not have hay for the entire night, as it was provided at 7.00 am and 7.00 pm, while during the day they were in paddocks and the hay is provided *ad libitum*. Moreover, because of the organization of horses' handling and the stable's personnel schedule, we were able to record animals in Big Box© only during early morning and late evening, and not during the entire night, so it can be considered a limitation in the calculation of the activity budget of the horse in BBP. The total of lying duration recorded was 11.41% of the entire resting duration. The study conducted by Raabymagle (2006) observed that the box size positively influenced the duration of lying behavior, with horses spending more time lying in recumbent positions in the largest boxes. Horses reach REM and slow-wave sleep in recumbent positions (Houpt, 1980). Sleep is important for thermoregulation and energy conservation; also, it is a restoration process that allows the recovery of a vigilance state and helps the consolidation of memories (Berger & Phillips, 1995; Dukas & Clark, 1995; Smith, 1995). An increase in sleeping time, especially for a sport horse, might lead to an improvement in their wellbeing (Belling, 1990). The Big Box© offers the animals a large area that allows them to spend a part of their time lying recumbent, allowing an opportunity to sleep. In our study, the lying behavior (resting and sleeping while recumbent) occupied 2.13% of the total time of observation, which is lower than the results reported both for feral and stabled horses by Kiley-Worthington (1990). The lack of observational data during all-night is a limitation of this study, and it could have influenced our results. However, according to the literature, the layout of the Big Box©, bigger than a conventional box, could positively influence the time spent in a lying position, the sleeping time and, therefore, the welfare of the horses. However, further investigations about this aspect should be conducted.

The part with the sand, which is in the back of the box, has been created to give to horses the possibility of rolling. Rolling is a behavior beneficial for coat care and it is a comfort behavior (Mills & McDonnel, 2005). This behavior was rarely recorded during the research. The frequency of swish/swat insect and stamp was higher in PP than in BBP, probably because the horses remained in the paddock during the warmer part of the day, and outside there was a higher presence of insects.

Alert behavior occupied 4.03% of the activity budget. The alert behavior was higher in BBP (5.91%±1.24%), as during the webcam recordings, the stable-boy usually prepared and distributed the hay and the feedstuffs. This is probably the same reason for the higher frequency of licking and chewing, yawning, stretching behavior, and pawing. An increase of alertness was noticed in the anticipation of a reward in rats (Van der Harst, Baars, & Spruijt, 2003), and also yawning and stretching were observed in other species (dogs and lions) associated with high arousal anticipatory situation, such as feeding (Baenninger, 1997; Gessa, Vargiu, & Ferrari, 1966). Also in horses, yawning, stretching, and pawing can occur during anticipatory situations, for example before the distribution of food ratio (Górecka-Bruzdaq, Fureix, Ouvrard, Bourjade, & Hausberger, 2016; Peters, Bleijenberg, van Dierendonck, van der Harst, & Spruijt, 2012). On the other hand, “licking and chewing,” which is a very misunderstood behavior, because is often wrongly interpreted as stress behavior, is a behavior that reflects a relaxation after a stressful situation. Indeed, it is associated with the return of salivation after a stress situation (the passage from the activation of the sympathetic nervous system to that of the parasympathetic nervous system) (Lie & Newberry, 2018). The arrival of food can determine relief for the animals, who show other behaviors, such as pawing, yawning, and stretching, usually associated with an excitation state. Anticipatory behaviors occur before the arrival of a reward during the appetitive phase, characterized by high arousal of the animal (Craig, 1918).

Other behaviors usually shown during anticipation are stereotypies (Peters et al., 2012), but our results showed a similar percentage of anomalous behaviors in PP (2.82%±2.82%) than in BBP (2.63%±2.63%). In this study, the anomalous behaviors occupy 2.74% of the time. The five horses considered in this study came from different traditional stables, and when they arrived at the Ethological Stable had already displayed anomalous behaviors, particularly, one of them an oral stereotypy (lip-smacking) and one a locomotor stereotypy (head shaking). In adult horses, stereotypies cannot be eradicated, and they become part of the behavioral repertoire of the individual, often elicited as a conditioned response (Waran, 2007). The duration and frequency of anomalous behavior can be reduced by the presence of a conspecific, identified as a social buffer. Indeed, in social animals, the presence of conspecifics significantly modifies the physiological and behavioral response to stress (Panzera, 2016). In the analyzed housing facility, the horses did not form a herd, usually were in paddocks alone, and only two of them sometimes shared the paddock. This situation resulted in a low frequency and duration of social behavior, and a higher frequency of vocalization in PP than in BBP. This kind of communication helps animals to keep in contact from a distance, and when they cannot see each other (Waran, 2007). Horses can perceive and localize sounds from distance (Fraser, 2010), and use vocalizations to express their emotional state. According to the frequency and the intensity used, the different vocalizations assume different meanings (Yeon, 2012). Even if the boxes were separated by grates and the horses could see each other, the social behaviors observed were fewer than in the paddocks. One of the behaviors with a higher frequency in the box than in the paddock was the “ears lied back/pinned,” an aggressive behavior, which can occur during competitions, when the resources are concentrated (Waran, 2007), as in our case during the distribution of hay to the horse. As the animals came from different stables and were kept isolated for most of their life, it means that the formation of a herd can be difficult because most of them do not have the social competencies necessary to live together. The Big Box© might facilitate the formation and maintenance of social bonds, but on the other hand, it is necessary to pay attention and put animals with close social bonds in adjacent boxes. Indeed, the social bonds or friendship is a social structure that is very important for the cohesion of the herd (Seyfarth & Cheneyal, 2012). These bonds can last throughout the lives of horses, and they can be between individuals of the same gender or not (Cameron, Setsaas, & Linklater, 2009). The paired bond can also involve more than two individuals and are identifiable by the index of closeness during the daily activities and the frequencies of mutual grooming (Feh & de Mazieres, 1993). Evolution has promoted the formation of social bonds for the advantage of the individual because the “friend” is both a physical obstacle and sentinel of the presence of a predator. Moreover, friendship means collaboration between animals (Seyfarth & Cheneyal, 2012).

CONCLUSIONS

Analyzing the daytime activity budget of the horses and comparing our results with the literature, in this facility the behavioral categories most represented are the same reported for feral horses (Table 1). Feral horses and domestic horses belong to a unique genus (*Equus caballus*). Genetic selection made by humans has modified some morphological features of horses, such as the color of the coat or the size, but their behavioral needs, such as the possibility of grazing for most of the time, are the same (Franchini & Worthington, 2013). The presence of a stable group and the acquisition of correct social competencies by the subjects can promote the creation of a single herd. The formation and maintenance of social bonds might be promoted by the presence of large paddocks, which can be shared by the animals, and by the layout of the boxes.

In conclusion, this kind of facility could be considered a good alternative to traditional management, because it tries to take into account the behavioral needs of the horse and the practical management needs of humans. This study represents preliminary work; indeed, it has some limitations. The stable used for the research is the only one in Italy and, at the beginning of the study, it had just started, so the sample of subjects was too small to conduct a formal comparison using statistical analysis. Moreover, because of the management of the stable itself, we were able to record animals in the Big Boxes© only in two limited-time bands. It would be interesting, for example, recording the animals during all-night, when they are in the Big Boxes©. Indeed, for the feature of these kind of boxes, it is necessary to consider the social links between the horses and pay attention to their behavior. Therefore, further studies are necessary to evaluate the welfare in the facility, also considering other parameters.

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TABLES

Table 1. Comparison between the activity budgets of feral and stabled horses measured in 24hr by *Kiley-Worthington, M. (1990) and the study's results. "Standing" includes standing while resting and sleeping and standing alert. PP = Paddock Period; BBP = Big Box Period.

	***Feral Horses (*Equus Caballus*)**		***Isolated Stabled Horse + restricted long fiber food**	**Ethological Stable**		
	Summer	*Winter*		*BBP+PP*	*PP*	*BBP*
Eating	56.17%	61.55	25%	67.76% ±2.51%	81.31% ±4.16%	49.17% ±1.81%
Standing	22.2%	24.2%	65%	20.67% ±1.58	9.09% ±2.78%	36.85% ±2.63%
Lying	11.6%	7.9%	10%	2.13% ±1.21%	0%	5.04% ±2.85%
Moving	9.5%	6.2%	0%	5.20% ±0.51	5.27% ±0.85%	5.13% ±0.41%

FIGURES

Figure 1. Big Box© – Picture of the Big Box© (http://www.laprosperacentroequestre.it/).

Figure 2. Paddock Paradise – Pictures of the Paddock Paradise made by Giulia Schiavoni and from http://www.laprosperacentroequestre.it/.

Figure 3. Daytime activity budget- The durations of the considered behavioral categories expressed as a percentage, calculated according to the total observational time, when the horse where in Paddock and in the Big Boxes (PP+BBP).

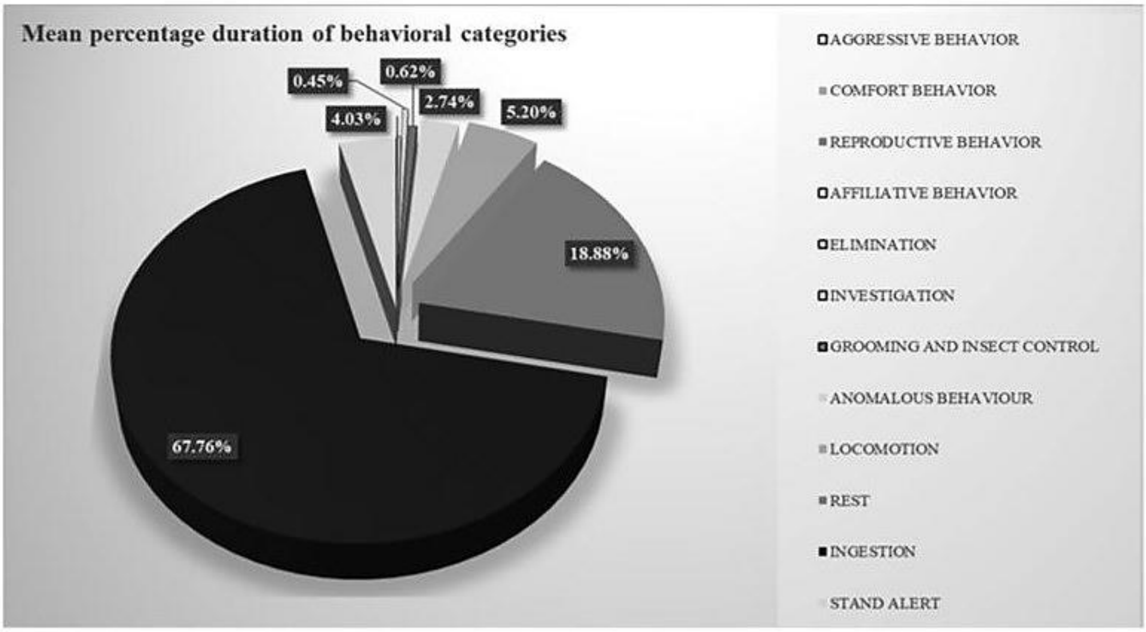


Figure 4. Comparison of the two periods- a) Comparison between the mean percentage durations and standard errors of foraging, resting and locomotion in paddock (PP) and into the Big Box (BBP); b) Comparison between the mean percentage durations and standard errors of affiliative behavior, aggressive behavior, reproductive behavior, comfort behavior, elimination, grooming and insect control, investigation and anomalous behavior in paddock (PP) and into the Big Box (BBP).

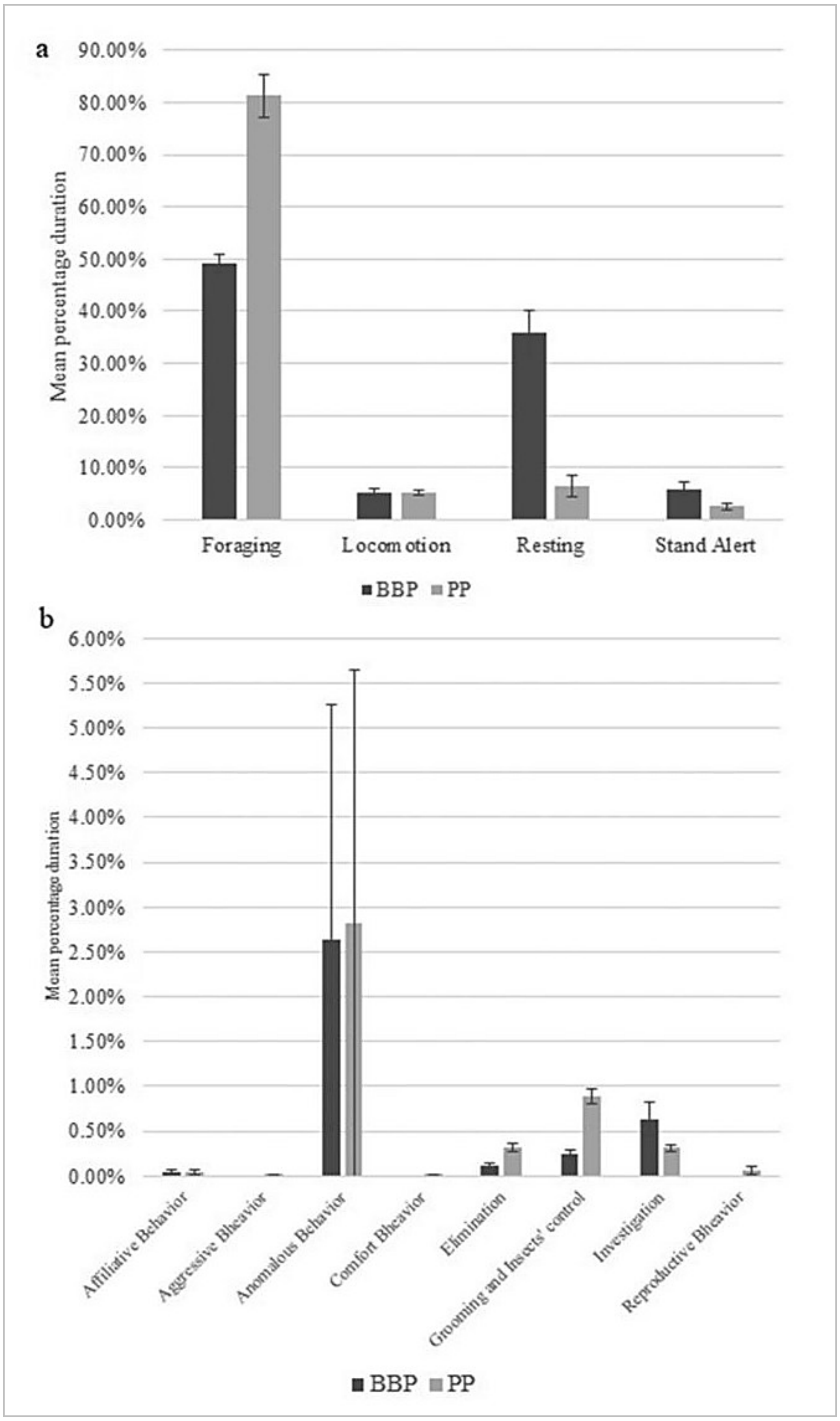


Figure 5. Mean frequencies and standard error of events of grooming and insect control category – a) Frequencies of stamp, roll and shake; b) Frequency of swish/swat insect. PP = Paddock Period; BBP = Big Box Period.

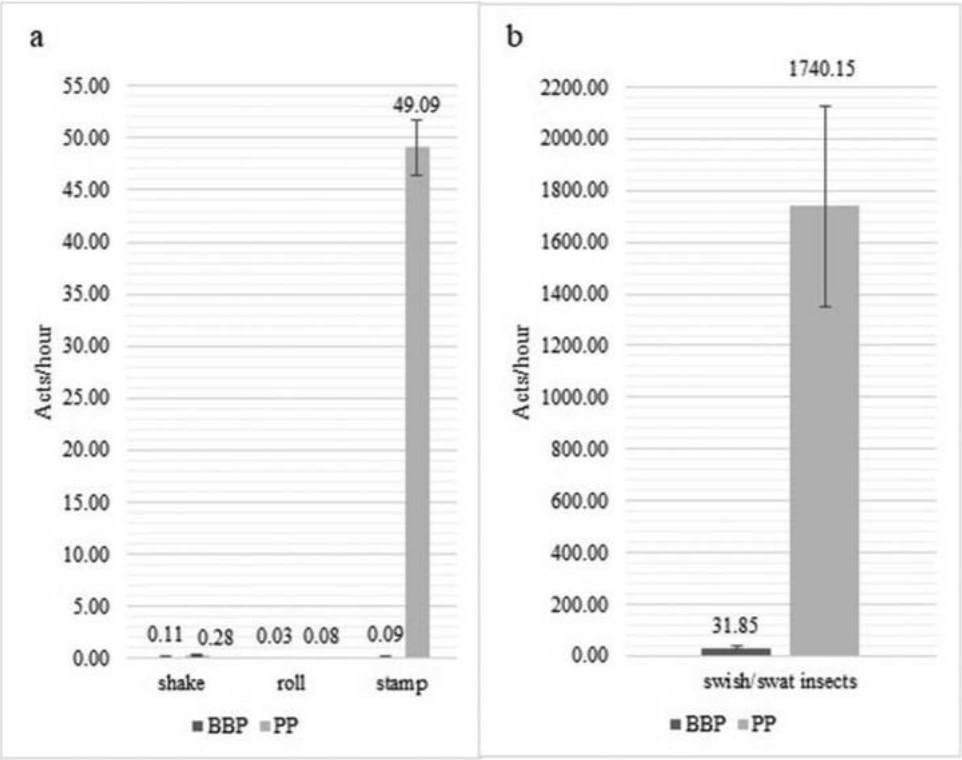
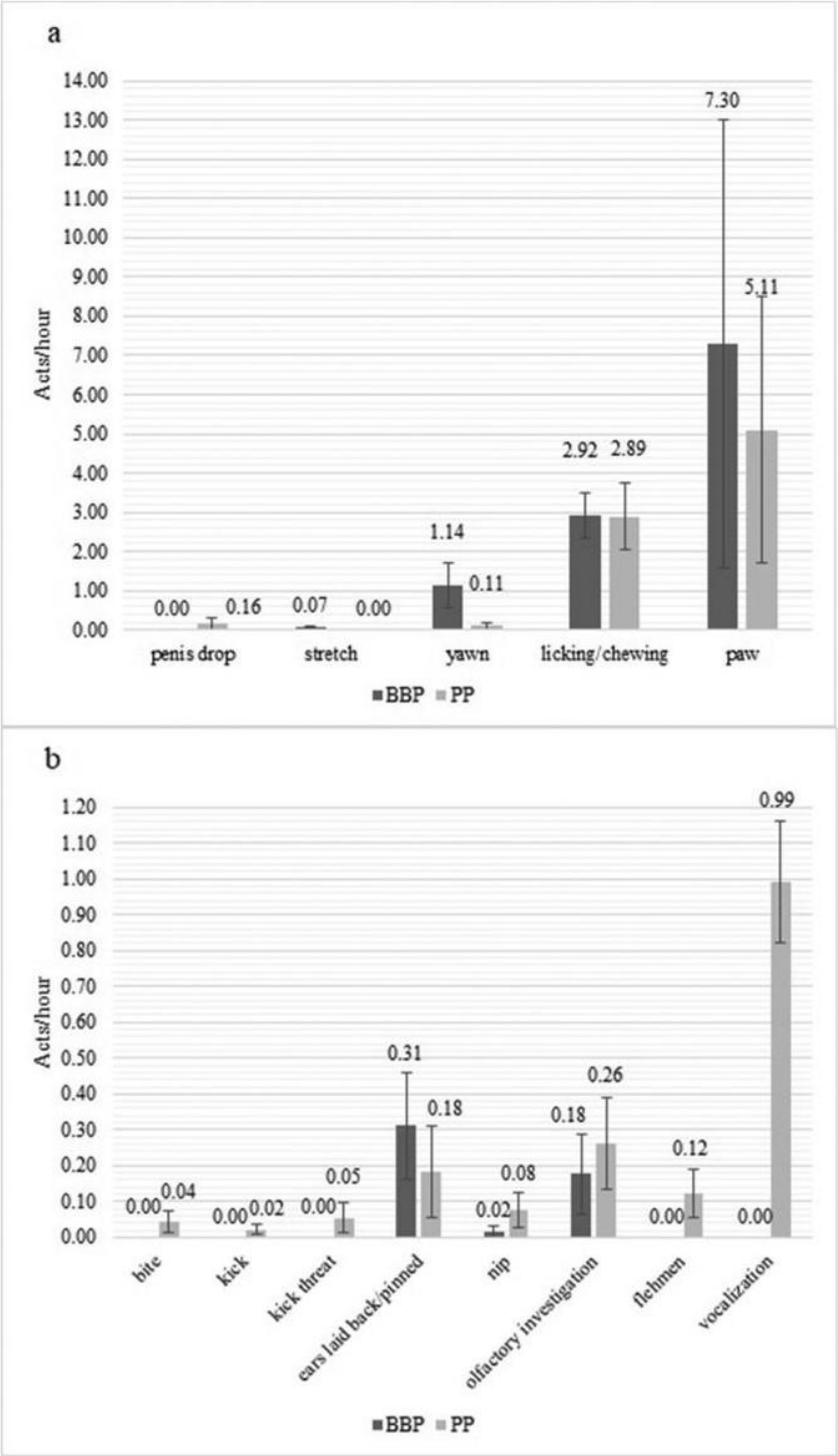


Figure 6. Mean frequencies and standard error of the other events observed a) frequencies of locomotor (paw and stretch), resting (yawn) and comfort and relief (penis drop and licking and chewing) behaviors; b) frequencies of general social communication events. PP = Paddock Period; BBP = Big Box Period.



