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Characterising stereotypies in horses: physiology, personality, learning abilities and ontogeny

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PhD Thesis submitted to:

The Faculty of Sciences, Institute of Biology
University of Neuchâtel, Switzerland

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Defended on the 29th of October 2018

10.35662/unine-thesis-2735

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Titre:

“Characterising stereotypies in horses: physiology, personality, learning abilities and ontogeny”

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Neuchâtel, le 4 février 2019

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SUMMARY

Stereotypies are characterised as repetitive, relatively invariant patterns of behaviour usually defined as having no apparent ultimate and proximal function. They exist in humans and also in a wide range of animal species in captivity. Animal welfare being of increasing public and scientific concern, it is important to assess whether or not stereotypies are adaptive, maladaptive or both, and thus to assess the consequences of such behaviour. Domesticated horses are an interesting species to study stereotypies, also because of the considerable economic implications of these behaviours. The most prevalent stereotypies in horses are crib-biting and windsucking, which were hypothesised to be caused primarily by current feeding practices.

The aim of this PhD thesis was to address open questions regarding stereotypies in horses, that is, their physiology and function, as well as their relation with personality and cognition. To investigate the physiology and function, I first compared crib-biting with normally behaving horses using an ACTH-challenge test. My results showed, firstly, that crib-biters reacted with a higher cortisol release than non-stereotypic horses, suggesting a higher sensitivity to stress, which could be linked to a potential genetic predisposition for stereotypic behaviour. Additionally, some crib-biters that did not perform the stereotypic behaviour during the ACTH-challenge test may have a higher cortisol levels than the controls, which was not the case for crib-biters showing stereotypic behaviour. I concluded that crib-biting might be, for some horses at least, an adaptive coping strategy helping individuals to reduce cortisol levels caused by stressful situations.

In a third chapter, I compared the personality profile of crib-biting and non-stereotypic horses, using five personality traits: reactivity to humans, tactile sensitivity, fearfulness/curiosity, social reactivity and locomotor activity. My results indicated that crib-biters were more sensitive to tactile stimulation than non-stereotypic horses. I suggested that this higher tactile sensitivity could be due to altered dopamine physiology, resulting from chronic stress exposition.

In order to assess the consequences of crib-biting behaviour, I conducted two different studies (Chapters IV and V) using cognitive tests, which serve as indirect measures of some aspects of the *basal ganglia* dysfunction, a neural structure, that has been suggested to be altered in stereotypies. In both studies, the performance of crib-biters during the various cognitive tasks was similar to the performance of non-stereotypic horses. Although in Chapter IV, all the tests involved spatial problems, with limited training, which may be ecologically relevant for horses and easier to solve, the results of Chapter V, challenge the hypothesis that crib-biting horses might be not flexible in learning tasks. Indeed, in Chapter V, all the crib-biters performed two reversal learning tasks, considered as measure of flexibility of learning. According to this result together with the results of previous studies, a new hypothesis can be risen suggesting that the *accumbens* reward pathway might be affected in crib-biters and not the *dorsal loop* as previously suggested. Such a dysfunction could be the cause of the higher stress sensitivity reported in the first study, conducted in the PhD, and would have no impact on reversal learning tasks.

Overall, my results seem to suggest that stereotypic behaviour is an adaptive coping strategy helping affected individuals to reduce physiological stress states, something that is especially prevalent in captivity. I thus conclude that it is counterproductive to prevent horses from executing crib-biting behaviour once the stereotypy has developed, but that it may be more advisable to alter their environment in adequate ways, such as by providing opportunities to display more natural behaviours. However, the ontogeny of stereotypic behaviours still remains elusive and further studies are needed to assess how they emerge.

Keywords: stereotypies, *Equus caballus*, ACTH challenge, cortisol, stress, personality, basal ganglia, learning capacities

RESUME

Les stéréotypies sont caractérisées par des comportements répétitifs, de formes constantes et sans objectifs ou buts apparents. Ces comportements se retrouvent chez les humains, ainsi que chez un grand nombre d'animaux détenus en captivité. Etant donné que le large publique et le domaine scientifique sont de plus en plus concernés par le bien-être animal, il est important d'investiguer si les stéréotypies sont des comportements adaptifs et/ou une atteinte neurologique, ainsi que les conséquences d'un tel comportement sur l'organisme. Le cheval domestique est une espèce intéressante pour étudier les stéréotypies car il est détenu en captivité et utilisé, donc potentiellement exposé à des situations de stress important ou stress chronique, qui semblent être à l'origine du développement de ces comportements. La stéréotypie la plus fréquente chez le cheval est le tic à l'air, dont les causes principales sont supposées être liées à la gestion de l'affouragement (type d'aliment, fréquence des repas) dans la détention de chevaux. Le but de cette thèse a été d'investiguer des questions ouvertes sur le tic à l'air chez le cheval, en lien avec la physiologie, la fonction, la personnalité et la cognition.

Afin d'étudier le lien avec la physiologie et la fonction de ce comportement, j'ai tout d'abord comparé des chevaux tiqueurs avec des chevaux non-stéréotypés dans un test ACTH challenge. Mes premiers résultats montrent que les chevaux tiqueurs ont sécrété une plus grande quantité de cortisol, en réponse au test, que les chevaux non-stéréotypés, et laissent donc penser que ces derniers sont plus sensibles au stress. Cette caractéristique pourrait être liée à une potentielle prédisposition génétique ou à une exposition à un stress chronique. Par ailleurs, dans cette étude, certains chevaux tiqueurs n'ont pas tiqué durant le test. Des analyses statistiques supplémentaires montrent que ces derniers ont en réalité sécrété une quantité beaucoup plus importante de cortisol que tous les autres chevaux. La conclusion de cette première étude est que le tic à l'air semble être, du moins pour certains chevaux, une stratégie adaptive les aidant à diminuer leur stress physiologique.

Dans une deuxième étude, j'ai comparé le profil de personnalité de chevaux tiqueurs et non stéréotypés pour cinq traits de personnalité: la réaction à un humain inconnu passif, la sensibilité tactile, la peur et/ou la curiosité, la réactivité sociale et l'activité locomotrice. Mes résultats indiquent que les chevaux tiqueurs se sont montrés plus sensibles aux stimuli tactiles que les chevaux non-stéréotypés. L'hypothèse qui pourrait expliquer cette plus grande sensibilité tactile serait une modification du système dopaminergique due à une exposition à un stress chronique.