Appendix 1. Ethogram by McDonnel, 2003 with the addition of three behaviors. The ethogram reports all the behaviors divided in categories and distinguished in States or Events.

Feeding behavior

BEHAVIOR	STATE/ EVENT	DESCRIPTION	REFERENCES
Graze	**State**	Ingest grassy vegetation. With the lips and tongue, vegetation is gathered into the mouth, broken off usually in clumps by jerking the jaw while chewing; and swallowed.	Feist, 1971; Tyler, 1972; Waring, 1983; Keiper, 1985; Boyd and Houpt, 1994; Moehlman, 1974; 1998; Gardner, 1983; Penzhorn, 1984
Browse	**State**	Ingest woody plants.	Feist, 1971; Tyler, 1972; Waring, 1983; Keiper, 1985; Boyd and Houpt, 1994; Moehlman, 1974; 1998; Gardner, 1983; Penzhorn, 1984
Drink	**State**	Ingest water, typically by using lips at or slightly below the surface of water and drawing water with sucking action through slightly parted lips and teeth and swallowing.	Feist, 1971; Tyler, 1972, Waring, 1983, Keiper, 1985; Boyd and Houpt 1994; Moehlman, 1974; 1998; McCort,1980; Gardner, 1983; Penzhorn, 1984
Pica	**State**	Ingest soil, by using the lips and tongue to draw soil into the mouth and swallow.	McDonnell, 2003

Elimination

Urination/ Defecation	**State**	Urination: With forelegs slightly extended forward and hind legs extended backward and slightly spread, expelling of urine through the urethra. The penis is typically partially or fully relaxed from the prepuce. With the back arched, the tail raised, and the hind limbs extended backward and separated (without squatting), expelling of urine through the urethra and vulva. Final small quantities are expelled with vulvar contractions that commence after the mainstream. Defecation: With tail raised, expelling of feces through the anus. The anal sphincter contracts rhythmically and the tail may be lashed vertically at the completion of passage of feces. The mare simply postures, defecates and walks off following defecation. The stallion sniffs and may paw at the site before and again after defecation, with Flehmen response, whether or not further elimination marking sequence behaviors are included.	McDonnell, 2003

Locomotion

Stand Alert	**State**	Rigid stance with the neck elevated and the head oriented toward the object or animal of focus. The ears are held stiffly upright and	Feist, 1971; Tyler, 1972, Waring, 1983, Keiper, 1985; Boyd and Houpt 1994; Moehlman, 1974; 1998;

		forward, the nostrils may be slightly dilated.	McCort,1980; Gardner, 1983; Penzhorn,1984; Duncan, 1985; Berger, 1986; Schilder and Boer, 1987.
Walk	**State**	Movement forward in the slowest (four beats) of the mammalian quadrupedal gaits.	Evans, 1989; Waring, 1983; Boyd and Houpt 1994; Moehlman, 1974; 1998; McCort,1980; Gardner, 1983
Backwards	**State**	Backward movement of the horse.	Kaiser, 2006; Denderen, 2011
Trot	**State**	Movement forward in a two-beat gait, in which diagonally paired feet touch and lift simultaneously.	Evans, 1989; Waring, 1983; Boyd and Houpt 1994; Moehlman, 1974; 1998; Gardner, 1983
Gallop	**State**	Running fast with a four-beat gait.	Evans, 1989; Waring, 1983; Boyd and Houpt 1994; Moehlman, 1974; 1998; Gardner, 1983
Trek	**State**	Two or more animals moving a distance together, typically following one after the other.	Feist, 1971; Tyler, 1972; Waring, 1983; Keiper, 1985; McCort, 1980
Jump	**Event**	With mostly hind leg propulsion, moving forward with the forelegs leaving the ground first followed by the hind legs. Jumping can be vertical to clear high obstacles or broad to span ditches or small streams.	Moehlman, 1974; 1998; Tyler, 1972; Waring, 1983
Stampede	**State**	Running of a group together as a unit at high speed.	Penzhorn, 1994
Shying	**Event**	Sudden veering to avoid novel or fear-provoking animate or inanimate stimulus	McBane, 1987
Paw	**Event**	A foreleg is lifted off the ground slightly, extended quickly in a forward direction, and followed by a backward, toe-dragging movement as if digging	Waring,1983; Moehlman, 1974; 1998; McCort,1980; Penzhorn, 1984
		Resting	
Rest Standing	**State**	Standing inactive in a relaxed posture, usually, with head slightly lowered, eyes partly or nearly closed, and often bearing weight on three legs (one hind leg slightly flexed). With deeper drowsiness (transition between wakefulness and sleep), the lips relax, and the ears rotate laterally.	Feist, 1971; Tyler, 1972, Waring, 1983, Keiper, 1985; Boyd and Houpt 1994; Moehlman, 1974; 1998; McCort,1980; Gardner, 1983; Penzhorn,1984
Sleep Standing	**State**	With eyes closed and head lowered below the back, light sleep in a standing position.	Waring, 1983, Boyd and Houpt 1994; Moehlman, 1974; 1998; McCort,1980; Gardner, 1983
Rest Recumbent	**State**	*Lying Down; Getting Up*; *Sternal Recumbency; Lateral Recumbency*	Blakeslee, 1974; Feist, 1971; Tyler, 1972; Boyd, 1980; Waring, 1983; Boyd and Houpt

		Rest or sleep while lying down with head up or with legs and head outstretched.	1994; Moehlman, 1974; 1998; McCort,1980; Gardner, 1983; Keiper, 1985; Penzhorn,1984
Sleep Recumbent	**State**	*Lying Down; Getting Up; Sternal Recumbency; Lateral Recumbency* Sleep while lying down with head up or with legs and head outstretched.	Blakeslee, 1974; Feist, 1971; Tyler, 1972; Boyd, 1980; Waring, 1983; Boyd and Houpt 1994; Moehlman, 1974; 1998; McCort,1980; Gardner, 1983; Keiper, 1985; Penzhorn,1984
Yawn	**Event**	Deep, long inhalation with mouth widely opened, with jaws either directly opposed or moved from side to side.	Feist, 1971; Tyler, 1972; Waring, 1983; Boyd and Houpt 1994; Moehlman, 1974; 1998.
Stretch	**Event**	Rigid extension of the limbs and arching of the neck and back.	Feist, 1971; Tyler, 1972, Waring, 1983; Keiper, 1985; Boyd and Houpt 1994; Moehlman,
Grooming and insect control			
Roll	**State**	Dropping from standing to sternal recumbency, then rotating one or more times from sternal to dorsal recumbency, tucking the legs against the body.	Feist, 1971; Tyler, 1972, Waring, 1983; Keiper, 1985; Boyd and Houpt 1994; Moehlman, 1974; 1998; McCort,1980; Gardner, 1983; Penzhorn,1984
Shake	**Event**	Rapid, rhythmic rotation of the head, neck, and upper body along the long axis while standing with feet planted.	Waring, 1983; Boyd and Houpt 1994; Moehlman, 1974; 1998; McCort,1980; Gardner, 1983; Penzhorn,1984
Autogroom	**State**	Nibbling, biting, licking, or rubbing a part of the body	Feist, 1971; Waring, 1983; Boyd and Houpt 1994; Moehlman, 1974; 1998; McCort,1980; Penzhorn,1984
Swish/Swat Insects	**Event**	Swish the tail, swing the head against the shoulder or abdomen, flex the chin to the chest, or flex a limb to remove insects.	Waring, 1983; Boyd and Houpt 1994; Moehlman, 1974; 1998; Hafez *et al.*, 1962
Stamp	**Event**	Sharply strike the ground with a hoof by flexing and raising and then rapidly lowering a fore or hind leg.	Waring, 1983; Boyd and Houpt 1994; Moehlman, 1974; 1998; McCort,1980; Penzhorn, 1984
Insect control - mutual	**Event**	While standing head-to-tail within close proximity, two or more animals swish their tails, effectively removing insects from the head and hindquarters of the participants.	Waring,1983; Boyd and Houpt 1994; Moehlman, 1974; 1998; Penzhorn, 1984
Investigation			
Mouth, Sniff, Lick, Paw	**State**	With nose near object of interest, draw air in through nostrils. Manipulate with open mouth, as if to gain touch or taste. Contact an object with the tongue. With an object as an apparent target, a foreleg is lifted off the ground slightly, extended quickly in a	Waring,1983; Boyd and Houpt 1994; Moehlman, 1974; 1998; McCort,1980; Penzhorn, 1984

		forward direction, and followed by a backward, toe-dragging movement as if digging.	
General social communication			
Nip	**Event**	Similar to a bite, but with the mouth less widely opened and the teeth closing on only a small piece of flesh. Nipping is seen during play-fighting, during mutual grooming, and during moderate to serious aggressive interactions.	McDonnell and Haviland,1995; Houpt and Wolski, 1982; Hoffmann, 1985
Vocalization	**Event**	Whinny (neigh), squeal and scream.	McDonnell, 2003
Affiliative behavior			
Interspecies Interactions	**State**	Apparent recognition and communication or interaction with animals of other species.	Keiper, 1985; Tyler, 1972; Penzhorn,1984
Mutual Groom	**State**	Herd mates standing beside one another, usually head-toshoulder or head-to-tail, grooming each other's neck, mane, chest, back, rump, or tail by gentle nipping, nuzzling, or rubbing.	McFarland,1987; Feh and de Mazières, 1993; Feist, 1971; Tyler, 1972; Keiper, 1985; Boyd and Houpt 1994; Moehlman, 1974; 1998; McCort,1980; Penzhorn, 1984
Head on neck, Back, or Rump	**State**	Mount	Schilder, 1988; McCort, 1980; Feist, 1971; Boyd and Houpt, 1994
Approach	**Event**	Forward movement at any gait toward another horse in a straight or curving path. The head may be elevated and ears forward or the head may be lowered and ears pinned back.	Welsh, 1975; Waring, 1983; Feh, 1988; Boyd and Houpt, 1994; Moehlman, 1974; 1998; McCort,1980;Penzhorn, 1984; Schilder, 1988; 1990
Follow	**State**	Move along behind another horse, usually at the same gait as the horse being followed. In contrast to a chase, there seems to be no attempt to direct the movement, attack, or overtake the leading horse.	Waring, 1983; Feh, 1988; Moehlman, 1974; 1998; Schilder, 1990
Snapping	**Event**	Moving the lower jaw up and down in a chewing or suckling motion, usually with the mouth open and lips drawn back exposing the incisors. A sucking sound may be made as the tongue is drawn against the roof of the mouth (Waring 1983). Typically, the head and neck are extended, with the ears relaxed and oriented back or laterally. This behavior is usually performed while approaching the head of another, usually on an angle.	Moehlman, 1974; 1998; McCort,1980; Feist, 1971; Wells and Von Goldschmidt-Rothschild,1979; Feh, 1988; Hogan et al., 1988; Boyd and Houpt, 1994; Schilder et al.,1984
Rolling	**Event**	Dropping from standing to sternal recumbency, then rotating one or more times from sternal to dorsal recumbency, tucking the legs against the body. Rolling typically occurs on dusty or sandy areas. Rolling is	McCort, 1980; Boyd and Houpt, 1994; Waring, 1983.

		usually preceded by pawing and nosing of the ground and followed by body shaking. Snort vocalizations may occur when the horse is nosing the ground or shaking.	
Olfactory Investigation	**State**	Olfactory investigation involves sniffing various parts of another horse's head and/or body. This behavior typically begins after horses have approached one another nose to nose. After mutually sniffing face to face, typically one horse works his way caudally along the other's body length, sniffing any or all of the following: neck, withers, flank, genitals, and tail or perineal region. During the investigation, it is common for one or both horses to squeal, snort, kick threat, strike threat, or bite threat.	Welsh, 1975; McCort, 1984; Feh, 1988; Boyd and Houpt, 1994; Moehlman, 1974; 1998; McCort, 1980;Schilder, 1990
Interference	**Event**	Disruption of horses' combat by moving between the combatants, by pushing, attacking, or simple approaching. One or more horses may simultaneously interfere with an encounter.	Keiper, 1985; Schilder, 1990; Bannikov, 1971
Rump Presentation	**State**	One horse positions his rump squarely in front of another horse's head, lifting the tail slightly, reminiscent of estrous presentation of mares. The horse to whom the rump is presented usually sniffs the perineal region, and he may push his shoulder against the hindquarters, and/or rest his chin or head on the rump, and he may mount.	McDonnell and Haviland,1995
		Aggressive behavior	
Head Bowing	**Event**	Head bowing is a repeated, exaggerated, rhythmic flexing of the neck such that the muzzle is brought toward the point of the breast. Head bowing usually occurs synchronously between two horses when they first approach each other head to head.	McDonnell and Haviland, 1995
Avoidance/ Retreat	**State**	Movement that maintains or increases an individual's distance from an approaching or following herd mate. The head is usually held low and ears turned back. The retreat can be at any gait but typically occurs at the trot.	Feist, 1971; Schilder and Boer 1987;Schilder, 1990; Houpt and Wolski, 1982; Syme and Syme, 1979; Miller, 1981, Keiper, 1985; Feh, 1988; Moehlman, 1974; 1998; McCort, 1980; Schilder, 1990
Arched neck threat	**Event**	Neck tightly flexed with the muzzle drawn toward the chest.	Miller, 1981; Boyd and Houpt, 1994;Berger, 1986
Ears Laid Back/Pinned	**Event**	Ears pressed caudally against the head and neck. Typically associated with intense aggressive interaction.	Feist, 1971; Waring, 1983; Keiper, 1985; Boyd and Houpt 1994; Moehlman, 1974; 1998; McCort,1980; Berger, 1986; Bannikov, 1971; Schilder *et al.*, 1984

Head threat	**Event**	Head lowered with the ears pinned, neck stretched or extended toward the target horse, and, often, the lips pursed. The pointing extension of the head and neck may be interrupted with momentary tossing, rotating gestures of the head.	Wells and Von Goldschmidt-Rothschild, 1979; Bannikov, 1971; Boyd and Houpt, 1994; Moehlman, 1974; 1998; McCort,1980; Waring, 1983; Feh, 1988; Berger, 1986.
Stomp	**Event**	Raising and lowering of a foreleg to strike the ground sharply, usually repeatedly. Stomping differs from pawing in that stomping is a vertical rather than horizontal movement of the leg.	Klingel, 1967; Welsh, 1975; Waring, 1983; Bannikov, 1971; Boyd and Houpt, 1994; Moehlman, 1974; 1998; McCort,1980; Berger, 1986
Posturing	**State**	Pre-fight head-bowing, prancing, stomping, olfactory investigation, as well as a stiffening of the entire body, including forelegs. The arched neck threat is also a major component, being held throughout most of the interaction. The participants may rub and push against one another.	Welsh, 1975;Boyd and Houpt, 1994; Moehlman, 1974; 1998; McCort, 1980; Feist, 1971; Berger, 1986; Waring, 1983; Penzhorn, 1984
Parallel Prance	**State**	Two horses, moving forward beside one another, shoulder-to shoulder with arched necks and heads held high and ears forward, typically in a high-stepping, slow-cadenced trot (passage, in dressage terminology). Rhythmic snorts may accompany each stride. Parallel prancing often immediately precedes aggressive encounters. Solitary prancing also occurs.	Boyd and Houpt, 1994; Feist, 1971; Berger, 1986; Waring, 1983
Lunge	**Event**	Swift forward thrust of the body from the rear position or charge from close range toward another horse (usually toward his fore body), most often displayed concurrently with a bite threat, with ears pinned.	Schilder, 1988; McCort, 1980; Moehlman, 1974; 1998; McDonnell and Haviland, 1995; Penzhorn, 1984
Balk	**Event**	Abrupt halt or reversal of direction with movement of the head and neck in a rapid, sweeping, dorsolateral motion away from an apparent threat while the hind legs remain stationary. The forelegs may simultaneously lift off the ground. Typically associated with an approach or lunge of another horse.	McCort, 1980; Moehlman, 1974; 1998; McDonnell and Haviland, 1995; Penzhorn, 1984
Head Bump	**Event**	A rapid lateral toss of the head that forcefully contacts the head and neck of another horse. Usually the eyes remain closed and the ears forward.	McCort, 1980; Moehlman, 1974; Penzhorn, 1984; Syme and Syme, 1979; Feist, 1971
Push	**State**	Pressing of the head, neck, shoulder, chest, body, or rump against another in an apparent attempt to displace or pin the target horse against an object.	Waring, 1983; McCort, 1980; Moehlman, 1974; Feist, 1971; Schilder, 1988
Bite Threat	**Event**	Similar to bite except no contact is made. The neck is stretched and ears pinned back as the head gestures toward the target. The miss appears deliberate as opposed to accidental or	Waring, 1983; Feh, 1988; Hogan et al., 1988; Boyd and Houpt, 1994; McCort, 1980; Moehlman, 1974;1998;

		successfully evaded by the target, thus giving the appearance of a warning to maintain distance. Bite threats are typically directed toward the target animal's head, shoulder, chest, or legs and may be performed during an aggressive forward movement such as a lunge, or toward the hind end of an opponent being chased or herded.	Bannikov, 1971; Schilder et al.,1984
Bite	**Event**	Opening and rapid closing of the jaws with the teeth grasping the flesh of another horse. The ears are pinned and lips retracted.	Feh, 1988; Hogan et al., 1988; Boyd and Houpt, 1994; McCort, 1980; Moehlman, 1974;1998; Bannikov, 1971; Welsh, 1975; Feist, 1971
Strike Threat	**Event**	A strike motion that appears deliberately abbreviated or gestured so as to miss contacting the opponent. Often a part of ritualized interactions between horses and frequently accompanied by a loud squeal or snort.	Klingel, 1967; Boyd and Houpt, 1994; Waring, 1983; Keiper, 1988; Penzhorn, 1984
Strike	**Event**	One or both forelegs rapidly extended forward to contact another horse, while the hind legs remain in place. The strike is typically associated with arched neck threat and posturing. A horse may also strike when rearing. The strike is often accompanied by a squeal or snort.	Klingel, 1967; Boyd and Houpt, 1994; Waring, 1983; Keiper, 1988;
Kick Threat	**Event**	Similar to a kick, but without sufficient extension or force to make contact with the target horse. The hind leg(s) lifts slightly off the ground and under the body in tense "readiness," usually with no subsequent backward extension of the hind leg(s). The horse may turn his rump and may back up toward the target. The tail may lash in accompaniment and/ or he may vocalize a harsh squeal. This action is often indistinguishable from the preparation for an actual kick.	Waring, 1983; Feh, 1988; McCort, 1980; Moehlman, 1974;1998; Bannikov, 1971; Berger, 1986; Keiper, 1988; Boyd and Houpt, 1994; Schilder, 1990; Penzhorn, 1984
Kick	**Event**	One or both hind legs lift off the ground and rapidly extend backward toward another horse, with apparent intent to make contact (in contrast to the kick threat described on page 137). The forelegs support the weight of the body and the neck is often lowered. It is common for two horses to simultaneously kick at each other's hindquarters, often associated with pushing each other's hindquarters.	Feh, 1988; Hogan et al., 1988; Boyd and Houpt, 1994; McCort, 1980; Moehlman, 1974;1998; Bannikov, 1971; Welsh, 1975; Berger, 1986; Schilder, 1990
Neck Wrestle	**Event**	Sparring with the head and neck. One or both partners may remain standing, drop to one or both knees, or raise the forelegs during a bout.	Feist, 1971; Tyler, 1972; Blakeslee, 1974; Keiper, 1985; Berger, 1986; Moehlman, 1974;1998; Penzhorn, 1984

Rear	**Event**	Raising of the forelegs into the air, supporting the body on the hind legs only, resulting in a near-vertical position (see also boxing, dancing). Two horses will rear in close proximity and while vertical attempt to bite one another on the head and neck or strike with the forelegs. The forelegs are typically flexed at the knee and fetlock in a more or less tucked position.	Boyd and Houpt, 1994; Berger, 1986; Moehlman, 1974;1998; McCort, 1980
Dancing	**State**	Two horses rearing and interlocking the forelegs and shuffling the hind legs, while biting or threatening to bite one another's neck and head.	McCort, 1980; Tembrock, 1968; Waring, 1983; Penzhorn, 1984
Boxing	**State**	Two horses in close proximity simultaneously rearing and striking out toward one another with alternate forelegs.	Waring, 1983; Penzhorn, 1984
Chase	**State**	Pursuit of another horse, usually at a gallop in an apparent attempt to overtake, direct the movement of, or catch up with the pursued horse. The pursuing horse typically pins the ears, exposes the teeth, and bites at the pursued horse's rump and tail. The horse being chased may kick out defensively with both hind legs. Chasing is usually a part of fight sequences.	Feh, 1988; Waring, 1983; Hogan et al., 1988; Boyd and Houpt, 1994; Keiper, 1985; McCort, 1980; Moehlman, 1974;1998; Bannikov, 1971; Welsh, 1975; Berger, 1986; Schilder, 1988
Herding	**State**	Combination of head threat and ears laid back with forward locomotion, apparently directing the movement of another horse(s).	Welsh, 1975; Feist, 1971; Miller, 1981; Boyd and Houpt, 1994; Keiper, 1985; Waring, 1983; Penzhorn, 1984
Pawing	**Event**	One foreleg is lifted from the ground slightly, then extended quickly in a forward direction, followed by movement backward dragging the toe against the ground in a digging motion. Most commonly, this action is repeated several times in succession. The horse's nose may be oriented toward the substrate at which he is pawing or, if the activity is exhibited as a direct apparent threat to another horse, the head will remain elevated and the neck arched. Pawing is frequently seen in aggressive encounters. It is also seen near fecal piles or dusty rolling sites, either as a solitary activity or during pair or group interactive encounters.	Feist, 1971; Odberg, 1973; Waring, 1983; Hoffmann, 1985; Keiper, 1985; Moehlman, 1974;1998
		Reproductive behavior	
Erection	**Event**	Extended, tumescent penis. Observed during mild to moderately intense aggressive encounters. Bachelors will mount one another with an erection, and anal insertion has been observed.	Bannikov, 1971; McCort, 1980; Moehlman, 1974;1998; Waring, 1983

Flehmen	**Event**	Head elevated and neck extended, with the eyes rolled back, the ears rotated to the side, and the upper lip everted exposing the upper incisors and adjacent gums while drawing air and fluids through the teeth. The head may roll to one side or from side to side. Typically occurs in association with olfactory investigation of urine or feces.	Schneider, 1930; Stahlbaum and Houpt, 1989; Feist, 1971; Tyler, 1972; Waring, 1983; McCort, 1980; Boyd and Houpt, 1994; Penzhorn, 1984
Elimination marking sequence	**Event**	A sequence in which an animal approaches and sniffs voided urine or feces, performs the Flehmen response, covers the urine or feces with urine and/or feces, and then sniffs again.	McDonnell, 2003
Distant Approach	**Event**	Approaching a female from a distance, usually vocalizing with a long, loud whinny. Most commonly seen in a bachelor to an approaching young mare, or occasionally in a harem horse upon returning to his harem after expelling an intruder to some distance away from the harem mares.	Feist, 1971; Keiper, 1985 Waring, 1983; Moehlman, 1974;1998
Head-to-Head Approach	**Event**	Horse and mare facing one another as they come into close proximity for precopulatory or copulatory interaction.	Henry et al., 1991; Feist, 1971; Keiper, 1985; Waring, 1983; Penzhorn, 1984
Head-to-rear Approach (receptive Mare)	**Event**	Horse and mare initiate precopulatory interaction by the horse approaching the mare from the rear. Mare typically turns head to face horse as he approaches	Henry et al., 1991; Feist, 1971; Keiper, 1985; Waring, 1983; McCort, 1980; Penzhorn, 1984
Teasing Sequence	**State**	Precopulatory investigation of the female by the male and sometimes of the male by the female. Sniff Tail; Sniff Shoulder; Sniff Flank; Flehmen Response.	Henry et al., 1991; Feist, 1971; Keiper, 1985; Waring, 1983; McCort, 1980;1984; Boyd and Houpt, 1994; Penzhorn, 1984.
Chin rest and Chest Bump	**State**	During precopulatory interaction, often immediately before mounting, the horse stands behind the mare with his chest against the hindquarters and his chin resting or pressing along the dorsal midline of the mare.	Henry et al., 1991; Feist, 1971; Keiper, 1985; Waring, 1983; McCort, 1980; Boyd and Houpt, 1994; Penzhorn, 1984
Mount Without erection	**State**	Without a penile erection, a horse raises his forelegs and fore body to rest the ventral abdomen and chest upon the hindquarters of the mare, grasping around the body of the mare with the forelegs and sometimes grasping onto the mane and crest of the mare's neck with the teeth.	Klingel, 1998; Henry et al., 1991; Keiper, 1985; Waring, 1983; McCort, 1980; Penzhorn, 1984
Penis drop	**Event**	Relaxation and protrusion of the penis from the prepuce.	Henry et al., 1991; Feist, 1971; Keiper, 1985; Waring, 1983; Penzhorn, 1984

Lean	**Event**	During precopulatory interaction, a horse and mare push against one another. Typically, the horse pushes with his chest and shoulder and the mare with her shoulder and flank.	Henry et al., 1991; Feist, 1971; McCort, 1980; Boyd and Houpt, 1994; Penzhorn, 1984
Lateral Mount	**Event**	A horse's lifting of his forelegs and fore body upon the mare with his legs and chest across the long axis of her back.	Henry et al., 1991; Feist, 1971; McCort, 1980; Keiper, 1985
Roll near Mare	**Event**	A horse's rolling sequence when tending a mare in estrus, either before or after precopulatory and copulatory interaction. Elimination marking behavior may accompany the rolling sequence.	Henry et al., 1991
Non-receptive Female responses	**State**	Any of a number of mildly to strongly aggressive threats or avoidance behaviors displayed by mares to precopulatory interactions of a horse. (ex. Kick Threat and Lunge, Strike, Strike threat).	Klingel, 1998; Henry et al., 1991; Feist, 1971; Keiper, 1985; Waring, 1983; McCort, 1980; McCort, 1980; Moehlman, 1974;1998; Penzhorn, 1984
Receptive Female responses	**State**	Backing into Horse; Mare Winking and "Breaking Down"; Saw Horse Stance with Stand for Mounting; Head Turned Back.	McDonnell, 2003
Copulatory Sequence Mount with erection and insertion	**State**	Standing squarely behind or with the fore body slightly to one side of the mare, the horse lifts his forelegs and fore body to rest his ventral abdomen and chest upon the back of the mare, grasps around the body of the mare with the forelegs and sometimes grasps onto the mane and crest of the mare's neck with the teeth.	Klingel, 1998; Henry et al., 1991; Feist, 1971; Keiper, 1985; Waring, 1983; McCort, 1980; McCort, 1980; Moehlman, 1974;1998; Penzhorn, 1984; Boyd
		Comfort behavior	
Licking/ Chewing	**State**	The horse moves their tongue around their mouth and lips or chewing movements with mouth.	Jaeger, 2017
Object Play	**State**	Contact and manipulation of an object. The target object may be either inanimate, such as an environmental object, or animate, such as the mane, tail, or other body part of a herd mate or even of an animal of another species.	McDonnell, 2003
Play Sexual Behavior	**State**	It is conspicuous and frequent in foals and young adolescents of both sexes and among young and adult bachelor stallions. The elements of the precopulatory and copulatory sequences may be out of order or exaggerated from that of a mature adult in a breeding context.	McDonnell, 2003
Locomotor Play	**State**	Any play behavior response or sequence that is performed while in motion at any gait. It can occur either in a solitary or social context.	McDonnell, 2003

Play Fighting	**State**	It involves behavioral elements and sequences similar to serious adult fighting behavior but with more of a sporting character than serious fighting. In contrast to serious fights, the cohorts appear to alternate offensive and defensive roles, spar on as if to "keep the game going," and stop short of injury. Initiation of play fighting has been observed to follow the head threat tossing motion in which the head is rapidly flipped up and down either while the animal is standing still or moving. Most play fighting occurs in a social context. However, certain elements such as stamp and rear occur also within solitary play bouts of young foals.	McDonnell, 2003
Masturbation **Spontaneous Erection and Penile Movements**	**Event**	Penile erection with rhythmic drawing of the penis against the abdomen, with or without pelvic thrusting.	Henry et al., 1991; Feist, 1971; Tyler, 1972; Waring, 1983; Keiper, 1985; Boyd and Houpt 1994; Moehlman, 1974; 1998; McCort,1980; Gardner, 1983; Penzhorn,1984

Anomalous behaviors

Separation Anxiety	**State**	When separated from herd mates, frantic locomotor behavior, typically with long, loud whinny vocalizations. Pacing or frantic running may focus at the gate or fence line barrier, thwarting the animal from re-joining herd mates.	Boyd and Houpt, 1994
Tongue Displacement	**Event**	Tongue hanging far out of the mouth, usually to the side where it dangles loosely, sometimes moving from one side to the other. This tongue hanging can occur when under bit or at rest in stall or pasture.	Waring, 1983; Fraser, 1992
Self-mutilation	**Event**	Flank-biting; Shoulder-biting Kicking; Chest-biting. Repeated self-injury by biting, kicking, or lunging into objects.	McDonnell, 1986; Houpt, 1994; Fraser, 1992
Non-nutritive Sucking	**Event**	Sucking on own or herd mate's body as if sucking the dam's udder. Common self-targets are abdominal coat, prepuce, penis, hind leg. Herd mate targets also include ears, tail, and immature udder.	Boyd and Houpt, 1994; S. McDonnell unpublished Observations
Coprophagy in Adults	**State**	Ingestion of feces. Normal in foals but not in adults.	Feist and McCullough, 1976; Waring, 1983; Boyd and Houpt, 1994.
Wood Chewing	**State**	Chewing and/or ingesting wooden objects such as fences or stall construction materials.	Waring, 1983; ; Boyd and Houpt, 1994; Boyd, 1991;
Trichophagia	**State**	Ingestion of hair.	Waring, 1983

Wall or Fence Kicking	**Event**	Deliberate extension of the hind leg to contact barriers, using one or both hind legs alternately or simultaneously.	Waring, 1983; Fraser, 1992; Houpt, 1994
		Oral stereotypies	
Cribbing	**State**	Unique equine aberrant behavior involving grasping of surface with incisors while arching the neck and drawing a gulp of air into the throat and then expelling it, repeated rhythmically in bouts typically lasting from minutes to as long as an hour.	Waring, 1983; McBane, 1987; Fraser, 1992; Boyd and Houpt, 1994
Tongue Lolly	**State**	Extraneous moving of the tongue in and out of mouth.	S. McDonnell unpublished Observations
Tongue Sucking	**State**	Sucking the tongue as a foal would suck a teat, typically with ears back and the neck and head extended level with the body or with the neck down and the head curved upward, typical of a foal reaching for the udder.	S McDonnell unpublished Observations
Lip smacking	**State**	Rhythmical smacking action of the lower lip toward the upper lip.	Houpt, 1993
Wind sucking	**State**	The horse opens its mouth, contracts the pharyngeal musculature, flexes the neck muscles and swallows or expels air. Usually, this behaviour is marked by a characteristic noise.	Kennedy *et al.*, 1993
Lip play	**State**	The horse moves its upper lip up and down without making contact with an object, or the horse smacks its lips together.	Fureix *et al.*, 2013
Tongue play	**State**	The horse sticks out its tongue and twists it in the air.	Fureix et al., 2013
Lip or teeth rubbing	**State**	The horse rubs its upper lip or its upper teeth repetitively against the box wall.	Fureix et al., 2013
Repetitive licking/ biting	**State**	The horse licks or bites the box walls, box grids or external part of the feeding trough.	Fureix et al., 2013
		Locomotor stereotypies	
Perimeter Walking, Pacing, Circling	**State**	Stylized repetitious locomotion at any gait, usually along aperimeter.	Waring, 1983; Prince, 1987; Fraser, 1992; Boyd and Houpt, 1994; S. McDonnell unpublished Observations
Weaving	**State**	Abbreviated pacing involving rhythmic, repeated side-to-side shifting of the weight on the forelegs. The vigor and speed of movement vary among individuals and within	Waring, 1983; Fraser, 1992; Boyd and Houpt, 1994.

		or between episodes from slow to frenetic. The front feet may remain “planted” in position, or in an extreme form, the horse “throws” its fore body from one side to the other of a doorway or narrow stall, sometimes contacting the walls with each motion.	
Stereotypic Pawing	**State**	Repetitive, seemingly aimless rhythmic dragging of a hoof in the same action as normal pawing, as is used in uncovering vegetation.	KA Houpt personal communication; Waring, 1983; Fraser, 1992; McDonnell unpublished Observations.
Stereotypic Stomping	**State**	Lifting and lowering of a hind leg as if deliberately striking the substrate, using one or both hind legs one at a time.	Waring, 1983; Fraser, 1992
Head Shaking, Bobbing, Tossing, **Nodding (Stereotypic)**	**State**	Repeated, rhythmic head movements.	Waring, 1983; McBane, 1987; Fraser, 1992.
Door kicking	**State**	The horse kicks the door of the box repeatedly with its forelegs.	Fureix *et al.*, 2013

PAPER 4

Is the judgment bias test a good tool to assess the quality of horse management?

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Is the judgment bias test a good tool to assess the quality of horse management?

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ABSTRACT

Animal welfare should include the possibility of animals experiencing positive emotions. Emotions influence the cognitive process, and judgment bias tests (JBTs) are employed in different species, to assess the optimistic or pessimistic expectation of an individual and its affective state. Only recently the JBTs have been applied to horses. This research aims to investigate the relationship between a spatial JBT and hypothalamic-pituitary-adrenal axis (HPA) chronic and acute activation in forty-one animals hosted in different kinds of environments: traditional stables (TS), natural boarding (NB), and ethological stable (ES). Fecal (FC) and horsehair (HC) cortisol concentrations were quantified for each subject through Radio-Immuno-Assay (RIA). Body condition score (BCS), as an indirect index of animal motivation towards food, and personality traits were measured to explore their possible influence on JBT results. Horses had to distinguish a positive position (P), where a bucket full of food was positioned, from a negative one (N), with an empty bucket. Then, 3 intermediate positions (Near Negative-NN, Near positive-NP, and Medium-M) with an empty bucket were presented to the subject one at a time. Only 20 subjects out of 41 completed the JBT and were included in the statistical analysis, and both BCS and P position, whether at the right or the left of the subject, seems to have influenced the inclusion rate. Only the ES group registered a significantly lower score in NN, suggesting a more optimistic affective state, whereas NB and TS did not significantly differ in their responses. Despite this, horses from NB recorded higher FC concentration than TS subjects during all the phases of the test, but lower HC levels, which could suggest a generally lower level of chronic stress but its interpretation presents several confounding factors. These results put into question whether JBT is indeed a good test to monitor the quality of the management, as it does not seem to reflect the chronic physiological state of the animals and could be influenced by a state of acute stress, caused by the test procedure. Due to these confounding factors, this procedure should be accompanied by other indicators. Finally, to include more animals and exclude possible biases, the structure of the JBT and the employment of food as a reward should be evaluated considering the peculiarities of the species and individual motivations.

Introduction

The consideration of emotional aspects in animal welfare science has increasingly gained importance (Broom, 2003). According to the 5 Domains Model, animal management should reduce situations that negatively affect animals and promote the experience of positive emotions (Mellor and Beausoleil, 2015; Mellor et al., 2020). However, the investigation of welfare is often focused on external circumstances, while affective states are difficult to explore (Duncan, 2006; Mellor et al., 2020).