Afin d’investiguer les conséquences du tic à l’air, j’ai ensuite conduit deux études différentes (Chapitres IV et V) en utilisant des tests cognitifs, qui servent de méthode indirecte pour mesurer certains aspects d’une dysfonction des *ganglions de la base.* Ce sont des structures neurales supposées être altérées dans les stéréotypies. Dans ces deux études, la performance des chevaux tiqueurs, dans des tests d’apprentissage variés, était similaire à celle des chevaux non-stéréotypés. Bien que, dans le Chapitre IV, les tests étaient tous uniquement constitués de tests d’apprentissage spatiaux, contenant un nombre d’essais limités et, par conséquent, facile à résoudre, les résultats du Chapitre V remettent en question l’hypothèse que les chevaux tiqueurs ne sont pas flexibles dans l’apprentissage. En effet, dans le Chapitre V, tous les chevaux tiqueurs ont réussi deux apprentissages inverses, considérés comme la mesure d’une potentielle dysfonction dans la flexibilité de l’apprentissage. En accord avec ces résultats et ceux des études précédentes, une nouvelle hypothèse peut être émise suggérant que seule une partie des *ganglions de la base* serait affectée chez les chevaux tiqueurs: la voie méso-limbique (au niveau du *nucleus accumbens*). Cette hypothèse contredit les théories précédentes qui supposaient qu’une autre partie des *ganglions de la base,* responsable du développement des stratégies d’habitude, était également impliquée dans le tic à l’air: le *striatum dorsal*. Une telle dysfonction pourrait également expliquer une plus grande sensibilité au stress, comme reporté dans la première étude de cette thèse (Chapitre II), et n’aurait ainsi aucun impact sur la flexibilité dans l’apprentissage.

Globalement mes résultats concordent sur le fait que le tic à l’air serait une stratégie développée pour réduire le stress physiologique, plus important chez les chevaux tiqueurs. Ainsi, le tic à l’air serait une stratégie adaptive. Je conclus finalement qu’il semble être contre-productif d’empêcher les chevaux de tiquer, une fois que le tic à l’air est développé, et qu’il serait en revanche plus adapté d’améliorer l’environnement en offrant l’opportunité aux chevaux d’exprimer plus de comportements naturels et donc de satisfaire leurs besoins. Toutefois, l’ontogénie des comportements stéréotypés reste une thématique importante à étudier dans le futur.

Mots clefs: stéréotypies, *Equus caballus*, test ACTH challenge, cortisol, stress, personnalité, ganglions de la base, performances d’apprentissage.

CONTENTS

CHAPTER I

GENERAL INTRODUCTION

DEFINITION OF STEREOTYPIES

Stereotypies are repetitive, relatively invariant patterns of behaviour usually defined as having no apparent ultimate and proximal function (Mason and Rushen, 2006) Stereotypies also exist in humans, can be either psychologically or environmentally induced, and are often associated with developmental disorders, such as autism, neurological disorders, obsessive compulsive disorder (OCD), Tourette's syndrome and severe psychiatric disturbances (e.g. schizophrenia) (McBride and Parker, 2015). In animals, stereotypies are commonly observed in a wide range of species, but so far only in captivity. They exist in various forms and are mainly caused by environmental deprivation. Cage-induced stereotypies exist among the 1,700 rodent species in captivity (kept in zoos or laboratories, or as pets), such as field voles (Fentress, 1976), bank voles (Ödberg, 1987), chinchillas, gerbils and different species of mice (Würbel and Stauffacher, 1994). Rodents show different forms of stereotypies, including bar-mouthing in mice, gerbils, bank voles and hamsters, jumping in mice, and digging and looping in gerbils and mice (Würbel and Stauffacher, 1994). In primates in captivity, the form and prevalence of stereotypies varies greatly across species. Self-injurious behaviour occurs from prosimians to apes (Trollope, 1977). Primate species also show regurgitation, reingestion and coprophagy behaviours. Captive carnivores commonly show locomotor stereotypic behaviours. Such stereotypies can change in form over time as the behaviour develops. Figure-eight pacing, for example, can develop from pacing back and forth along a fence line (Clubb and Mason, 2003). Locomotor stereotypies can exist in the form of swimming in circles and weaving from side to side as well (Hunter et al., 2002). Captive ungulates commonly show oral stereotypic behaviours, even if locomotors behaviours like weaving and pacing ("box walking") are common in horses. Oral stereotypic behaviours include bar-biting and sham-chewing by sows, tongue rolling by cows and crib-biting or windsucking in horses (Sambraus, 1985; McGreevy et al., 1995). Such oral stereotypies did also occur in exotic ungulates in zoos; for example object licking in antelopes (Ganslosser and Brunner, 1997) and tongue rolling in giraffes and okapi (Koene, 1999).

CAUSES OF STEREOTYPIES

Stereotypies are assumed to develop in the context of chronic stress, resulting from environmental restriction of species-specific behaviours (Cabib et al., 1998; McBride and Hemmings, 2005). Chronic stress is known to occur when a situation exceeds the natural regulatory capacity of the organism for an extended period, particularly in situations that include unpredictability and uncontrollability (Koolhaas et al., 2010). Chronic stress differs from acute stress, which can be defined as an animal's perception of a threat that challenges internal homeostasis and triggers behavioural and physiological adjustment to avoid or adapt to the stressor and return to homeostasis (Koolhaas et al., 2010). Moreover, the propensity to develop stereotypies seems to vary between individuals within a species, because of genetic predispositions and differences in strategies used to cope with stress.

GENETIC PREDISPOSITION

Some individuals seem to have a higher susceptibility to develop stereotypies than others, despite being exposed to similar environments. Such individual differences in propensity to develop stereotypies seem to be partially genetically determined. For example, in bank voles, there is a higher incidence of stereotypy performance in offspring of stereotyping mothers than in offspring of non-stereotyping mothers (Schoenecker and Heller, 2000). Evidence for a genetic determination of propensity to develop stereotypies also comes from strain or breed differences in form and level of stereotypy performance. In laboratory mice, differences in level of performance seem to be related to differences in general activity, which differs between strains. As a result, there seems to be a higher incidence of stereotypies in more active strains (Würbel and Stauffacher, 1994). In horses, despite the fact that no specific genes have been linked yet to stereotypies, oral stereotypic behaviour (i.e. crib-biting) has been reported more predominantly in certain breeds (Hosoda, 1950; Vecchiotti and Roberto, 1986). These studies concluded that the genetic transmission of this behaviour is similar to some human mental disorders involving polygenic inheritance. Recently, Hemmann et al. (2014) found an unusually high heritability of crib-biting in a small Finnhorse population, again suggesting that horses might inherit the behavioural susceptibility to develop such stereotypy. Moreover, behavioural frustration and expression of equine stereotypy seems to be linked with dopamine receptor, i.e. DRD4 (dopamine D4 receptor subtype) gene (McBride and Hemmings, 2005; Ninomiya et al., 2013). However, knowledge of the genes involved in the propensity to develop stereotypies is sparse, and more studies are needed on this topic.