Cortisol is commonly employed in animal welfare studies as a physiological indicator of stress. Nevertheless, the increase of this glucocorticoid can occur as a normal non-stress-physiological

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response or can be caused by both negative and positive situations, such as pain, anxiety, and fear as well as excitement or pleasure (Koolhaas et al., 2011; Ralph and Tillbrook, 2016). Therefore, it is always important to contextualize and try to understand what potentially has caused the activation of the hypothalamic-pituitary-adrenal axis (HPA), which leads to the increase of cortisol (Ralph and Tillbrook, 2016). Feces and horsehair are used as non-invasive methods for the determination of cortisol concentrations (Palme, 2012; Duran et al., 2017). While feces evidence the increase of plasmatic cortisol according to the animals' metabolism (in horses after about 24 hours) (Mostl et al., 1999), horsehair reflects the individual history, reflecting chronic stress conditions (Duran et al., 2017).

However, stress and, consequently, cortisol levels can be influenced by both emotional and physical stressors. Therefore, to investigate emotions in non-vocal animals, research relies on indirect indications of how the animal perceives the environment, considering that emotional states can influence memory, attention, and judgment (Duncan, 2006; Mendl et al., 2009). In particular, Judgment bias tests (JBTs), modulated by human sciences (Bethell, 2015), have been employed in animal research to investigate how the environment or routine procedures affect animals' affective states (Burman et al., 2009, Neave et al., 2013; Daros et al., 2014). In animal research, judgment bias represents the animal's response to a neutral stimulus and, evaluating if the individual expects from this stimulus either a positive or a negative outcome, it defines the subject as optimistic or pessimistic (Mendl et al., 2009).

During recent times, the JBT has been applied in horses (Freymond et al., 2014; Löckener et al., 2016). Domestic horses' welfare and mental health depend on both their relationship with human beings and their management (Hausberger et al., 2009; Freymond et al., 2014). Methods of horses' management are varied and differ in terms of freedom of movement, the possibility of interaction with conspecifics, and the kind of feeding and its routine. In some countries, domestic horses are traditionally housed in individual stalls, where they spend most of their time (Yarnell et al., 2015). This kind of management is considered practical for humans because, for example, it is easier to detect any diseases, control the consumption of water and food, and manage the horse for various routine operations (McGreevy, 2012; Ruet et al., 2019). The natural boarding system of the horse tries to reproduce the living conditions of feral equines. Horses usually live in a group in paddocks designed to stimulate movement, exploration, relationship with conspecifics, and body care (McIlwraith and Rollin, 2011; Saba et al., 2013). Finally, in Italy, there is a new kind of facility, called Ethological Stable, that has intermediate characteristics of the traditional stable and natural boarding system, and it's characterized by bigger stalls than traditional ones and separated by grates with windows, which allow the horses to see and touch each other (Marliani et al., 2021). Whichever management method is considered, it has a profound effect on the welfare of the individual (Yarnell et al., 2015; McGreevy, 2012; Ruet et al., 2019), and JBT could be helpful for the assessment of the welfare impacts of different housing systems, but its application in horses requires further investigation.

This research evaluated horses under 3 different management systems: traditional stable, natural boarding, and ethological stable. Each subject underwent a JBT, fecal and horsehair sampling, and an evaluation of personality traits and Body Condition Score (BCS). We tested if the type of management can influence horses' judgment bias. We also investigated the influence of other independent variables (personality, age, and BCS) on judgment bias, and we examined if JBT results correlated to cortisol levels found in feces and horsehair.

Material and Methods

Stables

Natural Boarding System (NB)

This system hosted 21 horses, living 24/7 together in a large paddock of about 6 ha. The paddock was composed of heterogeneous spaces with large areas and corridors, huts and muddy and sandy soil areas (according to season). Trunks and objects, where animals can rub, were scattered within the paddock, to stimulate the movement of animals, and the entire area was arranged in the shape of a labyrinth. Hay and water were available *ad libitum* and horses were occasionally allowed to access grassy lawns. This system was frequented daily by at least 1 person, from a few hours to the entire day. Most of the horses were used for occasional horse trekking and horse-riding activities in the sandy field (maximum 2 hr/day) by riders of varying ages and experience. Only 2 of the horses included in the statistical analysis were not involved in these activities. Horses were barefoot and ridden without a bit.

Traditional Stable (TS)

This facility housed 55 horses during the study. The stable was organized in 2 parallel corridors that had separate rows of individual stalls (3.00 m x 3.50 m). Each stall had wood chip bedding, a sliding door and a window that allowed the horse to look out into the corridor, facilitating visual contact with the other horses in the stable. The facility had individual paddocks (10.00 m x 10.00 m; or 20.00 m x 20.00 m) where horses were pastured for a different lag of time that varied depending on weather conditions and the owner's decisions, not every day and only during the daytime. Hay was provided at 7.00 am and 4.00 pm and a supply of mixed grains were provided at 8.00 am and 8.00 pm. Most of the horses housed in this stable were involved in sporting activities, in particular jumping, dressage, and vaulting with beginners and competitors. The stable was frequented every day and had a carousel, a round pen, and some sand courts.

Ethological Stable (ES)

The system was the same as described by Marliani et al., 2021. During the study, the stable hosted 11 horses. The stable included 12 stalls, called Big Box© (8.00 m x 5.00 m), arranged in rows of 6. Each stall was divided into 2 sections of $20m^2$: the front part consisted of high-density impact rubber mats that provided the horse with rigid support; the rear part consisted of a soft and semi-permanent element of river sand. This system of double litter allowed the horse freedom of movement and choice of the ground on which to rest. The walls of the Big Box© were of metal gratings, with windows between stalls, so that subjects were able to see and touch each other. There was a paddock of 1.50ha organized with large areas and corridors, and 6 paddocks of 2.00ha each. Horses, individually or in a small group, stayed in paddocks all day and were brought into the stall for the evening, or in case of bad weather. Hay was available throughout the day and cereals (barley, oats, and corn), apples, and carrots were provided at 7.00 a.m. and 7.00 p.m., according to a personalized food plan. There were also a round sand pen and a covered sand rectangular field. Horses were engaged principally in flatwork, country riding, and walks, and they were not used for competitions.

Subjects

Forty-one adult horses were involved in the study: 16 subjects from NB, 14 horses from TS, and 11 subjects from ES (Table 1). All horses had been resident in each stable for at least the last 3

Table 1
The number of total mares, geldings, and stallions for each stable and mean age ± standard deviation of each group considered for the study. In brackets the number of mares, gelding and stallions and mean age ± standard deviation of horses that completed the test.

	Natural Boarding	Traditional Stable	Ethological Stable
Mares	7 (4)	4 (0)	5 (1)
Geldings	9 (6)	9 (6)	6 (3)
Stallions	-	1 (0)	-
Age (years)	14 ± 6 (12± 6)	18 ± 5 (17± 4)	18 ± 6 (19± 3)

months. The history of each subject and the characteristics of the stables were collected using 2 questionnaires.

Body Condition Score (BCS) and Personality Evaluation

An evaluation of BCS was made for every horse. This is a nutrition assessment system, in which a score is assigned based on the visual (rib and bone prominence) and manual evaluation of the panniculus adipose in 6 main regions: neck, shoulder, withers, thorax, lumbar region, and tail attachment. First of all, the amount of fat present is visually evaluated, assessing the visibility or not of bone and rib prominences. The score is from 1 to 9, where 1 corresponds to excessive emaciation and 9 is a condition of excessive nutrition (Henneke et al., 1983). The BCS was collected and considered in the statistical analysis for all the horses, except for 3 (2 NB horses who suffered from Cushing and 1 ES individual).

To assess the personality of the subjects involved in the study, a translated version of the HPQ (Horse Personality Questionnaire) (Lloyd et al., 2007) was used. The HPQ was completed by the horse's owner or caregiver. For each of the 25 items, they were asked to assign a score on a Likert scale from 1 to 7, where 1 indicates that the trait was not at all manifested, while 7 corresponds to the extreme expression of the character trait. The data were analyzed according to Lloyd et al. (2007), obtaining a score for each of the 6 personality components (Dominance, Anxiousness, Excitability, Protection, Sociability, and Inquisitiveness).

Judgment Bias Test

The protocol employed in the study was a modification of those proposed by Freymond et al. (2014) and Henry et al. (2017). It was a Go/No-Go JBT with spatial stimuli. Black buckets were employed for the test, to prevent the horses seeing the contents. In each stable, the test was conducted in a fenced arena with a sandy substrate, familiar to the subjects and sufficiently far from disturbances.

In the arena, a start-line and a semicircular stop-line were drawn on the ground using the wood-chip, to allow the experimenter to see better when the horses passed the line to stop the stopwatch. The test was carried out by 2 trained experimenters. Experimenter 1 (E1) was in charge of preparing the bucket and placing it behind the finish line, 9 meters from the start line (Figure 1). Experimenter 2 (E2) had the task of leading the horse into the test arena and placing it behind the start line. Only when E1 had placed the bucket, the E2 release the horse and take a step to the side. Once the horse finished the trial, E2 retrieved the horse and brought it back to the start line. The trial was completed when the horse passed the finish line with one of the forelegs, or after 60 seconds, which was the maximum latency considered. E2 measured the time taken by the horse to reach and pass the stop line. While the horse completed the trial, the experimenters remained stationary and directed their gaze towards the ground to avoid influencing the behavior of the horse. The experimenter who led the horse was always the same for the same horse to get the horse used to the same person and minimize biases due to the possible stress caused by different experimenters. The JBT was divided into 4 consecutive days: habituation, training, and 2 sessions of the test.

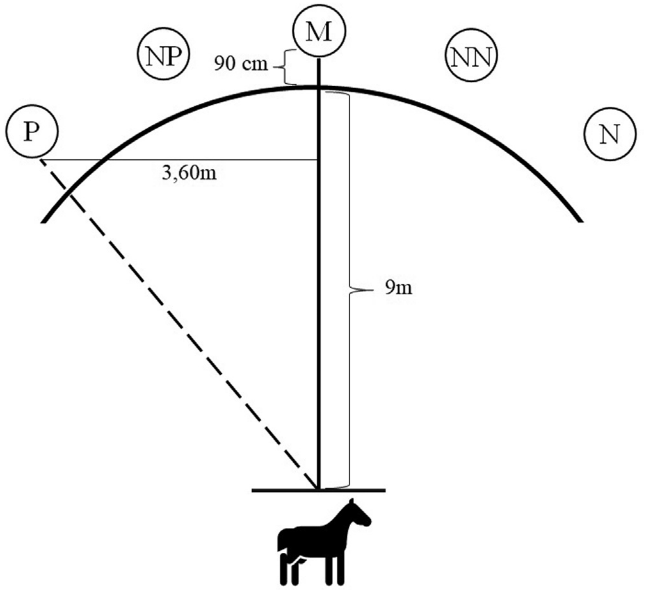

Figure 1. Representation of the test arena. P = Positive location; NP = Near Positive intermediate location; M = Medium intermediate location; NN = Near Negative intermediate location; N = Negative location. P and N were randomly on the left side or the right side of the horse.

Habituation

The first day was employed for the phase of habituation, during which horses got used to the setting. During this phase, 1 horse at a time was led into the arena by an experimenter, then the second experimenter positioned the bucket with a reward, which consisted of a small amount of oats, at the positive position (P). Oats was chosen as a reward because usually horses are highly motivated by them. The habituation was completed when horses, released at the start line, independently approached the bucket 3 consecutive times. The horses were randomly allocated to either the left or right position of the test apparatus.

Training

For the training phase the negative position (N), corresponding to an empty bucket, was set at the opposite in respect of the positive (P), 7.20 meters apart. The 2 positions were presented 1 at a time, and the same position was presented no more than twice in a row. The training always finished with P. E2 recorded the latency of each subject to reach and pass the finish line. If the horse didn't move towards the bucket in 60 seconds, E1 went towards the horse, took it, and led it again to the start position and the trial was considered completed. The training ended when the latencies to reach the last 3 positive buckets were lower than that to reach the last 3 negative ones (Mendl et al., 2010). Following the protocol suggested by Henry et al. (2017), each training session was composed of 3 positive positions and 2 negative locations, and finished always with the positive one. The minimum number of sessions employed was two. If the horse failed to distinguish the 2 positions after 5 training sessions, it was excluded from the JBT.

Testing

The day after the training, the testing phase was performed in 2 consecutive days. During the test, 3 intermediate positions, 1 at a time, between N and P were presented: NN (Near-Negative), M (Middle), and NP (Near-Positive). These were placed 1.80 meter from each other and the bucket was always empty. Before the beginning of the test, N and P were presented as a reminder of the training, a maximum of 6 times. Each session followed the scheme proposed by Henry et al. (2017), where ambiguous locations were preceded alternately by positive and negative locations:

- first session (first day testing phase) P-N-NP-P-N-M-P-N-NN-P
- second session (second day of testing phase) N-P-M-N-P-NN-N-P-NP

The second session always ended with an N position where the bucket was full of food, to exclude horses employed their sense of smell during the test (Mendl et al., 2010).

The test sessions were videotaped using a Sony HDR-CX240E camera placed on a tripod outside the arena. All videos were analyzed using the software Solomon Coder, to record precisely the time that horses employed to reach buckets.

To avoid biases caused by differences in baseline running speeds, due to size and/or age of individuals, raw latencies recorded to reach the intermediate positions were transformed into scores, according to the formula proposed by Mendl et al. (2010)

$$\text{Adjusted lateny} = \left(\frac{\text{mean latency to ambiguous location} - \text{mean latency to P}}{\text{mean latency to N} - \text{mean latency to P}}\right)$$

This formula returns 0 for the P and 100 for N.

Collection of Fecal and Horsehair Samples

Fecal samples were collected immediately after defecation, for a total of 4 samples for each horse that completed the test. The collection was made during:

1. the habituation (P1), which was representative of the baseline cortisol concentration before the beginning of the whole procedure;
2. the training (P2), which was representative of cortisol level during habituation;
3. the first session of the test (P3), which was representative of training;
4. the second session of the test (P4), representative of the first day of the test.

A total of 86 fecal samples were collected. Some samples were missing for the NB group. One horse was excluded because he suffered from Cushing disease, which alters cortisol concentration, and some could not be collected for other reasons (1 sample during the habituation; 4 samples during the training, and 1 sample for the first session of the test). Each fecal sample was placed in a non-sterile container identified with date, time, subject name, refrigerated to +4°C, transported to the laboratory and frozen to –20°C.

Horsehair samples were collected from the base of the mane of each horse who completed the JBT and stored at room temperature in identified non-sterile containers, for a total of 20 samples. The sample of the horse who suffered from Cushing was excluded from the statistical analysis.

Cortisol Assay

Cortisol concentrations were determined by Radio Immunoassays (RIAs). Cortisol was extracted from fecal specimens (500 mg, wet weight) with methanol-water solution (v/v 4:1) and ethyl ether, whereas for horsehairs 60 mg of trimmed horsehairs (1-3 mm) of a subject were put in a glass vial with 5 ml methanol (Accorsi et al., 2008). All samples were dried under an air-stream suction hood at 37°C and the dry-residue was dissolved into phosphate-buffered saline (PBS) 0.05 M, pH 7.5. Cortisol metabolites assay in both feces and horsehair were carried out according to Tamanini et al. (1983). The cortisol RIA was performed using an antiserum to cortisol-21-hemisuccinate-BSA (anti-rabbit), at a working dilution of 1:20 000 and 3H-cortisol (30 pg/tube vial) as a tracer. Validation parameters of analysis were: sensitivity 0.19 pg/mg, intra-assay variability 5.9%, inter-assay variability 8.7%. Radioactivity was determined using a liquid scintillation β counter and a linear standard curve, *ad hoc* designed by a software program (Motta and Degli Esposti, 1981).

Statistical Analysis

To analyze the collected data, non-parametric statistical methods were employed, given that the small size of the sample did not guarantee the reliability of the normality assumption. In particular, to assess the presence of differences among the management, the Kruskal-Wallis test with Dunn's adjustment for multiple comparisons was adopted. To test for the presence of correlation between numerical variables, Spearman's correlation coefficient was considered. To assess the influence of the position of nominal and numerical variables on the inclusion or the exclusion of the horses from the test, we employed a Chi-squared test and a Mann-Whitney test. To compare the fecal cortisol concentration during the different phases of the test we employed a Friedman test, with a Wilcoxon pairwise as post-hoc test. Statistical significance was set at $P < 0.05$. The whole statistical analysis was carried out in the R environment (R Core Team, 2021).

Results

Task Acquisition

Of the 41 horses considered, only 20 horses completed the JBT and were considered for the statistical analysis (NB n = 10; ES n = 4; TS n = 6). Eleven horses did not pass the habituation phase (NB n = 6; ES n = 1; TS n = 4) and 4 subjects the training (ES n = 4). Moreover, 6 did not maintain a significantly different latency between P and N during the reminders before the test phase (ES n = 2, TS n = 4). The rate of excluded animals was similar for the 3 types of management.

All the horses included employed the same time to reach N when there was both the empty and the full bucket (last position of the second test phase).

Twenty-two horses had the positive bucket on their left side, 19 on their right. Considering this variable and the entire group in a chi-squared test, there was a significantly higher probability that the horses who had the P location on their left side was excluded from the test ($\chi^2 = 5.47$, df = 1, P-value = 0.02). Indeed, considering the subjects included in the test, 13 had P on their right side and 7 on their left.

The BCS of the excluded horses was significantly (W = 104; r = -0.359; 95%C.I. [-0.614, -0.109]; P-value = 0.02) lower (Median ±IQR: 4 ± 1.5, n = 19) than that of the included subjects (5 ± 2.0, n = 19) (Figure 2).

JBT Scores

Comparing the 3 different stables, scores recorded in NP (χ^2= 4.2957, df = 2, P-value = 0.12) and M (χ^2 = 1, df = 2, P-value = 0.61) did not significantly differ between the groups, but there was

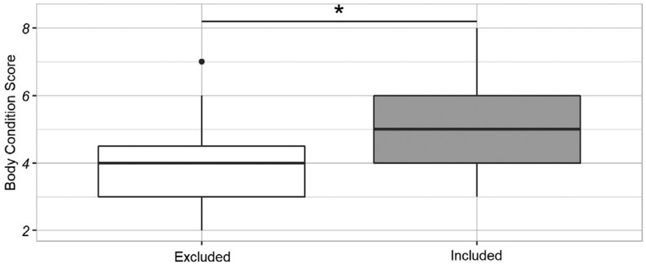

Figure 2. Boxplot of the Body Condition Score (BCS) of excluded and included horses. The box plots show the median and 25th and 75th percentiles; the whiskers indicate the values within 1.5 times the inter-quartile range, IQR. The outlier is indicated by the black circle; P-value < 0.05 is indicated by *.

a significant difference for NN position (χ^2= 8.3082, df = 2, P-value = 0.02). Indeed, the horses of ES had an NN score significantly lower (6.46 ± 5.94) than subjects of NB (100.00 ± 46.50; P-value = 0.01) and than those of TS (72.34 ± 35.75; P-value = 0.04) (Figure 3).

Considering the entire group of horses, the scores recorded in NP (Median ± IQR: 10.24 ± 44.63), M (2.88 ± 17.34), and NN locations (71.09 ± 67.00) were significantly different, according to the Kruskal-Wallis test with Dunn's post-hoc test (χ^2= 18.7236, df = 2, P-value $<$ 0.001). In particular, NN was significantly different from NP (P-value $<$ 0.01) and M (P-value $<$ 0.001), but NP and M did not differ (P-value = 0.34). No correlation was found between the scores recorded for the intermediate positions and personality traits and age, but there was a significant negative correlation between the score in NP and BCS (S = 1907.1, $P < 0.01$, rho = -0.67). Body condition score differs between groups (χ^2= 9.0149, df = 2, P-value = 0.01) and it was significantly higher in NB (6.22 ± 1.72) horses than in TS ones (3.83 ± 0.75, P-value = 0.01), while ES subjects recorded an intermediate value (5 ± 0), and did not differ neither from TS (P-value = 0.14) and NB (P-value = 0.64).

Cortisol Results

Fecal cortisol (FC) baseline level (P1) and its concentration during the habituation phase (P2), training (P3) and the first session of the test (P4) were determined from the fecal samples collected during the 4 days of the JBT. Comparing the FC concentration in P1, P2, P3 and P4, it was not significantly different in both TS groups (χ^2=2, df = 3, P-value = 0.57) and ES (χ^2= 8.1, df = 3, P-value = 0.051)

The FC baseline concentration was not significantly different between the 3 groups of horses (χ^2= 0.56, df = 2, P-value = 0.75). However, during habituation (χ^2= 6.67, df = 2, P-value = 0.04), training (χ^2= 11.59, df = 2, P-value $<$ 0.001) and the first day of the test (χ^2= 11.97, df = 2, P-value $<$ 0.001), subjects from NB registered a significantly higher concentration of FC than the TS group. The ES group showed a significantly higher concentration than TS horses in P3 (P-value = 0.03) and, with a significant tendency, in P4 (P-value = 0.07). (Table 2).

Considering horsehair cortisol (HC), its concentration resulted significantly different in the 3 groups (chi-squared = 13.93, df = 2, P-value $<$ 0.001). It was higher in TS horses than NB individuals (1.03 ± 0.16 pg/mg; 0.21 ± 0.19 pg/mg; $P < 0.001$), while the ES group, which had intermediate values (0.38 ± 0.14 pg/mg), did not differ significantly from either of the others (ES vs. NB, P-value = 0.20; ES vs. TS, P-value = 0.14). In addition, as suggested by Heimbürge et al., 2019, we analyzed the influence of age and sex. We found a significant difference for sex (W = 9, r = -0.54, P-value = 0.01), and female (0.25 ± 0.12) had HC levels than geldings (0.52 ± 0.66). In addition, HC concentration was significantly positively correlated with age (S = 615.39, P-value = 0.047; rho = 0.46), but the groups did not differ significantly for age (χ^2=5.1301, df = 2, P-value = 0.08) . No significant results were found considering the influence of sex (W = 27, P-value = 0.96) and age (S = 1025.2, P-value = 0.82, rho = -0.05) on the fecal cortisol baseline (P1).

Table 2
Results of the Kruskal-Wallis test with Dunn's adjustments for multiple comparisons of fecal cortisol concentration (pg/mg) recorded in each phase of the test, considering a time-lag of 24 hours from their collection. The median ± IQR were reported for each kind of management, and the letters indicate the different groups according to Dunn's test.

Phase	Ethological Stable	Natural Boarding	Traditional Stable
P1 (baseline)	3.04 ± 1.57^{a}	1.71 ± 4.40^{a}	2.33 ± 0.47^{a}
P2 (habituation)	3.06 ± 0.42^{b}	6.80 ± 1.86^{a}	3.26 ± 3.39^{b}
P3 (training)	5.92 ± 2.63^{a}	7.52 ± 5.21^{a}	3.49 ± 1.34^{b}
P4 (test1)	$7.08 \pm 4.22^{a,b}$	9.71 ± 3.12^{a}	2.45 ± 1.74^{b}

a,b = $p<0.05$

Discussion

The concept of animal welfare includes the possibility of animals experiencing positive emotions, whereas negative emotional and experiential states, such as fear, are sufficient elements to compromise it (Dawkins, 2006). The primary function of emotions is to guide individual decisions and they are strictly related to the cognitive process (Paul et al., 2005; Roelofs et al., 2016; Mendl et al., 2009). In particular, the judgment bias indicates how an individual judges a certain situation in dependence of their emotional state (Paul et al., 2005). The evaluation of judgment bias is considered useful for comparing the emotional state of individuals of the same species living in different environmental situations (Bethell, 2015). This research aimed to understand the usefulness of JBT in the evaluation of welfare in horses in different housing and management systems by comparing results between JBT and chronic HPA activation. We also considered other independent variables, such as BCS, age and personality traits, to determine their influence on the outcome of the test.

Only 20 on 41 horses completed all the phases of JBT, which poses serious doubts about the possibility to generalize the results (Roelofs et al., 2016). However, as the study of JBT in horses is relatively recent and the difficulty of this species in learning and memorizing the spatial discrimination between P and N has already been reported (Hintze et al., 2018), we consider this study to be a useful addition to the current literature. The learning processes of the subjects across the trial was not influenced by the type of management, similar to what is reported by Henry et al. (2017). However, we identified 2 other factors that could have influenced the exclusion rate: the position of the positive bucket and the BCS. As for the position of the bucket, we cannot exclude a phenomenon of lateralization. Lateralization in vertebrates is the different specialization of the 2 cerebral hemispheres and it can be observed both in motor and sensorial (olfaction and sight) activities (Vallortigara and Rogers, 2005). Horses have eyes in a lateral position, with decussation of 80-90% of optic fibres (Brooks et al., 1999). Similar to other species, they usually use the right eye, so the left hemisphere, to investigate novel objects, and the left eye to evaluate objects with an emotional negative valence (De Boyer Des Roches et al., 2008). The positioning of the positive bucket on their left side could have negatively influenced the value that the subjects have assigned to the object. Moreover, the positive position was the first and only position presented to the horse during the entire habituation phase. Another factor that seems to affect the inclusion/exclusion rate is the BCS of the horses. Indeed, the

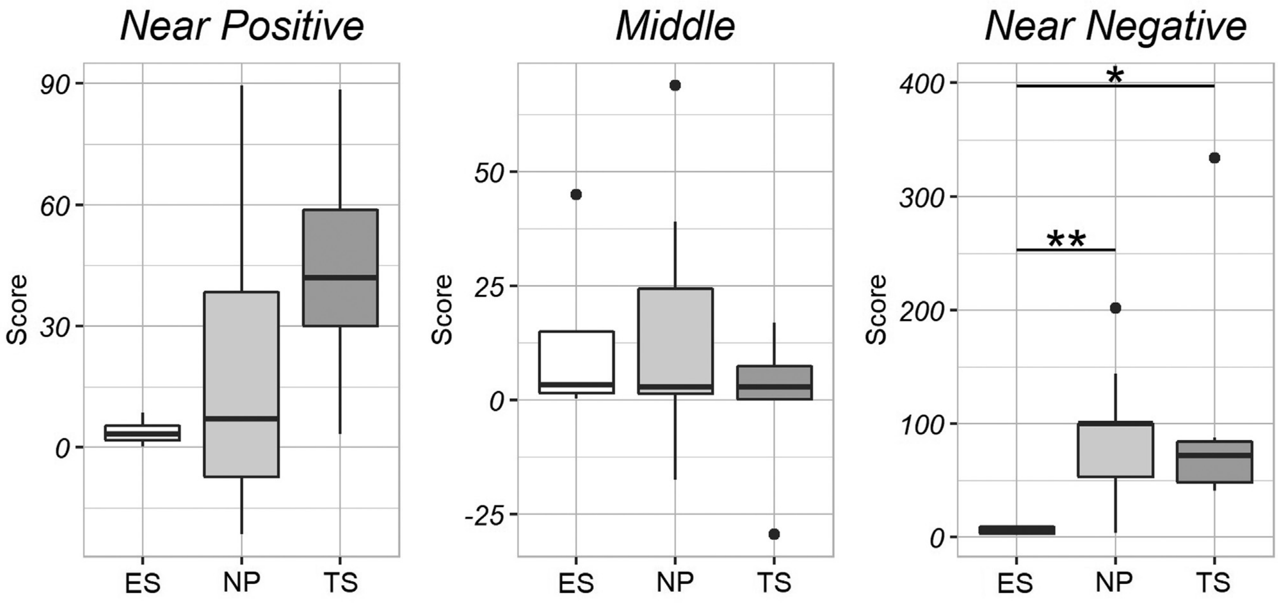

Figure 3. Boxplot of JBT results of each group (ES = ethological stable; NB = natural boarding system; TS = traditional stable). The box plots show the median and 25th and 75th percentiles; the whiskers indicate the values within 1.5 times the inter-quartile range, IQR. The outlier is indicated by the black circle. *P*-value = 0.01is indicated by **, and *P*-value $<$ 0.05 is indicated by *.

excluded subjects have a BCS significantly lower than the included ones. BCS is an indirect measure of the adequate nutritional intake and health state of the individuals, and a potential index for the presence of pathologies. A healthy equid should have a BCS between 4 and 6 (Henneke et al., 1983; Yngvesson et al., 2019) and in this range fall the median values registered in all the 3 stables. However, if we consider the BCS as a potential indicator of motivation towards food (Henry et al., 2017), horses with a lower BCS could be less motivated and this can negatively influence the learning of a discrimination task based on food rewards (McCall, 1989). In addition, we should consider the different management and the different activities the horses are used for. Indeed, in the 3 management we noticed that NB horses had a significant higher BCS than TS. Not only feeding routine, health status and environment can influence BCS, but also the type of exercise (Christie et al., 2006; Zoller et al., 2019). Therefore, the differences that we found in the 3 systems can be also due to the differences of equestrian activities in which horses were involved.

The 20 horses included in the test obtained different scores for each intermediate position, demonstrating that they can differentiate NP, M, and NN. All individuals included showed the same latency to reach N also in the last trial when the bucket was filled, demonstrating that they did not use their sense of smell to deal with the JBT (Mendl et al., 2010).

The first of our predictions was that independent variables, such as body condition score (BCS), age and personality traits, do not influence the JBT results. No correlation was found between JBT scores and both age and personality traits. Regarding personality traits, this result agrees with Lalot et al. (2017) that did not evidence any correlation between personality traits and an optimistic bias in canaries. On the contrary, in another study by Barnard et al. (2018), it was found that dogs with higher sociability and excitability approached faster the ambiguous probes of a spatial JBT than those characterized by high separation-related anxiety and dog-directed fear. Therefore, the relationship between personality and JBT seems to be complex as its outcomes are influenced not only by personality traits but also by transient affective states (Whittaker and Barker, 2020), which are difficult, if not impossible, to describe in a non-vocal species. Moreover, the HPQ was validated in English and we employed a translated version of the HPQ, so we cannot exclude the presence of possible bias due to the use of a different language. Instead, a negative correlation was found between BCS and the score in NP, so the latency to reach the near-positive position was lower for horses with a higher BCS. This result is in agreement with the previous result about the inclusion rate and confirms the fact that a different motivation among individuals can influence the JBT result.

Secondly, we predicted that different management styles would have affected JBT results. Only the ES group showed a significantly lower latency to reach NN, suggesting that those horses had greater anticipation of a positive outcome at that location, and thus experienced a general more positive affective state. NN is usually associated to negative outcome and a slower latency to reach this probe location is usually interpreted as index of fear and anxiety, so of a pessimistic affective state (Barnard et al., 2018). Indeed, our results are similar to those of Karagiannis and colleagues (2015) that found that dogs suffered from separation related problems approach significantly slower NN probes but not the other locations. However, this result should be considered with caution, because only 4 of 11 horses completed the JBT in the ethological stable. Contrary to our predictions, NB and TS did not score differently to reach intermediate positions, although these 2 management systems are extremely different. It cannot be excluded that the manipulation by strangers of NB horses and, overall, the separation from their herd caused a mild state of distress, which could have altered the results of JBT. This hypothesis can be partially confirmed by the analysis of fecal cortisol, which evidenced an increasing level of glucocorticoids in the feces (representative of daily levels of stress) and a significantly higher FC concentration in NB in respect to TS subjects during the JBT procedure. According to Roelofs et al. (2016), involving social animals individually in JBT could increase stress perception. Indeed, it is well known that social isolation induces stress in most gregarious species, manifested through behavioral and physiological changes such as increasing vocalization, locomotion, heart rate, and plasma cortisol levels (Mal et al., 1991; Wolff, Hausberger and Le Scolan, 1997; Lansade and Bouissou, 2008), and the horse is a social animal that forms preferential bonds within the herd to guarantee social stability (Beaver, 2019). Therefore, the procedure itself could have been stressful for NB horses, which are also usually less used to being manipulated by strangers, and this can have determined that they did not differ from the TS group in their latency to reach intermediate positions. All these elements suggest that JBT may not be reliable to evaluate the quality of the management style for these individuals because it is affected by contingent confounding factors.

Finally, we predicted that acute and chronic activation of the HPA axis would be different according to the management system and this would be reflected in the JBT results. As for the acute activation, the fecal cortisol results are partly in accordance with the JBT results, as explained in the previous paragraph. In any case, we recommend collecting fecal samples at least 2 days after the end of the JBT procedure, to check the level of cortisol also during the second day of testing and at the return to the baseline. Fecal cortisol concentrations help us to measure not only cortisol base line, but also animal's stress response, avoiding the invasiveness of blood collection. It reflects an average of the plasma cortisol secreted and metabolized by the animal over the course of a species-specific period (in horses about 24 hours) (Mostl et al., 1999; Sheriff et al., 2011). Therefore, the daily collection of fecal cortisol allowed us to monitor the stress of animals possibly caused by the entire procedure of JBT, considering also that it could be a stressful disruption of their daily routine.

We obtained surprising results when we considered horsehair cortisol concentration, whose results, if matching with those of JBT, would have reinforced the role of JBT as a useful method to assess welfare in managed horses. Indeed, HC concentration indicates the chronic activation of the HPA axis and it is commonly employed to assess the chronic stress conditions in animal welfare studies because the concentration of cortisol in the hair reflects the history of the subject (Prandi et al., 2010). We found that NB horses had significantly lower horsehair cortisol level than TS horses, suggesting a lower activation of the HPA axis in the long term, whereas ES horses recorded intermediate values of HC and did not differ significantly from the others, which is in line with the management style that we could consider "intermediate" between TS and NB. These results are in accordance with Placci et al. (2020), who found a most favorable endocrine framework in horses kept in natural boarding, suggesting that this kind of management style matched most with the ethological and physiological needs of the species. From these results, we would have expected that NB horses also had the best judgment bias, and vice versa for the TS group, whereas NB and TS did not score differently to reach intermediate positions. Therefore, in our study horsehair cortisol did not correlate with JBT results, which is quite surprising given that JBT is considered a helpful method in the assessment of good management. Cortisol level should be discussed considering several limitations. In particular sex, breed and seasons (Aurich et al., 2015; Heimbürge et al., 2019) are the principal influencing factors that was not possible to control in this study, both for the availability of the structures and for the exclusion of most of the horses from the JBT. Analyzing also our statistical results, female and younger animal seems to have a generally lower HC level. This could have influenced the lower HC found in NB group, considering in TS group there were only geldings. Also, the season could be another potential confounding factor. Indeed, NB was tested in spring, while TS and ES in autumn and summer. Mazzola and collegues (2021) found higher cortisol concentration in the hair of horses during summer and autumn, in accordance with Aurich et al. (2015), which found that salivary cortisol level was lower in March and April. In the feces baseline, we did not find any difference between sexes and no correlation with age. Also, the season seemed to not have any influence, because baseline cortisol level did not show any difference among the 3 groups.

Conclusion

This is the first research using JBT in horses which also considers fecal and horsehair cortisol analysis. Considering the endocrinological results, with all their limitation, we cannot exclude that the procedure of the JBT itself can induce acute stress to some subjects and this is a potential confounding factor for the evaluation of the living environment on the immediate affective state of these animals. Moreover, the horsehair cortisol, which reflects the accumulated stress in the long term, did not match JBT results, thus posing even more doubts on the reliability of this test *per se* as applied to the evaluation of management. However, horsehair cortisol evaluation presents several limitations and potential confounding factors. According to our results, the use of the JBT test on horses seems much affected by individual motivation for food, as emerged by the high exclusion rate and by the fact that horses highly motivated for food (and present both in the natural boarding and the traditional stable management) had the best results for NP score, thus reducing the possibility to impartially evaluate the effect of management on the individuals.

In conclusion, we strongly suggest further research about the application of JBT in this species, both to ensure a higher rate of inclusion by reducing confounding factors, and to avoid the evaluation of management on its sole basis. We also recommend the selection of a multidisciplinary approach in the welfare assessment of horses.

Ethical Considerations

The study was not invasive or the animals and was evaluated and approved by the scientific Ethics Committee for Animal Welfare of DIMEVET (Department of Veterinary Medical Sciences) of the University of Bologna. The trial was monitored for Animal Welfare.

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Conflict of Interest

The authors declare no conflict of interest.

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PAPER 5

Limitations of spatial judgment bias test application in horses (*Equus ferus caballus*).

animals

Article

Limitations of Spatial Judgment Bias Test Application in Horses (*Equus ferus caballus*)

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Simple Summary: Public awareness about animal welfare is increasing and new research interest in this field is the evaluation of affective states. This research aimed to highlight possible limitations in the application of a spatial judgment bias test for the assessment of affective states in horses, considering the influence of personality traits, stress levels, and the structure of the test. Horses were trained to distinguish between a positive position, where they found a bucket with food reward, and a negative position where the bucket was empty. The training ended when the subjects approached the positive position faster than the negative one. Then, three ambiguous positions (Near Positive, Middle, Near Negative) were presented to them, and the latency to reach these three positions allowed their classification as pessimistic or optimistic. Our results showed that some personality traits can influence horses' responses to ambiguous cues. In addition, the spatial nature of such a test seems inappropriate for horses, which use lateralization when evaluating new objects. Therefore, this preliminary study suggested that this type of test should be modified considering species-specific and individual peculiarities.

Abstract: Affective states are of increasing interest in the assessment of animal welfare. This research aimed to evaluate the possible limitations in the application of a spatial judgment bias test (JBT) in horses, considering the influence of stress level, personality traits, and the possible bias due to the test structure itself. The distinction between two positions, one rewarded (Positive) and the other not (Negative), was learned by 10 horses and 4 ponies,. Then, the latency to reach three unrewarded ambiguous positions (Near Positive, Middle, Near Negative) was measured. Furthermore, the validated Equine Behavior Assessment and Research Questionnaire (E-BARQ) was employed to assess personality traits. Fecal and hair cortisol levels were measured through radioimmunoassay (RIA), and the frequency of behavioral stress indicators was recorded. Results showed that horses that had the rewarded position (Positive) on the right approached Near Negative and Middle faster than those that had Positive on the left. Certain personality traits influenced the latency to reach Middle and Near Positive, but chronic stress did not seem to affect horses' judgment bias. This preliminary study highlighted several limitations in the employment of spatial JBT for the assessment of affective state in horses and that personality traits can partially influence the cognitive process. Further research is needed to refine the use of this test in horses, considering the peculiarities both of species and of individuals.