COPING STYLE - PERSONALITY

Since genetic differences result in individual variation in behaviour (i.e. personality, behavioural syndromes or coping strategies), it is possible that predispositions to develop stereotypies originate from differences in the way individuals respond to chronic stress situations (Joshi and Pillay, 2016). According to the coping predictability concept, evolution has shaped general adaptive response patterns in response to everyday challenges in natural habitats. When encountering a sufficiently powerful stressor, individuals will adopt a certain coping strategy. Coping styles can be defined as a coherent set of behavioural and physiological stress responses that are consistent over time and vary between individuals (Koolhaas et al., 1999). We can distinguish the stress sensitivity, which determines the threshold at which a coping response is triggered (Koolhaas et al., 1999), and the coping response itself, which functions to minimise stress once the stress threshold is reached. There are two main different stress response patterns; proactive and reactive coping responses. In the presence of a stressor, proactive copers will try to escape or remove the stressor ('fight-or-flight' response), while reactive copers will show no sign of being affected (conservation-withdrawal response, (Seaman et al., 2002)). Overall, proactive individuals are characterised by lower levels of immobility, more aggression and more persistence than reactive individuals. By contrast, reactive individuals are characterised by low levels of aggression and more flexibility (Koolhaas et al., 1999). As a consequence, proactive individuals have difficulty coping with a variable environment that they cannot predict, and especially with false expectations and wrong outcome expectancy (Koolhaas et al., 2010). Coping strategies are also related to underlying physiology (Koolhaas et al., 2010). In reaction to stressor, two different pathways are activated, the hormonally based hypothalamo-pituitary-adrenocortical (HPA) axis and the neural sympatho-adreno-medullary (SAM) axis. Proactive individuals tend to have a lower HPA and higher SAM axis reactivity than reactive ones (Koolhaas et al., 2010). It has been proposed that stereotypic behaviour reflects a proactive coping response to stress and that, in reactive individuals, there is an opposite response, namely depression (Cabib and Puglisi-Allegra, 2012; Ijichi et al., 2013). However, results to date have been controversial and the coping function of stereotypies is highly debated.

ENVIRONMENTAL FACTORS

Environmental causes of the development of stereotypies are only partially known but thought to be linked to the stress state resulting from the need to perform highly motivated behaviours, due to environmental restrictions in captive conditions. Ethological models of behavioural motivation have been used to understand the causal and functional aspects of stereotypies (Hughes and Duncan, 1988). Research across a wide range of species suggests that stereotypies arise from the persistence and/or thwarting of highly motivated behaviours, largely unfulfilled under captive conditions. The type of unfulfilled behaviours that can lead over time to stereotypies depends on each species' internal motivations or needs. These unfulfilled behaviours defined as essential for the animal (i.e. behavioural or ethological needs), are mediated by reward systems (Hughes and Duncan, 1988). Such behaviours include, for example, foraging, retreat from aversive stimuli, and reaching a desired resource (e.g. food, novelty, potential mates or social partners). In rodents, the causes of stereotypies seem to emerge from more basic behavioural motivations, such as to gain access to conspecifics or to seek shelter, which leads to repetitive attempts to escape from the cage. In carnivores, highly motivated foraging behaviours that are usually unfulfilled in captivity include the appetitive search phase of the hunt, food searching, chasing or capture. In ungulates, stereotypies might originate from frustrated foraging behaviours. In fact, in nature, ungulates spend most of the time eating and moving from one food point to another, while in captivity, they are often given certain amounts of food that are consumed in half or even a third of the time dedicated to eat in the wild. The long periods of food restriction result in high levels of frustrated feeding motivation. As a result, captive ungulates often develop oral stereotypic behaviours that are similar in form and pattern to natural foraging, and that are often in close temporal association with food delivery (Mason and Mendl, 1997). The thwarting of highly motivated behaviours is thought to trigger sustained appetitive behaviours that seem highly resistant to habituation and are repeated over and over. At the same time, the stress engendered by the initial thwarting of highly motivated behaviour may facilitate stereotypy development through sensitising of the neural system. This may lead to dysfunction of the neural system involved in neural control (for more details, see below Section *brain dysfunction*).

An ontogenetic model of stereotypic behaviour

The mechanisms underlying the development of stereotypies could be summarised as follows; animal behaviours are driven by motivational states that are determined by an array of internal and external factors. Motivated behaviours often have an appetitive preparatory phase, followed by a species-typical consummatory phase. If the consummatory phase is not attained, the internal motivation to consume can normally be reduced through negative feedback, such as the performance of the appetitive behaviour. The failure of such negative feedback loop is often thought to be the cause of stereotypies. Such failure can occur when constraints applied by the captive environment regularly leave animals in a state of high motivation to perform an unfulfilled behaviour, which results in frustration-related stress states. A number of behavioural phenomena can emerge from such states, such as "intention behaviour" (e.g. restless attempts of migratory birds confined to a cage (Dawkins, 1988)), “redirected movements” (e.g. sucking that calves direct to pen-mates in the absence of a teat (Jensen, 2003)), “vacuum activities” (e.g. “mimed” dustbathing movements of hens kept on bare wire floors (Lindberg and Nicol, 1997)), and “displacement activities” (e.g. “out of context” preening by birds during conflict situations (Tinbergen, 1952)). If sustained, such activities could develop into stereotypies. In addition, more complex patterns of feedback could also be involved and the performance of appetitive behaviours per se may also serve to reduce motivation, while the performance of consummatory behaviours could sometimes instead increase the motivation via positive feedback (Toates, 2001). Overall, three possible mechanisms have been proposed to underlie the stereotypies: high, maintained motivation for an unfulfilled behavioural sequence; lack of inhibitory feedback to decrease the motivation; and positive feedback loop, in which the performance of the stereotypy is self-reinforcing, and dominates the action selection process.

BRAIN DYSFUNCTION

The neurobiological bases of sensitisation may represent a common substrate for different mental and biological disturbances across species (e.g. mouse stereotypies, schizophrenia, addiction in humans (Cabib et al., 1998). However, such pathological adaptation could also occur in caged animals showing no stereotypic response.