Keywords: judgment bias test; equine; E-BARQ; cortisol; affective state

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1. Introduction

In the discipline of animal welfare science, affective states of animals are becoming of increasing interest [1]. The Treaty of Amsterdam on the Protection and Welfare of Animals officially recognized them as sentient beings [2]. In addition, the Five Domain Model for animal welfare evaluation has introduced the "mental domain", as a result of affective states inferred by external circumstances (environment, nutrition, health, and behavioral interactions) [3].

Animal sciences are increasingly focusing on the exploration of affective states. Emotional experiences are characterized by valence and a state of activation or arousal. In animals, emotional states arise in response to a potential reward that enhances fitness or punishment that threatens fitness. The arousal and valence of emotions are determined by the success or failure of the animal in acquiring a reward or avoiding a punishment [4]. In particular, it is possible to recognize discrete emotions, which are short-lasting and dependent on a certain stimulus, as well as long-lasting emotional states (moods), which are indicative of the environment where the subject lives in terms of possibilities of punishments and rewards and how it copes with those [4]. Often the emotions of animals are investigated using behavioral tests, usually assessing anxiety or fear, and physiological parameters [5,6]. For example, the activation of the hypothalamic–pituitary–adrenal (HPA) axis, which can occur in different situations, can be a good indicator of emotional arousal, but it does not give suggestions about the valence of the underlying emotion (punishing or rewarding) [6]. Therefore, to solve this problem, physiological indicators are considered together with behavioral measurements, but sometimes these parameters can be incongruent and their interpretation can be difficult [4,5]. In addition, often the physiological and behavioral response of an individual facing certain stimuli used in a test can vary according to its subjective experience and mood. Consequently, in non-human animals, the investigation of long-lasting emotional states is challenging, but human psychology gives good solutions to obtain an approximation for it. In particular, it has long been recognized that cognition and emotional state, especially mood, influence each other, and thus cognitive processes, such as memory, decision-making, and attention can be useful tools in the assessment of emotions [4,5,7]. Considering the hypothesis that the effect of emotions on cognitive function has an adaptive value [8,9], the exploration of cognitive indicators of emotional states also becomes valuable for animals [7]. Indeed, different emotions arise in response to threats and opportunities and they influence thoughts and actions in response to them. For example, an individual that is in a state of fear or anxiety will be more prone to negatively judge a neutral stimulus or to better memorize a threatening situation [3,8].

In recent decades, many studies employed cognitive tests to investigate emotions in animals. The most popular is the judgment bias test (JBT), which is based on the decision-making process when facing ambiguous cues [10]. The JBT includes a period of training, during which animals learn to distinguish between two stimuli, one with positive valence and the other with a negative one. Once the animals have learned the difference, they are presented with ambiguous stimuli [11]. The animals that respond as if they expect a negative outcome from the ambiguous cues are considered to have a negative affective state, while animals that have positive expectations and behave accordingly are presumed to be in a positive emotional state. According to their operational response, the individuals can be defined as "pessimistic" or "optimistic" [12]. However, the JBT results can be indicative of a long-term affective state, but there can be other influential factors such as recent positive or negative experiences, particular training conditions, and the nature of the task [10,13]. In addition, there is a strong relationship between personality and emotional states. Stable personality traits can affect mood and this can influence the decision-making process and JBT results [14–17]. For example, proactive pigs seem to have an optimistic judgment of ambiguous cues [16], while fearful and inactive heifers are more pessimistic than inactive non-fearful heifers [15]. In addition, dogs scoring higher in negative personality traits (fear, aggression, and anxiety) are more likely to judge an ambiguous stimulus negatively, while dogs with higher sociability scores are more optimistic [14].

In a recent review, Hausberger and colleagues [18] indicate the horse as a potential animal model for the study of interaction between welfare conditions and cognition. Moreover, several studies used horses as a model to be studied under stressful conditions, such as dietary challenges [19–21]. However, the research of the JBT in horses broadened only recently, especially as regards the effect of the environment and training method on the affective state of this species [22–25]. Nevertheless, further research is warranted because of the lack of a standardized protocol and insufficient studies on the influence of personality traits on JBT results in horses. Other studies have evidenced how several factors, such as lateralization and temperament, can influence test results in horses [26]. Sensorial lateralization, for example, emerges when they have to evaluate situations with different emotional valences [27,28]. Therefore, these aspects should be taken into account when we want to standardize a test protocol in this species.

This research aimed to investigate the limitations of the application of a spatial JBT in horses, considering the influence of their stress level and personality traits, and the test structure itself. For this, we explored the acute and chronic activation of the HPA axis (by measuring fecal and horsehair cortisol levels [29,30]) and assessed the horses' personalities (by the Equine Behavior Assessment and Research Questionnaire—E-BARQ [31]). We hypothesized that both acute stress, potentially caused by the test procedure itself, and individual chronic stress could influence the horses' responses during the JBT, causing a negative judgment bias. Furthermore, considering the influence of personality traits on the JBT, we predicted that more anxious animals may show more pessimistic bias than highly sociable and confident individuals (as previously observed in dogs [14]). Finally, we investigated if the structure of the spatial JBT employed could have had limitations in its application in horses.

2. Materials and Methods

2.1. Ethics Declarations

The study was evaluated and approved by the scientific Ethics Committee for Animal Experimentation of the University of Bologna, the experiments were not invasive and were performed following regulations of Legislative Decree no. 26/2014.

2.2. Animals and Housing

The study involved eleven adult horses and five ponies from the same facility, to avoid variables due to different environmental conditions. To have a sufficient number of animals, we were not able to select for sex and/or breed. The sample comprised two stallions, eleven mares, and three geldings. The age of the subjects ranged from five to 25 years (mean age = 14 ± SD 6.6 years).

The overall population of 20 horses of the studied facility was housed in individual boxes (3.0 × 3.5 m), with daily access to individual outdoor paddocks during favorable weather conditions. Natural lighting was provided by windows, and the boxes were equipped with automatic waterers. Depending on their individual needs, the horses were fed with hay (7–10 kg per day) divided into three meals (at 7:30 a.m., 12:30 p.m., and 6:00 p.m.), supplemented by pelleted food with maize and carob (0.8–1.0 kg) in the evening meal. The center of the yard was occupied by two rectangular sand arenas (12 × 20 m and 15 × 50 m), a grass field (25 × 65 m), and a round sand pen (18 m diameter). All the horses were used for riding lessons for adults and children.

2.3. Personality Assessment

The owners and handlers filled out the online E-BARQ for each horse. This validated questionnaire is composed of 100 question items, divided into 13 categories for the ridden horse: Trainability (TR), Rideability (RID), Boldness (BOLD), Handling Compliance (HC), Working Compliance (WC), Easy to Stop (ETS), Human Social Confidence (HSC), Non-Human Social Confidence (NHSC), Novel Object Compliance (NOC), Touch Sensitivity (TS), Independence (IND), Easy to Load (ETL). Items indicating negative traits were scored on a 5-point scale, where Never = 5, Rarely = 4, Sometimes = 3, Usually = 2, Always = 1. A reversed scale was used for positive items, where Always = 5 and Never = 1. Therefore, a higher score in each category is always desirable. The total score for each category was automatically calculated upon completion of the online questionnaire (website: https://e-barq.com/ accessed on 25 November 2021) [31,32].

The ETL category was excluded during the data processing because most respondents did not answer its included questions.

2.4. Judgment Bias Test (JBT)

A Go/No-go spatial JBT has been employed, considering the protocol previously used by Freymond et al. [22] and Henry et al. [24]. To provide a familiar environment, one of the rectangular sand arenas (12 × 20 m) of the facility was used. Each horse was always handled by the same experimenter (exp. 1), who remained neutral and did not give any indication during the JBT to not interfere with the decision-making process of the horse. To prevent the horses from seeing their content, black plastic buckets were chosen. The JBT procedure occupied four days during the week: the first day was dedicated to the habituation, the second day to the training, and, after one day off, two consecutive days were devoted to the two sessions of the test.

On the first day, the subjects were familiarized with the setting. One subject at a time was conducted in the arena by exp. 1. In the arena, a start line was delimited by two red cones. A second experimenter (exp.2) positioned the bucket with a food reward on the ground in the positive position (P), 9 m from the start line (Figure 1). The habituation was completed when horses, released at the start line, independently approached the bucket three consecutive times. Half of the horses had the positive cue to their right and the others to their left. During the second day, horses learned to discriminate between the P and the opposite negative position (N), where the bucket was empty. N was 12 m from P (Figure 1). Each trial lasted a maximum of 60 seconds and P and N were presented one at a time in a pseudo-random order. No more than two consecutive trials of the same position occurred. For each horse, the latency to reach the bucket was recorded, and the subject succeeded in the training phase when the longest latency to reach the last three positive buckets was shorter than that of the three last negative ones [33]. The training phase was followed by a day off.

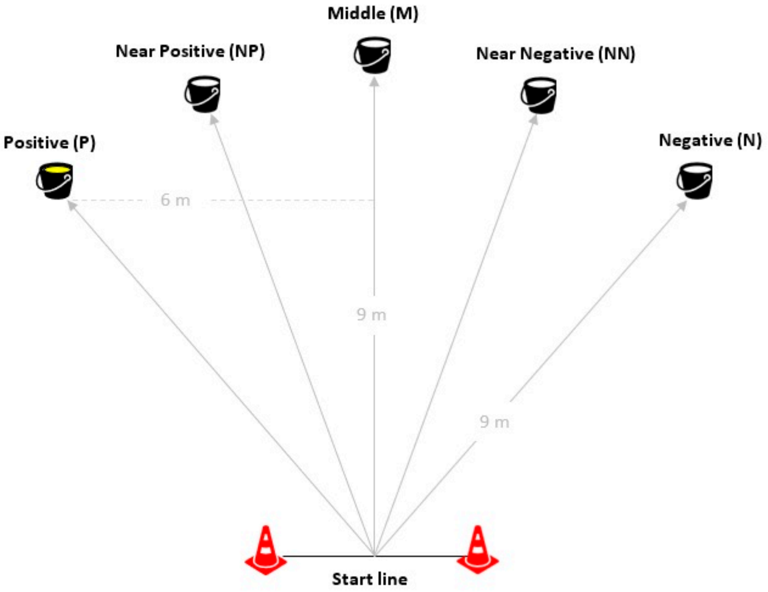

Figure 1. The setting of the spatial judgement test. P is the Positive position (bucket with food) and N is the Negative position (empty bucket). The ambiguous positions (empty bucket) are Near Positive (NP), Middle (M), and Near Negative (NN).

Two sessions of testing were performed on two consecutive days. Spread along a semicircle, three intermediate positions between N and P were presented one at a time: Near Negative (NN), Middle (M), and Near Positive (NP). In the intermediate positions, the bucket was empty. Each session followed the scheme proposed by Henry et al. [24], where ambiguous locations were preceded alternately by positive and negative locations (Test 1: P-N-NP-P-N-M-P-N-NN-P; Test 2: N-P-M-N-P-NN-N-P-NP) (Figure 1).

All positions were marked on the ground with small cones and were 9 m from the start line. The start line was in the north part of the arena for five subjects, while in the south part for the other five.

2.5. Video Recording and Analyses

Training and test sessions were videotaped using a Sony HDR-CX240E camera. The camera was placed on a tripod outside the arena beginning with the habituation phase to familiarize the horses with it. All videos were analyzed by the experimenters, previously trained, using the software Boris v. 7.9.19 [34], to record precisely the time needed by the horses to reach the buckets and to register their stress-related behaviors. The behavioral analysis was carried out using a specific ethogram [35–40] (Supplementary Material Table S1 and the frequency (acts/minute) for stress behaviors was calculated.

2.6. Collection of Fecal and Horsehair Samples and Cortisol Assay

The fecal cortisol level was monitored for one week in each horse, while the only variation in their daily routine was the habituation and training for the test and the test procedure itself, four days out of the seven. For all 14 subjects considered in the JBT, feces were collected every day: on the habituation day, on the training day, on the day off between the training phase and the testing phase, on the two testing days, and on the two following days. This testing schedule has been chosen to reflect the HPA activation on the day before, as recommended by Möstl et al. [41].

A total number of 98 fecal samples (seven per horse) have been collected from physiological (spontaneous) defecation, about 24 h after the tests, usually in the morning.

Each fecal sample was placed in a labeled non-sterile plastic bag and frozen at −20 °C. The horsehair samples (n = 14) were collected from the base of the tail of each horse, except two, for which we used the mane. The horsehair was collected during the habituation day. All samples were stored at room temperature.

Cortisol concentrations were determined by radioimmunoassays (RIAs) based on the binding of 3H-steroid by competitive adsorption [42]. After filtration and lyophilization of the feces, the extraction methodology was modified considering Schatz and Palme [43]. Cortisol was extracted from 500 mg of feces with methanol–water solution (v/v 4:1) and ethyl ether. The portion of ether was vaporized under an airstream suction hood at 37 °C. The dry residue was finally dissolved again into 0.5 mL PBS (0.05 M, pH 7.5). The extraction of the cortisol from the hair was performed as described by Accorsi and colleagues [44]. Cortisol metabolite assay was carried out according to Tamanini et al. [45]. The cortisol RIA was performed using an antiserum to cortisol-21-hemisuccinate-BSA (anti-rabbit), at a working dilution of 1:20,000 and 3H-cortisol (30 pg/tube vial) as a tracer. Validation parameters of analysis were: sensitivity 0.19 pg/mg, intraassay variability 5.9%, interassay variability 8.7%. Radioactivity was determined using a liquid scintillation β counter and a linear standard curve, ad hoc designed by a software program [46].

All concentrations were expressed in pg/mg of hair and fecal matter.

2.7. Data Analysis

To avoid biases caused by differences in baseline running speeds, due to the size and/or age of individuals, raw latencies recorded to reach the intermediate positions were transformed into scores, according to the formula proposed by Mendl et al. [33]:

$$\text{Adjusted latency} = \frac{(\text{mean latency to ambiguous location} - \text{mean latency to P})}{(\text{mean latency to N} - \text{mean latency to P})} \times 100.$$

This formula returns 0 for the P and 100 for N.

The whole statistical analysis was carried out in the RStudio environment [47].

The data distribution was assessed with the Shapiro–Wilk test. To compare the recorded scores in intermediate positions and fecal cortisol levels during the week, we conducted Friedman tests and pairwise Wilcoxon rank tests with post hoc Holm correction as. A Mann–Whitney U test was used to check if the latency to reach the intermediate positions was affected by P positions. Then, we compared the score obtained from E-BARQ of horses with P on their left and subjects with P on their right through a Mann–Whitney U test. Finally, we checked the Wilcoxon effect size (r) and 95% confidence intervals (CIs) for significant results.

To investigate the influence of horsehair cortisol levels on JBT results, a simple linear regression was employed, and different multiple linear regressions were used to model scores for each intermediate position as functions of (1) fecal cortisol levels and behavioral signals, (2) E-BARQ scores of personality categories. Collinearity was checked using multipanel scatterplots, Pearson correlation coefficients, and variance inflation factors (VIFs). We used backward selection based on the Akaike information criterion to find the optimal model. To verify the underlying assumptions of homoscedasticity, normality distribution, and independence of residuals, we employed the Breusch–Pagan, Shapiro–Wilk, and Durbin–Watson tests, respectively. When the model normalization was necessary, we used the Yeo–Johnson transformation, because of the presence of negative values.

The statistical significance was set at $p < 0.05$. Graphs were realized using the package ggplot.

3. Results

Two subjects were excluded from the statistical analysis because they failed the inclusion criteria of the JBT, and 10 horses and 4 ponies composed the final sample. Those that succeeded in reaching the criterion learned the task in an average of 16.5 trials (min 11–max 23).

According to the Friedman test, the latency of the subjects seemed to be significantly influenced by bucket position ($\chi^2 = 35.543$, df = 4, $p < 0.0001$). In particular, there was a significant difference between the latency to reach N (56.8 ± 8.51sec) and all the other positions ($p < 0.01$), while no significant difference was found between the latency to reach P (8.38 ± 1.27 s) and all the intermediate positions. According to the post hoc test, the subjects approached NN (13.6 ± 25.3 s) significantly slower than M (8.50 ± 0.62 s; $p < 0.01$, r = 0.88, CI (0.88, 0.89)). In addition, they approached NP in a mean of 8.76 ± 1.73 s (Figure 2).

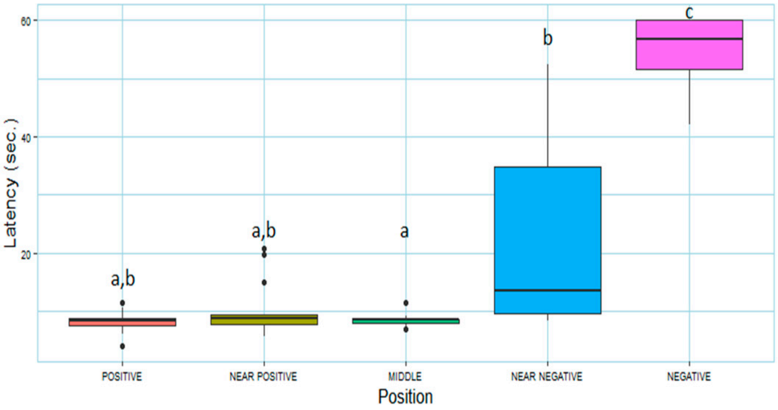

Figure 2. Latency in seconds (sec.) to reach the bucket in the five locations. Results refer to the two days of the test. The bar within the box represents the median, the borders of the box are upper and lower quartiles, the bottom and top whiskers signify the lowest and highest cases within 1.5 times the interquartile range (IQR), and outliers are shown through black full circles. a, b, and c indicate significant results ($p < 0.05$).

Due to the variation of size and baseline running speed between the subjects, raw latencies recorded to reach the intermediate positions were transformed into scores. The position of the positive cues (P) seemed to have influenced the speed of horses to reach M and NN. The seven horses that had the P at their right side registered significantly lower scores to reach NN (right-P = 2.31 ± 8.33; left-P = 55.92 ± 39.40; W = 3, $p < 0.01$, r = −0.735, CI (−0.845, −0.38)), and a lower score in M with a significant tendency (right-P = −0.50 ± 2.36; left-P = −0.65 ± 10.80; W = 3, $p = 0.07$).

3.1. Influence of Stress on JBT Results

During the week of the test, cortisol concentration remained constant (Figure 3). Considering the multiple linear regression model, the possible acute stress induced by the entire test procedure and assessed through fecal cortisol and stress-related behaviors (Supplementary Material Table S1) did not significantly influence JBT results (NP, F (2,11) = 1.615, p= 0.24, adjusted-R^2 = 0.09; M, F (2,11) = 1.83, $p = 0.21$, adjusted-R^2 = 0.11; NN, F (2,11) = 1.073, $p = 0.37$, adjusted-R^2 = 0.01).