The proximate mechanisms underlying the performance of stereotypies have been studied notably by testing the capacity of psychostimulants to induce these repetitive behaviours. Randrup and Munkvad (1967) were the first to show that systemic amphetamine (dopamine agonist) administration induced stereotypies in a range of species (including humans). Later on, other studies showed that administration of dopamine antagonists could attenuate spontaneous stereotypies. This was shown in a range of species, including bank voles, chickens, pigs and horses (McBride and Hemmings, 2005; McBride and Parker, 2015). It has been shown that psychomotor stimulant drugs, such as amphetamine and apomorphine, can directly induce stereotypic behaviour by activating the dopaminergic system in the *basal ganglia*. The behaviour becomes more stereotyped, intense and rigid with increasing doses of the drugs (Lyon, 1975). For example, dopamine or a dopamine agonist (i.e. elevated dopamine, e.g. apomorphine) injected into the *corpus striatum* part of the *basal ganglia* induced stereotypic behaviour in rats (Ernst and Smelik, 1966). On the other hand, intrastriatal administration of glutamate receptor antagonist (i.e. reduced dopamine) n-methyl-D-aspartate could attenuate some stereotypies (Karler et al., 1997). Therefore, the *basal ganglia* was demonstrated to be involved in the mediation of repetitive behaviours (Ernst and Smelik, 1966). Direct physiological measurements did also show the association between stressors, stereotypy development and alterations to dopamine receptor function in the *basal ganglia*. Receptor-based alterations were shown, for example, in rodent models of spontaneous stereotypy (Cabib et al., 1998) and in crib-biting horses (McBride and Hemmings, 2005; McBride and Hemmings, 2009). In addition, it was shown that pharmacologically induced stereotypies were aggravated by stress in many species (Randrup and Munkvad, 1967; Wei-Min et al., 2008). However, until now, it is not known if such alteration are present before or after the emergence of stereotypic behaviour (Roberts et al., 2017).

DEVELOPMENT OF STEREOTYPIES

EMANCIPATION - ONTOGENY

Stereotypies develop over time until they become fully established or emancipated and are then difficult to eliminate (Nagy et al., 2010). It is thought that animals learn to associate a behavioural response (response to initial conflict or thwarting) and some positive outcome (e.g. performance of appetitive behaviours). They then become highly motivated to use this strategy. Over time, this strategy can extend to situations other than the originally eliciting one (Nagy et al., 2009). At this point, the stereotypic behaviour is considered to be emancipated and it is then fixed and difficult to eliminate (Nagy et al., 2010). However, the reason why stereotypic activities become so time-consuming or ritualistic remains unknown. This question could be investigated using longitudinal studies of stereotypic behaviour. Yet, such studies, which would also allow to determine when a stereotypy is fully established or not, are lacking (Pell and McGreevy, 1999; Clegg et al., 2008; Wickens and Heleski, 2010). The comprehension of the evolution of the frequency of the performance of the stereotypic behaviour over time is also missing. It is therefore unknown if, for example, this frequency changes over time according to the developmental stages of the stereotypy or not.

CONSEQUENCES OF STEREOTYPIES

BRAIN DSYSFUNCTION - LEARNING ALTERATION

A deeper understanding of the role and function of the *basal ganglia* may help to provide a more complete understanding of the causes and consequences of stereotypies. Due to its primary role in allowing animals to learn about outcomes associated with objects, events and situations, and to select the most appropriate motor response for survival (action selection), dysfunctions of the *basal ganglia* might correspond to aberration in motivation during learning task performance (McBride and Hemmings, 2009). The *basal ganglia* plays an important role in instrumental learning, which consists in a transition from goal-directed control, also called response-outcome learning, toward habitual control, also termed automatisation, following extended practice (McBride et al., 2017). Response-outcome learning involves encoding the causal relationship between a response to a stimulus and the motivational value of the outcome (McBride et al., 2017), while habitual control, also called stimulus-response or "habit" learning, involves encoding the relationship between a stimulus and consecutive responses, independently of the outcome of the response (McBride et al., 2017). Action selection, which allows the animal to execute the most appropriate action (action selection) in the presence of different cues, is heavily affected by the learnt associations between previous actions and rewarded or aversive events (McBride and Parker, 2015). Once learnt, the cues grab attention and elicit motivation to re-attain the same goal. A dysfunction in the *basal ganglia* leads to behavioural abnormalities, as a result of an impaired response selection. The animals can no longer perform or acquire actions, in order to earn specific reward or avoid aversive stimuli (Yin and Knowlton, 2006).

ASSESSMENT OF LEARNING ALTERATION – INDIRECT BEHAVIOURAL MEASURES

Cognitive tests and/or indirect behavioural observations or measures have been developed to understand the consequences of stereotypies, given the ethical dimensions of direct physiological measurements (McBride et al., 2017). In this area, many studies have shown that for example, stereotypy levels (frequency of stereotypy performance) are strong predictors of the latency to extinguish a conditioned response (i.e. previously learnt response), called perseveration (Garner and Mason, 2002). Such results were shown in bank voles (Garner and Mason, 2002), bears (Vickery and Mason, 2005) and in Orange-Wing Amazon Parrots (Garner et al., 2003) and horses (Hemmings et al., 2005) . Similarly, impaired *basal ganglia* function, primarily in terms of dopaminergic regulation were implicated in human mental disorders stereotypies, such as schizophrenia, autism and induced by drugs (e.g. amphetamine). In autistic subjects, poor abilities to suppress behaviour (perseveration), due to a *basal ganglia* dysfunction, have been shown, although high stereotypic levels do not always correlate with higher perseveration (Lopez et al., 2005; Boyd et al., 2009). Other behavioural changes, such as an enhanced rate of behavioural initiation (impulsivity), have been shown to be associated with stereotypies in humans. These changes have been attributed to a general disinhibition of behavioural control mechanisms in the *dorsal basal ganglia* (Garner, 1999; Garner and Mason, 2002). Because dopamine agonists also increase the rate of behavioural initiation (i.e. the number of behavioural transitions performed in a given period) (Garner and Mason, 2002), measurements of this phenomenon have been used to understand the neural pathways implicated in stereotypy performing animals. Such measurements have been used for example in horses performing locomotor and oral stereotypy, behavioural initiation rate (BIR) (Roberts et al., 2015). Other basic measures of dopamine transmission, recording the spontaneous blink rate (SBR), used to determine striatal functioning in stereotypy performing humans were also applied to animals (Roberts et al., 2015).

FUNCTION OF STEREOTYPIES

CONSEQUENCES FOR WELFARE DECISIONS

It is commonly argued that stereotypies have no obvious function (Mason and Latham, 2004). However, some studies propose that stereotypic behaviour may serve as a coping mechanism, hence reducing stress or providing the animal with some form of control over its environment (Mason, 1991). However, while stereotypies may develop to cope with frustration, they often persist after the environmental stress has ceased. If stereotypies are indeed a coping mechanism, the animals should show signs of stress in a frustrating situation in which the execution of the abnormal behaviour is blocked by prevention methods (Nagy et al., 2009). However, results of experimental studies, which used prevention methods, on the association between stereotypic behaviour and stress-coping functions, have been contradictory to date (McGreevy and Nicol, 1998; McBride and Cudderford, 2001; Mason and Latham, 2004; Wickens and Heleski, 2010; Sarrafchi and Blokhuis, 2013). Animal welfare being of increasing public and scientific concern, it is important to assess whether or not stereotypic behaviour has an adaptive function (e.g. coping strategy) and/or if it is a malfunction or maladaptation, and to assess the consequences of such behaviour.

A more balanced view is that stereotypic behaviour might be beneficial, provided it is performed during only a small percentage of time, while it should be considered as a malfunction when it is performed during a high percentage of time, and therefore prevents the execution of other important behaviours. Additionally, it has been suggested that stereotypic behaviour should correlate with malfunction behaviour or behavioural pathology, resulting in "bad welfare", if dopaminergic dysfunction is present (Mason and Latham, 2004). In their review, Mason and Latham (2004) suggest taking into account the degree of perseveration of animals to determine whether stereotypies could be considered as maladaptive or pathological, in terms of being caused by a dysfunction of the nervous system. As consequence, more studies are needed in this area, to help to determine whether stereotypies should be considered as adaptive and/or maladaptive in different species and perhaps also between animals of the same species. In fact, there seems to be a large heterogeneity in the consequences of stereotypies between and among species.

ANIMAL MODEL: HORSES

LIFE STRATEGY – DOMESTICATION

Horses are social, steppe-dwelling animals. They rely on early predator detection and flight as primary defence mechanism. For this reason, their survival strategy relies on the formation of cohesive and stable social bonds (Mills and Nankervis, 2013). In feral conditions, family band size can vary from 2 to 35 horses (Boyd and Keiper, 2005). Solitary males usually form bachelor bands not exceeding 17 individuals (Boyd and Keiper, 2005). According to Hale (1969), horses were easily domesticated for the following reasons; 1) they have a social organisation with a clear dominance hierarchy and a harem system; 2) offspring are precocious and stay for a relatively long period of time (2-3 years) with both parents; 3) they are highly susceptible to dietary and environmental stressors. Changes due to domestication can be found in decreased reactivity and flight distance when exposed to fearful situations and increased adaptation to human husbandry systems (Price, 1999). Above all, the response thresholds at which animals show their defence mechanisms or stress reactions have changed whereas the basic organisation of behaviour has not (Price, 1999; Mills and Nankervis, 2013). It has been suggested that horses could easily go back to the wild life, because species-specific behavioural and physiological characteristics have remained relatively unchanged (Koene and Gremmen, 2002).

STEREOTYPY IN HORSES

In horses, different forms of stereotypies exist. Common are two locomotor stereotypies, *weaving* and *box walking* and two oral stereotypies, *crib-biting* and *windsucking behaviour.* The most prevalent stereotypy in horses is crib-biting behaviour. A crib-biting horse grasps a fixed object with its incisors, pulls back, and draws air into its oesophagus, without evidence of air passing it into the stomach, while emitting a characteristic pharyngeal grunt. Windsucking is an essentially similar behaviour, but it occurs without the grasping of an object. The prevalence of crib-biting and windsucking among horses reported in Europe and Canada is 2.4% - 8.4% (Wickens and Heleski, 2010). The domesticated horse is an interesting model to study stereotypies, because horses are often confined, individually with poor movement for extended period considered as severe restriction for this species (Williams and Randle, 2017) and therefore might develop stereotypy. Moreover, a better understanding of stereotypic behaviour and its consequences is also a very important theme for this species, because the appearance of stereotypic behaviour also has financial consequences for the horse owners, in term of loss in economic value. Such loss results from a common public perception or assumption that stereotypic behaviour is related to impaired performance (Randle, 2018), as well as with higher incidence of gastric ulcers, colic and dental pathologies (Marsden, 2002; Nicol et al., 2002; Archer et al., 2004; Wickens and Heleski, 2010; Wickens et al., 2013). However, the causal or correlational association between presence of stereotypies and risks of developing colic or gastric ulcers, and whether stereotypies are in fact a response to stomach pathology, remains not clear (Bachmann and Stauffacher, 2002; Wickens et al., 2013; Williams and Randle, 2017).

Several studies have been carried out on horse stereotypies. Management and feeding practices have been identified as the main factors influencing aspects of normal behaviour such as foraging, and contribute to the development of redirected or oral stereotypic behaviour. The initiation of crib-biting or windsucking is thought to be associated with diet and feeding management (Nicol, 1999; Roberts et al., 2017). Grazing fibrous food is the predominant activity of free-living horses; studies in feral and primitive Przewalski's horses under near-natural conditions reported it to be performed during around 52% of the time (Nicol, 1999). Horses are therefore adapted to eat forage for the majority of the time. However, under domestication eating habits of stabled horses are limited by the timing of meal and forage provision (Hothersall and Nicol, 2013). However, it was shown that crib-biting behaviour increases on a low-forage or high-grain diet (McGreevy et al., 1995; Bachmann et al., 2003; Nicol et al., 2005). By contrast, the use of straw bedding, which could serve as an additional source of dietary fibre, helps to decrease the development of stereotypic behaviour (McGreevy et al., 1995; Christie et al., 2006). For example, a kilo of hay is eaten by chewing 3,400 times in 40 minutes, while the same weight of oat is consumed in 10 minutes with 850 jaw movements (Hothersall and Nicol, 2013). Since chewing gives the opportunity to moisten food with alkaline saliva essential for digestion, it has been proposed that wood-chewing, which could be considered as a redirected behaviour, and crib-biting, are attempts to stimulate saliva production and reduce acidity in the stomach (Nicol, 1999). Chewing should indeed be especially important in horses that, as herbivores, secrete acid into the stomach continuously (Hothersall and Nicol, 2013).

THESIS OBJECTIVES

The aim of my PhD was to characterise horse crib-biting behaviour, the most prevalent stereotypy in horses. In order to extend knowledge in this domain, I investigated the function of this behaviour, if individual variation in behaviour may predispose animals to develop crib-biting behaviour, the consequences of this behaviour and the ontogeny.

In the course of my PhD project, I investigated the following characteristics of *crib-biting and windsucking* behaviour in horses

1. The physiological reaction of crib-biting horses, using a standard ACTH challenge test, in order to better understand the functions of stereotypies;
2. The personality and/or coping style of crib-biting horses;
3. The consequences of crib-biting for spatial learning tasks;
4. The consequences of crib-biting for more complex visual learning tasks
5. The development of stereotypic behaviour during weaning

My PhD thesis is divided in four data chapters (Chapters II–V), each designed to address one of the above questions, plus an annex to partially address the fifth question (Annex 1). Some of the individuals were tested in several Chapters (II – V); see Table 1, Annex II). In Chapter II, crib-biters and non-stereotypic horses were tested in an ACTH challenge test, with the aim of determining differences in physiological reaction linked to the stereotypy (Briefer Freymond et al., 2015). In Chapter III, horses were subjected to several personality tests, in order to establish whether a certain personality profile might predispose horses to develop stereotypies (Briefer Freymond et al., in preparation). In Chapter IV, horses were exposed to several challenging tasks potentially inducing stress and frustration, in order to test their capacity to solve tasks under these conditions (Briefer Freymond et al., in preparation). In Chapter V, horses were tested in an even more challenging cognitive task, based on visual cues, in order to assess the consequences of crib-biting on learning performance, and whether this stereotypy could be linked to a dysfunction of the nervous system (Briefer Freymond et al., 2018). Finally, I followed some foals at weaning time and collected faecal samples, in order to assess by measuring faecal cortisol metabolites, if the foals with the higher stress level developed stereotypic behaviour (Annex I).