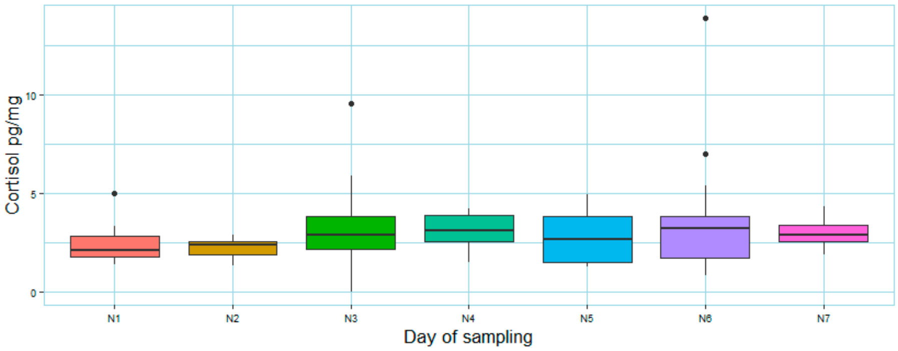

Figure 3. Fecal cortisol (pg/mg) measured from samples collected every day of the JBT week (N1 = habituation, N2 = training, N3 = resting day, N4 = test1, N5 = test2, N6 = resting day after the last test day, N7= second resting day after the last test day). The bar within the box represents the median, the borders of the box are upper and lower quartiles, the bottom and top whiskers signify the lowest and highest cases within 1.5 times the IQR, and outliers are shown through black full circles.

In addition, we did not find significant influence of the chronic activation of HPA (Figure 4), assessed using horsehair cortisol concentration, on JBT results (NP, F (1,12) = 1.202, $p = 0.29$, adjusted-R^2 = 0.01; M, F (1,12) = 0.008, $p = 0.93$, adjusted-R^2 = −0.08; NN, F (1,12) = 0.95, $p = 0.35$, adjusted-R^2 = −0.003).

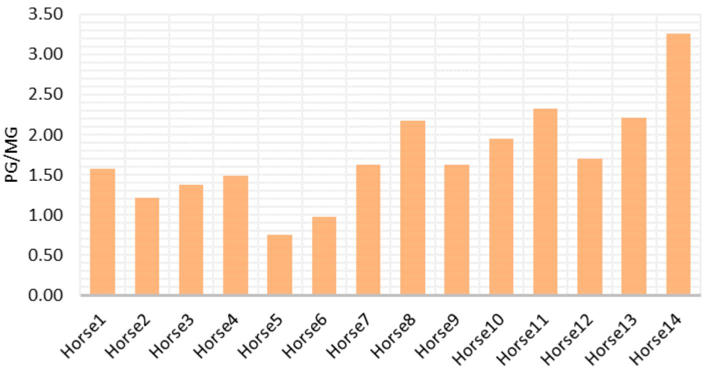

Figure 4. Cortisol level in horsehair (pg/mg) of each subject considered in the research.

3.2. Influence of Personality Traits on JBT Results

Comparing the E-BARQ scores of horses that have P on their right and those with P on their left, no significant difference was evidenced.

The analysis of the E-BARQ results (Figure 5) revealed collinearity between (1) TR and RID; (2) BOLD and NOC, HC, FG, NC; (3) HSC and TS. Therefore, for the model construction, we excluded RID, NOC, HC, WC, FG, and TS.

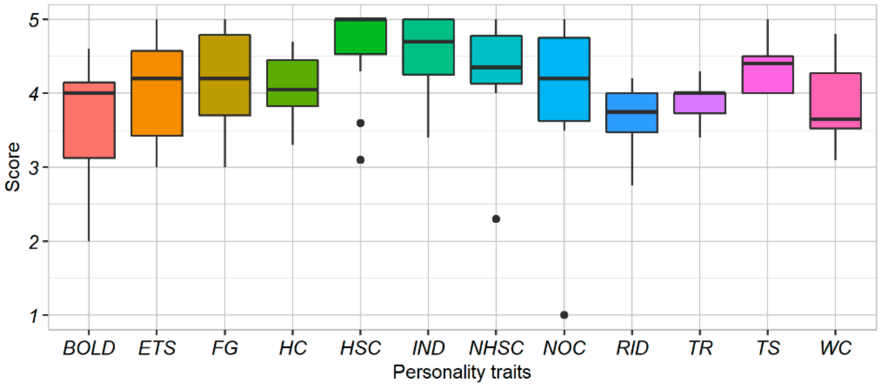

Figure 5. E-BARQ score for each personality trait. The box plots show the median and 25th and 75th percentiles; the whiskers indicate the values within 1.5 times the interquartile range, IQR. The outliers are indicated by the black circles.

We perform multiple linear regression to model NP, M, and NN scores as a function of TR, BOLD, ETS, HSC, NHSC, and IND. Finally, we used backward selection based on the Akaike information criterion to find the optimal models.

The model that included HSC and IND was the best one (AIC = 38.58) to explain a significant amount of the NP score (F (2,11) = 7.919, $p < 0.01$, adjusted-$R^2 = 0.52$). The model revealed that the NP score was positively predicted by IND ($\beta = 7.740$, t = 3.891, $p = 0.002$) and HSC, with a significant tendency ($\beta = 3.477$, t = 1.997, $p = 0.07$).

The model that included BOLD, ETS, HSC, and IND was the best one (AIC = 33.49) to explain a significant amount of the M score (F (4,9) = 4.962, $p = 0.02$, adjusted-$R^2 = 0.55$). The model revealed that the M score was negatively predicted by ETS ($\beta = -4.354$, t = -2.953, $p = 0.02$) and positively predicted by BOLD, with a significant tendency ($\beta = 2.572$, t = 2.249, $p = 0.05$).

None of the personality categories seems to have influenced the score in NN.

4. Discussion

The first relevant result of our study underlines a possible bias due to the position of the positive cue that can influence the latency of horses towards the NN position, disturbing JBT results. Horses who have P on their right were faster to reach NN than horses with P on the left. One hypothesis can be the presence of environmental or sensorial disturbances near the arena that could be relevant for the horses but that humans do not notice or cannot control, such as particular odors, sounds, or the presence of other subjects [48] in the stalls that were near one side of the arena. However, we randomized the position of P and, as specified in the Materials and Methods section, we positioned the buckets in the north part of the arena for nine horses, while for the other five we positioned the buckets in the south part. The second hypothesis was the presence of the phenomenon of lateralization that can cause biases in motor behavior or perception of stimuli [49]. In the setting of tests that employ spatial tasks, laterality should be considered, especially for prey animals, because lateralization can influence their performance. In particular, studies show that horses usually prefer the use of the left eye to evaluate and explore a new environment and novel object with an emotional valence, both positive and negative [27,50]. The use of the left eye is associated with rapid reaction [28] and with both positive and negative associations, while the right eye is used to examine neutral objects [27]. We cannot exclude that the presence of NN to the left of the horse has possibly led to a different evaluation of the intermediate position. We think that further research is necessary to explore lateralization as a possible source of disturbance in the horse spatial JBT.

According to statistical analysis, there was no difference in latency to reach P and ambiguous locations, even if the latency to reach NN recorded a high variability within the group and was different compared to those in front of NP and M. NP and NN often are perceived as P and N training cues by animals. However, the value associated with P and N can change the animal's decision to take a risk. Therefore, the lack of a reward in the N position, as in our case, is not so costly for the individual, and it can also decide to respond positively in front of a potential negative cue [7,10].

Similar to our results, Müller and colleagues [51] found that half of their dog population had similar latencies in approaching NP and M, but went slower to NN, whilst the other half approach the ambiguous locations from NP to NN increasingly slowly. They justified this discrepancy with a difference in personality traits [51]. Indeed, in our study we found that some of the horses' personality categories influenced the response toward NP and M. However, we did not find any relationship between the E-BARQ scores and latency to reach NN. In particular, horses with higher scores in IND and HSC, with a significant tendency, approach NP slower, whilst the subjects with lower scores in BOLD, with a significant tendency, and higher scores in ETS approach M faster. The traits IND and BOLD are general categories including anxiety, separation anxiety towards conspecifics, and avoidance behavior in a novel situation. Therefore, low scores in these two personality traits can indicate an anxious and fearful subject [28]. Considering that, our results disagree with previous studies, where animals characterized by anxiety, fear, and aggression traits approach intermediate positions slowly, expecting a negative outcome, and show a pessimistic bias [15,30,52,53]. However, our results could have been influenced by several factors, such as the small sample size and the lack of homogeneity for sex and breed in the sampled population, the spatial nature of the test (as stated previously), and the fact that the horses involved in the test, also for safety reasons, were not aggressive subjects. The other two results are more easily justifiable. The fact that subjects with lower HSC scores reached the NP faster can be justified by the fact that horses that are less confident towards humans may prefer to increase the distance from the handler, especially in front of the NP cue, which often is associated with P and, thus, is associated with a high expectation of a reward [10]. Finally, the trait ETS considers how the horse responds to human signals to stop. Therefore, we cannot exclude that those horses with higher ETS tend to respond better to human signals, so when they were released, they immediately started to approach M. Considering this last result, even if in this study we tried to avoid possible human influences and interactions with horses, future applications of this test should take this factor into account.

The effect of personality traits on JBT is difficult to explore because this test can also be influenced by both long-term mood and stressful or rewarded recent experiences [4,54–56]. We employed horsehair and fecal cortisol to investigate chronic and subacute HPA activation, and to evidence particular stressful situations. Cortisol is a useful tool to investigate animal welfare [6], but on its own it is not exhaustive of animal emotion and the welfare status. In particular, fecal cortisol concentration represents an integrated average of the circulating hormone over a species-specific amount of time, which in horses is about 24 h [29,41,57,58], and fecal sampling is non-invasive. On the contrary, plasma and saliva cortisol levels are prone to circadian patterns and short-term fluctuations, represent point-of-time estimates, and can be altered by the sampling procedures, which can cause stress in animals that are not trained for them [41,57,58]. Therefore, collecting fecal samples from each subject throughout the entire week allowed us to monitor the stress level of animals possibly due to the entire procedure of the JBT, which could have represented a disruption of their daily routine, and to compare cortisol levels during the test procedure and in the baseline condition. We did not record significant differences in fecal cortisol concentration during the days of the test procedure. In addition, both fecal cortisol concentration and relative frequency of stress behaviors seemed to not influence JBT results. Therefore, the test itself probably did not induce particular stress in the studied subjects, but it should be considered that testing social animals, such as horses, individually for the JBT could increase stress [59].

This aspect should always be considered because, to avoid biases in the decision-making process of the animal, it is important that habituation, training, and testing phases do not cause stress.

Considering horsehair cortisol, this represents the chronic activation of HPA and can be a potential indicator of welfare and long-term stress [29]. Indeed, cortisol is continuously incorporated into the hair during its growth. The cortisol concentration in this matrix can be a useful indicator of the HPA activity during the weeks preceding the sampling [60]. Each individual, also according to different personality traits, copes differently in the same environment, and in a group of animals that came from the same condition, we can find different levels of chronic stress [61]. This has been well represented in our sample population, where each individual presents a different concentration of horsehair cortisol. Our prediction was to find a positive relationship between horsehair cortisol level and ambiguous position score, finding that subjects with higher horsehair cortisol concentration show a more pessimistic bias. However, this hypothesis has been refuted and this aspect should be deepened by further research, enlarging the sample size, using a multidisciplinary approach in horse welfare evaluation, and refining the JBT structure considering horses' characteristics.

5. Conclusions

Considerations of personality traits and cortisol levels in horses' JBTs are the innovative aspects of this research. However, this preliminary study evidenced some limitations of the use of spatial Go/No go JBT tasks in horses. In particular, the spatial nature of this test and individual personality traits can influence the responses of horses to intermediate positions and alter the interpretation of the JBT. Further research is needed to find a standardized JBT for horses that takes into account setting, species peculiarities, and individual motivations. Finally, future application of the JBT to assess affective states in horses should check for stressful situations and personality traits.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani12213014/s1, Table S1: Ethogram used to assess horses' stress during the JBT [32–37].

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Data Availability Statement: Data available on request.

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animals

Article

Limitations of Spatial Judgment Bias Test Application in Horses (*Equus Ferus Caballus*)

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Table S1. Ethogram used to assess horses' stress during JBT test [32-37].

Stress-related behaviours	
Neck Turn	The horse turns its head and neck from the side opposite to the stimulus to which it is relating. During this movement, the trunk and limbs are still. Both ears are facing forward, or one ear is facing forward and the other is towards the stimulus.
Head Turn	The horse turns its head left or right away from the stimulus independently from its handler.
Vacuum Chewing/Licking and Chewing	The horse moves its mouth and chews, and sometimes it protrudes its tongue (licking and chewing), without any stimulus in its mouth.
Neck Shake	Rapid rhythmic rotation of the head and neck
Body Shake	Rapid rhythmic rotation of the entire body
Yawning	Deep long inhalation with mouth widely open and jaws either directly opposed or moved from side to side
Head toss	The horse makes an oscillation or rotational movement with the head. It can be emitted from a standstill position or during locomotion.
High Tail	Tail is raised with its fleshy part horizontal or above horizontal.
Clenched Tail	The tail is pressed under the region inguinal or between the hind limbs.
Tail Swishing	Quick and lateral movements of the tail.
Ears moving or scanning	The ears are continuously moved back and forth at varying speeds and sometimes one ears points forward and the other backward.
Ears Pinned Back	Ears pressed caudally against the head and neck.
Head Raised	The horse extends slightly its neck, helds the head higher than the normal carriage, and the nose is extended upward
Defecation	Expelling of feces
Rolling	The horse lyes on the ground and roll side to side, rubbing its back on the ground
Self-mutilation	Bite movement directed at own body usually the flanks or the chest and limbs (3)

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Scratching	Rubbing part of the body against surface of object, fance or stable, using also mouth and hind limb.
Pawing	Movement of one front limb back and forth on the ground or horizontal surface
Sniffing	Exploratory behaviour towards the floor or an object
Liking Object	Exploratory behaviour with tongue towards and object

4. GENERAL DISCUSSION

Horses evolved as social prey species that live in open plains and adopt early detection and flight response as first antipredator strategies (Goodwin, 2007; VanDierendonck and Spruijt, 2012). Domestication and breeding selection have affected only a limited number of genes that regulate behavioural characteristics, such as fear or reactivity, but the original natural behaviour and their physical and mental adaptations have remained the same as their ancestors. For example, the necessity to detect and flee from predators has shaped the sensory system, morphology and behaviour of horses, and this can influence their relationship with human beings (Goodwin, 1999; VanDierendonck and Spruijt, 2012). Even if the plasticity of their behaviour has facilitated the domestication process, many of the traits and ethological needs that have remained unaltered from their ancestors are difficult to accommodate in a domestic environment (Goodwin, 1999). Different housing systems and practices exist in the equestrian world, but each of them, without proper management, can present different threats that can be a source of psychological and physical stress for horses and can mine their welfare (Mills and Clarke, 2007).

On these bases, this PhD thesis aimed to assess the welfare of horses in different management systems, adopting a multidisciplinary approach and considering the three criteria of animal welfare: health and functioning, natural life and affective state (Fraser, 2008). In particular, we have tried to identify management practices and housing characteristics that can enhance or compromise horse welfare. Finally, we have critically analysed methods and measures which can potentially be useful in horse welfare evaluation, discussing their limitations and proposing innovative ones.

From the point of view of humans, it is important to guarantee food, shelter, protection and veterinary care to their horses, in a safe, clean and cost-effective environment. However, not always, the implications of a housing system for the health and welfare of animals are considered. For example, many housing systems are associated with confinement and isolation, to which the horse has an inherent aversion (Mills and Clarke, 2007; Goodwin, 1999). Stabling horses, especially sport horses, in individual stalls is really common in Europe and North America, where it is one of the main kinds of housing systems (Mills and Clarke, 2007; Ruet et al., 2019). This gives to humans the possibility to control horses' diet, easily catch and handle them and keep them clean. On the other hand, it offers horses social isolation, vulnerability, limitation to movement and foraging time, and restriction of sensory input, so prevention of predator detection and escape. Therefore, this kind of management conflicts with the natural survival strategies of these animals and can be psychologically detrimental for them, which can develop frustration and abnormal behaviours, such as stereotypies (Mills and Clarke, 2007; Goodwin, 1999). This is aligned with our results that have identified prolonged confinement in individual boxes as a potential risk factor for the development of behavioural problems in horses. In addition, we have found that also the composition of the diet and a high number of working hours can contribute to the development of undesired behavioural. These results are confirmed also by other studies that identify feeding composition and routine, time spent in paddocks or boxes, social opportunities and riding activity (in terms of time spent in working activities and riding style and discipline) as influential factors for horse welfare and the development of undesired behaviours (Christensen et al., 2002; Lesimple et al., 2016, 2011; Normando et al., 2011; Ruet et al., 2019; Visser et al., 2008). For example, a diet high in energy, rich in concentrate and low in fibre and roughage divided into a few meals during the day can be detrimental to the horse's health and can lead to the development of behavioural abnormalities, such as oral stereotypies. Indeed, the physiology of horses is adapted to a high fibre/low energetic diet, and they usually graze while walking for most of the daytime, and this underlies also the importance of free movement (Cooper et al., 2005; Harris, 2007; Houpt, 2005; Tinker et al., 1997). Another potential risk factor for the development of abnormal behaviour is the limitation of social interactions. Indeed, the possibility of visual contact with conspecifics can bring some positive effects but sometimes the impossibility of direct interactions can cause frustration. It was observed that the presence of grills or bars between the stalls, which allow close contact with neighbours, and the possibility of living together with other horses can be beneficial and reduce the development of undesired behaviour (Christensen et al., 2002; Mills and Clarke, 2007; Lesimple et al., 2016; Mcgreevy et al., 1995; Visser et al., 2008). Giving the possibility to horses of accessing pastures or paddocks and increasing social inputs is undoubtedly beneficial for horse welfare (Lesimple et al., 2016; Rivera et al., 2002). However, keeping them in a group in paddock systems with open shelters can be problematic. In this case, social isolation and movement restrictions are minimised, but, for example, particular attention should be paid to the soil that can be poached and spoiled in wet and cold weather conditions. Therefore, it can be important to follow precise instructions and principles of construction and design (Mills and Clarke, 2007). In addition, in non-voluntary groups of domestic horses, the individuals have not chosen to be in this group with this composition but cannot leave. Furthermore, a domestic group usually did not reflect the natural social organization of horses, and also the motivations and interactions can be different due to the presence, for example, of castrated males with intact females, which instead maintain their reproductive motivation. In a group of horses, aggressions can verify and this is one of the main concerns of owners and welfare bodies, which ask to find strategies to cope with large non-voluntary domestic groups, reduce the risk of injury and safely introduce new individuals or mix adult horses (Mills and Clarke, 2007; Hartmann et al., 2009; VanDierendonck and Spruijt, 2012).