CHAPTER II

Based on:

Stereotypic behavior in horses function to reduce stress

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Published in Physiology and Behavior

ABSTRACT

Stereotypies are repetitive and relatively invariant patterns of behavior, which are observed in a wide range of species in captivity. Stereotypic behavior occurs when environmental demands produce a physiological response that, if sustained for an extended period, exceeds the natural physiological regulatory capacity of the organism, particularly in situations that include unpredictability and uncontrollability. One hypothesis is that stereotypic behavior functions to cope with stressful environments, but the existing evidence is contradictory. To address the coping hypothesis of stereotypies, we triggered physiological reactions in 22 horses affected by stereotypic behavior (crib-biters) and 21 non-crib-biters (controls), using an ACTH challenge test. Following administration of an ACTH injection, we measured saliva cortisol every 30 min and heart rate (HR) continuously for a period of 3 h. We did not find any differences in HR or HR variability between the two groups, but crib-biters (Group CB) had significantly higher cortisol responses than controls (Group C; mean ±SD: CB, 5.84±2.62 ng/ml, C, 4.76± 3.04 ng/ml). Moreover, crib-biters that did not perform the stereotypic behavior during the 3-hour test period (Group B) had significantly higher cortisol levels than controls, which was not the case of crib-biters showing stereotypic behavior (Group A) (B, 6.44±2.38 ng/ml A, 5.58±2.69 ng/ml). Our results suggest that crib-biting is a coping strategy that helps stereotypic individuals to reduce cortisol levels caused by stressful situations. We conclude that preventing stereotypic horses from crib-biting could be an inappropriate strategy to control this abnormal behavior, as it prevents individuals from coping with situations that they perceive as stressful.

Keywords: stress physiology; stereotypy; coping strategy

INTRODUCTION

Physiological reactions are triggered during both positive, rewarding stimuli and negative, aversive stimuli [1,2]. Normal physiological reactions are thought to impact positively on individuals due to the release of energy-mobilizing glucocorticoids (GCs) and behavioral diversification [3]. Following such reactions, two systems are activated to help the individual to regain homeostasis (or steady state [3]); the hormonally based hypothalamo-pituitary-adrenocortical (HPA) axis and the neural sympatho-adreno-medullary (SAM) axis. In response to increased physical and psychological demands, the adrenocorticotropin hormone (ACTH) is released from the anterior pituitary gland, subsequently triggering a release of glucocorticoids (cortisol) from the adrenal cortex, epinephrine (adrenaline) from the adrenal medulla, and norepinephrine (noradrenaline) from the sympathetic nerves.

Chronic stress (or "stress") occurs when environmental demands produce a physiological response that, if sustained for an extended period, exceeds the natural regulatory capacity of the organism, particularly in situations that include unpredictability and uncontrollability [2].Whether a threatening situation is perceived as a stressor, however, appears to differ between individuals due to variation in coping abilities [4]. Despite its adaptive fight-or-flight function on the short-term, long term or chronic release of stress hormones can be detrimental. If one or both axes are persistently activated [5], individuals can be affected by cardiovascular diseases, depression or immunosuppression. In captive and domesticated animals, chronic stress can be provoked by unnatural husbandry practices, such as early weaning, social isolation, or dietary restriction, which can negatively affect the HPA-axis [5,6]. It can also trigger stereotypies and other behavioral disorders, which can be used as indicators of welfare problems, if they persist after the situation of chronic stress [7–10].

Stereotypies have been defined as repetitive, relatively invariant, patterns of behavior with no apparent goal or function [11]. They occur in various forms and contexts and have been observed in a wide range of species in captivity. In horses and other ungulates, different forms of stereotypies exist, including crib-biting, windsucking, weaving and box-walking [12]. The performance of stereotypic behavior varies between horses in terms of the percentage of time occupied by the activity, as well as the vigor and the persistence of the behavior [13]. The prevalence of crib-biting or windsucking among horses reported in Europe and Canada is 2.4–8.4% [14,15]. The causes of stereotypies are difficult to identify but have been linked to chronic stress, management factors and genetic predispositions [7,9,14].

An important problem is whether or not stereotypic behavior has an adaptive function or whether it is a functionless behavioral abnormality [11,16]. For example, stereotypies may function to cope with high levels of frustration, but the fact that stereotypic behavior often persists after the cause of frustration has been removed contradicts this hypothesis [16]. Another line of argument is that stereotypic behavior functions as a coping mechanism to reduce chronic stress or to provide animals with some form of control over their environments [7,11,17–19]. The main prediction of this argument is that the physiological response of animals should increase if they are being prevented from displaying the stereotypic behavior in response to a frustrating situation [16,20]. To our knowledge, however, there is no agreement between studies regarding the coping function of stereotypies [18,20–22].

One way to understand the nature of stereotypic behavior is to link it to coping styles. Coping styles have been defined as "a coherent set of behavioral and physiological stress responses that are consistent over time and which are characteristic of a certain group of individuals" [23,24]. The main idea is that, as soon as some "stress" threshold is reached, the coping response acts to minimize "stress" [24]. Two different coping styles have been distinguished: proactive copers try to escape or remove the stressor ("fight-or-flight" response), while reactive copers show no signs of being affected (conservation-withdrawal response) [25]. Proactive individuals tend to have a lower HPA and higher SAM axis reactivity than reactive ones [4].

One hypothesis is that stereotypic behavior reflects a proactive coping response, while depression is more typical of reactive individuals [6]. In this study, we experimentally induced a physiological stress response, in horses affected by stereotypic behavior (crib-biters) and a comparable number of non-crib-biter individuals (controls), using an ACTH challenge test, which consists of administering adrenocorticotropin [26]. To assess the relative reactivity of the HPA and SAM axes, we measured cortisol released from the adrenal cortex, as well as heart rate related measures [27]. If proactive individuals are more prone to developing stereotypies than reactive individuals, we predicted that stereotypic horses should have lower initial cortisol levels, smaller cortisol responses and higher sympathetic activity and reactivity to the ACTH challenge test than control horses [7,19,28].

METHODS

Subjects and management conditions

The study was carried out on 22 crib-biters and 21 control horses (total = 43 horses) of various breeds, sex (mares, geldings and stallions) and ages (3 to 24 years old), housed in 19 different farms in Switzerland, between April and July 2013 (Table 1). Thirty-two horses were privately owned, and 11 horses were owned by the Swiss National Stud Farm. All the horses had been at their respective farms for at least one year. To be eligible for inclusion in the study, crib-biters were required to have demonstrated crib-biting behavior for a minimum of one year, as reported by their owners. The numbers of years that crib-biters had been observed performing the stereotypy were estimated by the horse owners to range between at least 1 and 15 years. Controls were horses that had never been observed crib-biting or performing other kinds of stereotypies (e.g. weaving or box-walking). For each crib-biting horse, we tried to find a control horse that was of similar breed, sex and age, and that was housed in the same conditions, either individually or in a group, in single box or in box with paddock, and if possible in the same farm (Table 1). Routine care was provided by the owners. The study was approved by the Federal Veterinary Office (approval number VD 26777 bis; Switzerland).

Table 1. Characteristics of the horses used in the experiment. Sex (M= mare; G = gelding, S = stallion), Group (CB = crib-biters; C = non-crib-biters (controls)), age, breed, housing system (loose housing, paddock, box; alone or in group) and place (each letter refers to a given farm). Horses 1–15 (Group A) correspond to the crib-biters that did crib-bite during ACTH challenge test, and horses 16–22 to the ones that did not crib-bite (Group B).

horses	sex	crib-biters or control	age	breed	housing system	alone/ group	place
1	M	CB - A	13	Shetland	loose housing	group	u
2	M	CB - A	6	Swiss halfbred	box paddock	alone	c
3	M	CB - A	22	Criollo	box	alone	g
4	M	CB - A	16	Franches-Montagnes	box	alone	y
5	M	CB - A	9	Hispano-arabian	box paddock	alone	b
6	M	CB - A	5	Quarter horse	box	alone	s
7	M	CB - A	9	Paint horse	box	alone	r
8	M	CB - A	5	Paint horse	box paddock	alone	k
9	G	CB - A	9	Franches-Montagnes	box	alone	d
10	G	CB - A	11	Swiss halfbred	box	alone	g
11	G	CB - A	23	Franches-Montagnes	box paddock	group	n
12	G	CB - A	11	Franches-Montagnes	box	alone	bo
13	S	CB - A	9	Franches-Montagnes	box	alone	h
14	S	CB - A	17	Franches-Montagnes	box	alone	h
15	S	CB - A	15	Franches-Montagnes	box	alone	h
16	M	CB - B	5	Franches-Montagnes	box paddock	group	m
17	M	CB - B	19	Swiss halfbred	box paddock	alone	w
18	G	CB - B	19	Haflinger	box paddock	group	se
19	G	CB - B	18	Swiss halfbred	box	alone	a
20	G	CB - B	7	ONC	box paddock	alone	v
21	G	CB - B	10	English thoroughbred	paddock	group	d
22	S	CB - B	11	Franches-Montagnes	box	alone	h
23	M	C	7	Quarter horse	box paddock	alone	s
24	M	C	20	Friso-arabian	box	alone	y
25	M	C	14	Swiss halfbred	loose housing	group	h
26	M	C	18	Apaloosa	box paddock	alone	b
27	M	C	14	Swiss halfbred	loose housing	group	h
28	M	C	16	Trotter	box	alone	h
29	M	C	18	Franches-Montagnes	loose housing	group	h
30	M	C	10	Swiss halfbred	box	alone	g
31	M	C	19	Swiss halfbred	box paddock	alone	w
32	G	C	4	Franches-Montagnes	box paddock	group	n
33	G	C	24	ONC	box paddock	alone	v
34	G	C	22	English thoroughbred	paddock	group	d
35	G	C	7	Quarter horse	loose housing	group	k
36	G	C	6	Franches-Montagnes	box paddock	alone	di
37	G	C	8	Franches-Montagnes	box	alone	d
38	G	C	15	Swiss halfbred	loose housing	group	h
39	G	C	11	Swiss halfbred	box	alone	h
40	G	C	12	Frison	box paddock	alone	se
41	S	C	3	Shetland	loose housing	group	u
42	S	C	17	Franches-Montagnes	box	alone	h
43	S	C	7	Franches-Montagnes	box	alone	h

Experimental procedure

We performed an ACTH challenge test by injecting a synthetic adrenocorticotropic hormone (Synacthen® Tetracosactid 0.25 mg/l) intravenously [29]. The amount of Synacthen injection was calculated according to the weight of the subject (1 µg/kg). The cortisol secretion follows a circadian rhythm, with secretion peak occurring in the early morning.

These rhythms may be influenced by exercise, copulation, learning, excitement and stressors, such as venipuncture or the removal of an animal from its familiar environment [30]. For these reasons, the injection was always carried out at 13:00 ± 10 min local time, and the subject had not been exercised in the morning on the day of the test. All the horses were housed in their usual conditions during the test.

The procedure was similar for all subjects and lasted between 3 h 20 min and 3 h 30 min. The subject's weight was estimated following the method described in Carroll and Huntington [31]. Then, a noninvasive, wireless heart-rate monitor attached to a surcingle (see below) was placed around the horse, and a camera was installed to record the behavior. After 15 min of habituation to the test conditions, the first saliva sample (sample 1) was collected to determine the subject's initial cortisol level ("Cortisol1") before injecting the synthetic adrenocorticotropic hormone (Fig. 1). Then, a brief (15 min) clinical evaluation was performed by a veterinarian to assess body temperature, heart rate, respiratory rate and venous filling, in order to identify any potentially dangerous anomalies, such as cardiac arrhythmias or signs of febrile infectious disease that could potentially interfere with the horses' ability to respond to the ACTH challenge test. If the subject passed the health test (43 of 44 originally selected subjects), the veterinarian injected the substance intravenously. Eleven crib-biters and respective control horses housed in the same farm were tested on the same day within 10 min of each other, and 21 horses were tested individually on different days.

During the post-injection period, lasting 3 h, six further saliva samples were collected (samples 2–7) every 30 min (Fig. 1), while the ECG trace was continuously measured with the heart-rate monitor. We also monitored the behavior of the horses continuously via video recording using a Sony HandycamHDR-CX700. In total, we obtained 7 saliva samples, as well as the ECG trace and video recordings, corresponding to the 15-min habituation period (sample 1 — period 1), and 6× 30-min periods of test post-injection (samples 2–7—periods 2–7; Fig. 1).