Most of the ethological needs mentioned above have been taken into account in the design and management of the Ethological Stable system described in the results of this thesis. Indeed, even if our study presents some limitations, such as the impossibility of studying the animals during the night, our observations have reported a general alignment with the activity budget reported in the literature for feral horses (Duncan, 1980; Kiley-Worthington, 1990; Ransom and Cade, 2009). On the contrary, the activity budget of individual stabled horses differs from the natural one, with an increase in time spent in rest while standing and a decrease in foraging (Kiley-Worthington, 1990). This suggests that the housing and management systems proposed by Ethological Stable can be considered positive for horse welfare, but some critical points should be taken into account, such as the absence of a real herd. This can be because horses that were in the structure during the study had recently arrived from different housing systems and did not know each other. However, after the study, we have suggested implementing gradual and positive introductions and finding solutions to favour the creation of affiliative social bonds between individuals and the formation of a group. This can facilitate also the assignment of stalls to the horses. Indeed, this structure has stalls divided by grills and bars and, even if, as previously stated, this solution can have positive effects (Mcgreevy et al., 1995) and can facilitate the close interaction between neighbours, on the other hand, it can be risky. In support of this, our results have shown that horses inside boxes show more threats and aggressive behaviours. Therefore, a good solution can be assigning preferably adjacent stalls to horses that have a preferential affiliative bond and providing the boxes with partial visual barriers, allowing animals to hide from the conspecifics. As reported above, the increase of social inputs and the improvement of the social environment could be beneficial also in the reduction of the percentage of stereotypies and abnormal behaviours which were shown by some of the animals housed in the Ethological stable, even if their percentage was already low and probably the stereotypies were already present on their arrival in the structure. The presence of stereotypies is commonly associated with poor welfare, but it is not always the case (Mason and Latham, 2004). Indeed, stereotypies can function as “do-yourself enrichment”, and can become a coping mechanism or assume a calming effect, which ameliorates the welfare in sub-optimal environmental conditions. On the other hand, stereotypies can be “scars” of a past condition that have elicited them and they can persist even when their causal factors are removed or even if the welfare conditions have been improved. This happens for two mechanisms: one is called “central control”, so, with repetition, the behaviour becomes an automatic response that does not necessitate a cognitive process or sensory feedback and that can be triggered by several situations; the other is perseveration, which is usually associated with central nervous system dysfunctions that do not allow proper regulation of the behaviour. When the perseveration is severe, stereotypies are difficult to extinguish, even if the environmental and welfare conditions are improved (Mason and Latham, 2004). In horses, crib-biting is an example of stereotypy that, with the alteration of the dorsal and ventral striatum, can become a habitual response which persists even if the consummatory goal is achieved and its causal factors have been solved (Roberts et al., 2017). Because they are “coping mechanisms”, preventing an animal to perform stereotypies can have detrimental effects and it is better to understand what causes their expression and act on it. In addition, because of their difficulty to be extinguished, it is highly suggested to directly avoid their development by offering the animals adequate housing and management conditions from the beginning (Mason and Latham, 2004; Roberts et al., 2017). Therefore, there is not a straight link between the expression of stereotypies and the present welfare conditions. Furthermore, not always aversive conditions lead to the development of stereotypical behaviours. Thus, considering only this behavioural parameter as the only indicator of good or bad welfare can be risky and can induce wrong interpretations and conclusions. This can be avoided by considering other parameters, such as other behaviours or physiological measures (Mason and Latham, 2004). This is one of the reasons why in our assessment of horse welfare we have always tried to use more than one single parameter. For example, when we have compared horses with behavioural problems and “healthy” horses we have also considered their endocrinological framework, in particular considering cortisol and DHEA.

Cortisol, DHEA and their ratio have been considered in the first and second articles presented in this thesis. The use of glucocorticoids (cortisol and corticosterone) measured in different matrices, has been widely employed in the study of stress response and welfare in animals (Mormède et al., 2007). However, especially human studies propose the measurement of DHEA and its sulfate metabolite (DHEA-S) and the glucocorticoid/DHEA ratio to obtain a complete picture of HPA activity (Whitham et al., 2020). Available data suggest that DHEA and DHEA-S have an antagonistic action toward glucocorticoids, enhancing immune function and exerting a neuroprotective effect. However, the co-action of these hormones in the physiology of the animals should be deepened. The impact and role of DHEA and glucocorticoid:DHEA(S) ratio on stress, welfare and emotional, physical and mental health of non-human animals need further studies (Gabai et al., 2020; Whitham et al., 2020). To our knowledge, the researches presented in this PhD thesis are some of the first and few studies that propose and validate the measurement of DHEA in horsehair, considering a non-invasive method for the evaluation of the concentration of this hormone. The HPA axis activation causes a hormonal cascade that is important for the maintenance of the basal homeostatic state. Emotional, mental and physical stressors can trigger the activation of the HPA axis (Whitham et al., 2020). However, we should always consider that stress response is an adaptive and physiological mechanism, thus short-term acute stress is not necessarily negative (Ralph and Tilbrook, 2016). On the other end, repeated stress or long-term/chronic stress can lead to a dysregulation of the HPA axis response and detrimental effects on health (Ralph and Tilbrook, 2016; Whitham et al., 2020). Individuals can have different physiological and behavioural responses in front of the same environmental challenges, so it is difficult to define stress univocally. Usually, when researchers use the term stress in animal husbandry studies it assumes a negative connotation and refers to health and welfare impairment (Gabai et al., 2020). The activation of the HPA axis starts from the hypothalamus, which produces corticotropin-releasing hormone (CRH) that stimulates the anterior pituitary gland and the secretion of adrenocorticotropic hormone (ACTH). The ACTH stimulates the conversion of cholesterol to pregnenolone, which is the precursor to all steroid hormones, including glucocorticoids and DHEA, which are both excreted by the zona reticularis of the adrenal cortex. Only glucocorticoids and not DHEA(S) exert negative feedback on the HPA axis but prolonged or repeated stress can cause a dysregulation of the above-mentioned mechanisms (Guilliams and Edwards, 2010; Kamin and Kertes, 2017; Whitham et al., 2020). In the first phase, chronic or intense stress can cause an increase in glucocorticoids and a decrease in DHEA. After a prolonged period under stress, which can last also years, “adrenal fatigue/exhaustion” can verify, leading to low cortisol and DHEA level. This seems an adaptive mechanism that probably initiates in the hypothalamus and pituitary gland and propagates to the periphery to prevent the damage caused by a high level of glucocorticoids, such as immune suppression and increasing catabolic pathway. However, this causes hypocortisolism, which is equally harmful and can lead to pathophysiological consequences, which involve immune, mental and emotional health (Guilliams and Edwards, 2010; Whitham et al., 2020). The HPA axis dysregulation can be better investigated considering the cortisol:DHEA(S) ratio than using only glucocorticoids. However, DHEA(S) can be produced by different organs other than adrenal glands, such as gonads, placenta and nervous system. While in humans it is known that the circulating DHEA(S) derives primarily from adrenals, and only a portion is produced by gonads, in non-human animals it is difficult to extrapolate how much gonads and placenta, in pregnant animals, can contribute to the level of circulating DHEA(S). Therefore, when we study the activation of the adrenal glands employing DHEA(S), we should consider that also gonads and, in pregnant animals, the placenta can contribute to the circulating level of these hormones and this could bias our interpretations. In stallions, for example, the low DHEA concentration founds in seminal plasma, suggests that the contribution of the testis to circulating DHEA is scarce. In addition, we should always take into account that these hormones can have species-specific biological functioning, synthesis and regulation (Gabai et al., 2020). In certain species, DHEA(S) affects cognition, immune function, mental health, and survival and reproductive behaviours. Therefore, the cortisol:DHEA(S) ratio has been employed not only for the general welfare assessment but also as an indicator of immune function, mental health and cognitive performance (Kamin and Kertes, 2017; Whitham et al., 2020). Our first article reported differences in horsehair concentration of glucocorticoid, DHEA and cortisol/DHEA ratio between horses under different management conditions. In particular, horses housed in the traditional system present higher glucocorticoids and lower DHEA concentrations in horsehair and, consequently, higher cortisol:DHEA ratio in this matrix. We have interpreted this result as a potential indicator of chronic stress, suggesting that the natural boarding system could be potentially a better management system that tries to respect the physiological and behavioural needs of the animals. This hypothesis is supported by other studies that found similar results in the hair of cows and in the saliva of piglets, where detrimental environmental conditions were associated with an increase in cortisol:DHEA ratio (Fels et al., 2019; Peric et al., 2017). Interesting to notice the similarities with the study made by Peric and colleagues on the quantification of glucocorticoids and DHEA in the hair of cows (2017). Indeed, also these authors found that during the summer when the animals were on pasture, they found an increase in hair DHEA concentration compared to that registered when they were in the tie-stall system of the barn. As reported in this research, probably the increasing activity and the interaction with other animals, which stimulate limbic cortex and hippocampus activity, can influence DHEA concentration (Peric et al., 2017), as in humans where muscular activity has been correlated with an increase of this hormone (Tremblay et al., 2004). Also in our case, in the natural boarding system animals have more possibilities of interacting with conspecific and fewer movement restrictions compared to the traditional system. However, in our first article, against our expectations, we did not find any difference in the immune system parameters of the two groups of horses considered, even if both DHEA(S) and cortisol affect immune function, often acting in opposition (Kamin and Kertes, 2017; Phillips et al., 2007). A high concentration of cortisol and prolonged exposure to this condition, as in chronic stress, usually cause immunosuppression. Glucocorticoids block T-cell proliferation, reduce the extravasation of inflammatory cells, and cause the decrease of proinflammatory molecules (IL-1, TNF, GM-CSF, IL-2, IL-3, IL-4, IL-5, IL-8, prostaglandins, leukotrienes) and the apoptosis of lymphocytes and eosinophils. On the contrary, DHEA(S) stimulates the production of proinflammatory cytokines (IL-2; IL-3; IFN-γ), increase T-cell number and cytotoxicity, decreases the apoptosis of peripheral blood mononuclear cells and enhanced neutrophil function (Kamin and Kertes, 2017; Phillips et al., 2007). However, we cannot exclude that other factors, such as exercise, could have influenced our results. For example, in humans, moderate and regular exercise could ameliorate the detrimental effect of chronic stress on health and immunity (Dhabhar, 2014; Phillips et al., 2007), and this can be the case with the horses in our study that were all employed in different sporting activities. In the same article, we did not find any difference in DHEA and cortisol levels in plasma. This can be caused by the acute stress of capture and sampling procedures (Whitham et al., 2020), which can have masked what we have found in horsehair. Indeed, in welfare studies, the employment of non-invasive sampling assessing these hormones' concentration in alternative matrices is generally preferable (Whitham et al., 2020). In the second article we found higher cortisol:DHEA ratio in the plasma of horses that showed behavioural problems than in "healthy" horses, but the same results were not found in horsehair. In humans, higher cortisol:DHEA ratio has been associated with anxiety and acute stress response in long-term stressed subjects (van Niekerk, Huppert and Herbert, 2001; Kamin and Kertes, 2017) and the same could be in horses. Indeed, the undesired behaviours of horses are often due to anxiety, such as separation anxiety or avoidance behaviours, and the solutions that owners implement are often punitive and increase the state of anxiety (Hothersall and Casey, 2012). However, why we did not find the same result in horsehair can have several explanations. As explained in the article, several influential factors could have altered the dosage of this hormone in horsehair, such as the colour of the matrix or the age and sex of the animals (Lelláková et al., 2022; Whitham et al., 2020). In addition, as stated above, the manifestation of stereotypical behaviours is not necessarily the expression of a present suboptimal environment, but can develop in a past condition and persist in the present even if the causal factor has ceased (Mason and Latham, 2004). Indeed, several studies that try to correlate glucocorticoids and stereotypes have led to contrasting results. Some of them support the thesis that stereotypies can have a coping and adaptive role, reducing stress physiological response and cortisol levels (McBride and Cuddeford, 2001; Pell and McGreevy, 1999). Other researchers do not recognise the same coping role of these behaviours. For example, one study did not find a difference in plasma and faecal cortisol levels of stereotypic and non-stereotypic horses in the same sub-optimal environment, and another research found higher plasma cortisol concentration in stereotypic animals than in non-stereotypic ones (Fureix et al., 2013; McGreevy and Nicol, 1998). We cannot exclude that the coping activity of stereotypies can have influenced the hormonal concentration in horsehair. However, further research is needed to deepen this aspect and the use of horsehair as a matrix in the evaluation of DHEA. In addition, evaluating all the possible influential factors and considering separately oral and locomotor stereotypies and other undesired behaviours could help to clarify these results.

To have a better overview of horse welfare in different management systems, we have considered also the animals' affective dimension, which was evaluated by employing a spatial go/no-go judgement bias test. Also, in this case, we have decided to consider more than one parameter: faecal and horsehair cortisol to understand the activation of the HPA axis (Duran et al., 2017; Palme, 2012) during training and test procedures and in the long-term; BCS that can give information about health status, welfare, and, potentially, food motivation of horses (Henry et al., 2017; Lesimple, 2020; Yngvesson et al., 2019); and personality traits. In our first research, we applied the spatial task proposed by Henry et al. (2017) and Briefer Freymond et al. (2014). We compared the JBT results in three groups of animals that were managed in three different management systems (natural boarding system, traditional stable and Ethological stable). Even if these systems are really different from each other, we did not find any difference in the response to the three ambiguous cues, except for the Ethological stable horses that were the fastest in reaching the near negative ambiguous position, showing a more optimistic bias. However, this result should be interpreted considering that this group was less numerous than the others. In addition, several factors that could have influenced the JBT emerged. First of all, the position of positive and negative training cues and food motivation could have influenced the exclusion of horses during the training. Furthermore, faecal cortisol measurement suggests that horses from the natural boarding system were more stressed during the entire JBT procedure than traditional stable horses. Indeed, testing individually social animals that are used to living in a group can induce stress and this can alter JBT responses or their learning ability during the training (Roelofs et al., 2016). Because of these potentially influential factors, we have decided to deepen the use of this kind of test to assess the affective state in horses considering one group of subjects housed in the same stable. Contrary to the proceeding study, we found that behavioural stress parameters observed during the entire JBT procedure and faecal cortisol levels did not influence JBT results, suggesting that this test had not induced particular stress in these animals. Probably, in this case, horses, which lived in individual paddocks and stalls of a riding school, were more used to working individually and being handled by different people. However, similarly to the previous results, the positions of training Positive and Negative cues seem to have influenced the speed of approaching Near Negative and Middle ambiguous cues. Considering together the results of our articles, it cannot be excluded that in horses the phenomenon of visual lateralization could influence the performance of horses in this spatial task. Indeed, horses use a preferred eye according to the emotional value of the object that they are analysing and visual lateralization can affect also the rapidity of response (Austin and Rogers, 2007; De Boyer Des Roches et al., 2008; Farmer et al., 2010; Rogers, 2002). Furthermore, our last research showed that also some personality traits can influence the judgement bias of the subjects. This result is not necessarily in contrast with the previous study where we did not find any correlation between personality traits and latency to reach ambiguous cues. Indeed, we used in the first article the HPQ (Horse Personality Questionnaire) by Lloyd et al. (2007), while in the second project we preferred the E-BARQ (Equine Behaviour Assessment And Research Questionnaire), which was validated in 2020 (Fenner et al., 2020) just before the beginning of our research. Individual motivation and personality traits can influence judgement bias and should be always taken into account during the interpretation of JBT results (Roelofs et al., 2016). Interesting to notice that both our research did not find any relationship between horsehair cortisol concentration, potentially indicative of chronic stress conditions, and JBT results. However, both articles show that the use of a spatial go/no-go JBT in horses has several limitations. In addition, in the first research, only 20 out of 41 horses passed the habituation phase, reached the learning criterion and completed the JBT. This high rate of exclusion can limit the generalization of results (Roelofs et al., 2016). In the second research, only two out of 14 animals did not pass the habituation and training, probably because we did not pose any limits on these phases. However, extending the habituation and training phase could have an enrichment effect inducing a positive affective state and influencing the judgement bias of animals. In addition, a prolonged pre-testing phase can make this test difficult to apply in welfare studies (Mendl et al., 2009; Roelofs et al., 2016). To find an alternative to a spatial task, we tried to employ a visual task in the same group of horses that were involved in the second article, but these results remained unpublished. In this task, the animals have to learn to distinguish between white and blue boxes containing or not a food reward. For the three different ambiguous cues were created three intermediate colours (25% blue + 75% white, 50% blue + 50% white, and 75% blue + 25% white). Any of the horses reach the learning criterion during the training phase, even if horses can distinguish blue and white (Blackmore et al., 2008). Probably, in this case, they would need an extensive training period, which, as previously stated, is problematic. Indeed, horses seem to give particular importance to the positions of objects and spatial cues are more discriminable than other characteristics, such as colour or shape. In front of a discrimination task, horses learn more easily and faster using spatial cues than object characteristics (Hothersall et al., 2010; Nicol, 2002). This probably finds an explanation in their evolution. Indeed, having familiarity with the surrounding space leads to several advantages, for example, finding resources, such as food, or a place where to escape from predators. In addition, knowing familiar routes can improve the ability to move safely and more rapidly (Nicol, 2002). Therefore, differently from other species, like hens or calves (Daros et al., 2014; Deakin et al., 2016), employing visual discrimination tasks in horses' JBT is not recommended. Also, this result underlines the importance to take into account species-specific characteristics in the development of a JBT

5. CONCLUSIONS AND FUTURE RESEARCH

This PhD research project has underlined the importance of considering several parameters and having a multidisciplinary approach in the study and evaluation of welfare, considering health, natural life and affective dimension. Evaluating behaviour, endocrinological parameters and immune activity can be useful to explore sub-optimal welfare conditions and identify chronic stress situations. The DHEA(S) can be a promising indicator that can help to examine the HPA axis activation. Its measurement in horsehair proposed in this thesis can be advantageous to examine long-term conditions through a non-invasive sampling procedure. Future research is needed to deepen the use of this matrix in DHEA measurement and which are potential influential factors. The biological role, synthesis and regulation of DHEA in horses need further clarification, focussing on its potential role in stress and immune regulation and its impact on cognition and emotional dimensions. The affective state should be always considered in welfare research, but its evaluation in non-vocal animals is challenging. The judgement bias test can be a useful tool to investigate affective states in non-human animals, considering the strict relationship between affective and cognitive dimensions. However, in horses, this kind of test has not been standardized. According to our results, a specific test should be designed considering both species-specific and individual characteristics. The employment of visual cues is not recommended for horses, but also a spatial task has demonstrated some limitations.

The different management systems of horses should be taken into account the behavioural needs of these animals, paying particular attention to their sociality, the possibility of free movement, the diet composition and foraging routine. In addition, the type of activity and amount of work that humans require from horses can affect their welfare. Because still today housing and management systems do not always fulfil the species-specific needs of these animals, the implementation of legislation that regulates both management and activity of horses could be the next step for the protection of horse welfare. However, it should be considered always the individual. Different subjects can have different coping strategies in the same environment and their biological features (i.e sex and age), health status, social position, motivations, personality and life experiences can influence not only physiological, immunological and behavioural parameters, but also their emotional and cognitive dimension

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