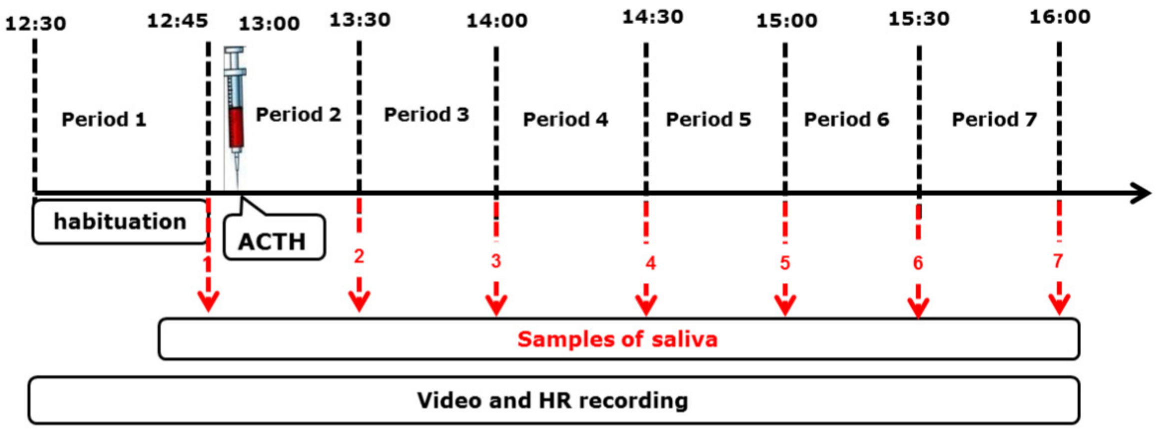

Fig. 1. Experimental procedure for the ACTH challenge test. The black dotted lines indicate the time at which each period started and ended (periods 1–7). The syringe indicates when the ACTH injection took place. The red dotted lines designate when the saliva samples were collected (1–7). Video and ECG trace were recorded continuously, as indicated by the black bar.

Response measures

Physiological measures

We assessed physiological measures, linked to both the hypothalamic– pituitary–adrenal (HPA) pathway and the sympathomedullary (SAM) pathway, which were likely to be affected by the ACTH challenge test [29], at least over short-time scales. Concerning the HPA axis, we collected salivary cortisol, which has been demonstrated to be affected by ACTH challenge test [29]. Saliva was collected with Salivette cotton rolls placed loosely onto the tongue of the horse for 1min using forceps. At the end of the test, the Salivettes were centrifuged for 6 min at 5000 rpm with a Hettich EBA 20, and were then maintained at −20 °C until they were sent to the laboratory for analyses (Salimetrics, USA). Concentrations of cortisol were determined with a direct enzyme immunoassay without extraction and validated for equine saliva [35]. The Salimetrics High sensitivity salivary cortisol enzyme immunoassay kit was used for the analyses.

Concerning the SAM axis, we measured heart rate (HR) and root mean square of successive inter-beat interval difference (RMSSD) [27]. Both measures were collected using a wireless, non-invasive monitor (MLE120X Bioharness Telemetry System, Zephyr), fixed to a surcingle placed around the horse's heart girth, to obtain the ECG trace, which produces more reliable HR measures compared to alternative methods [29,32]. ECG gel was applied on the electrodes before each use. The data were then transmitted and stored in real time to a laptop using LabChart software v.7.2 (ADInstrument) for later analyses. During the tests, one experimenter was entering comments in the software indicating when the ACTH challenge test started, and when each saliva sample was collected (Fig. 1). This allowed us to measure the physiological parameters precisely for each period. We analyzed HR and RMSSD from good-quality sections with clearly visible heartbeats on the ECG trace. Section durations submitted for analyses were comparable between crib-biters and controls (crib-biters, 646.62 ± 371 s; controls, 704.63 ± 406 s per horse). We ensured visually that the software tracked the heartbeats properly before extracting HR and inter heart beat (RR) intervals (ms). RR intervals were then used to calculate RMSSD (ms). If atrioventricular blocks were observed in the signal, we excluded the respective sections of the ECG trace [33,34].

Behavioral measures

Crib-biting events were scored directly during the tests. One experimenter was scoring as a crib-biting event the following behavior; the horse grasped a fixed object with its incisors, pulled back, contracted the neck muscles and drew air into its esophagus, emitting an audible grunt [9]. Instances when the horse performed the same behavior, but without grasping an object were also considered (“windsucking” [9]). Then, for each horse, we calculated the frequency of occurrences of crib-biting events per minute for every period (1–7; Table 2).

Table 2. Crib-biting events performed by the crib-biters for each period. Mean ± SD of occurrences of crib-biting events per minute for each period (period 1 (habituation) - period 7; *N* = 22 horses; indicated in number of events per min)

crib-biting (nb/min)		
Period	**Mean**	**SD**
1	1.55	2.22
2	0.66	0.91
3	0.69	1.04
4	0.96	1.35
5	0.77	1.13
6	0.79	1.02
7	0.73	1.25

From the videos of the tests, we scored the physical activity (movements) of the horse, because this behavior can potentially affect physiological parameters [27,30,36]. The duration of body movements was scored continuously ("State Events") using the Observer software XT v.11 (Noldus), and considered when the horse performed more than two steps [37]. We then calculated the proportion of the total time spent performing the behavior.

Statistical analysis

To compare the physiological reaction of crib-biters and controls to the ACTH challenge test, we tested for group differences in cortisol increase (HPA axis), HR and RMSSD (SAM axis), using linear mixed effects models (LMM; lme function, nlme library; [38,39]).

To calculate the cortisol increase (hereafter "Icortisol") in response to the ACTH challenge test, for each horse and each period, we subtracted the initial value, Cortisol1 (sample 1 — period 1) from its value measured at the end of each 30-min period 2–7 (i.e. after injection; samples 2–7 in Fig. 1; [40]). Cortisol1 is the value for cortisol after habituation and before Synacthen injection (Fig. 1). In order to test for group differences in HR and RMSSD while controlling for initial values before injection, we calculated HR and RMSSD ratios (hereafter "rHR" and "rRMSSD" respectively), by dividing the average HR and RMSSD values for each period (2–7) by the initial values before injection (HR1 and RMSSD1; period 1). The HR1 and RMSSD1 values are the average values of HR and RMSSD for period 1 (Fig. 1).

First, we carried out a series of models on the initial values before injection (Cortisol1, HR1 or RMSSD1). These LMMs included Cortisol1, HR1 or RMSSD1 as a response variable (3 separate models), the sex and age of the horses, the housing system (control factors) and the group (crib-biters or controls) as fixed factors. To control for differences between farms, the identity of the farms where the horses were housed was included as a random factor.

Second, we carried out another series of models on the values collected after injection (Icortisol, rHR or rRMSSD). These LMMs included Icortisol, rHR, rRMSSD, or movements as a response variable (4 separate models). The sex, the age and the housing system of the horses (control factors), the period (1–7) and the group (crib-biters or controls), as well as the interaction term between period and group, were included as fixed factors. Finally, to control for repeated measurements of the same subjects and for farm differences, the identities of the horses nested within the farms where they were housed were included as random factors. As the frequency of crib-biting varies between and even within horses [13], it turned out that seven crib-biting horses did not perform the stereotypic behavior during the ACTH challenge test. We thus then reran the same LMMs including the Icortisol, rHR or rRMSSD, and movements as a response variable (4 separate models), and the same fixed and random factors as mentioned above, to compare the crib-biters that did crib-bite during the test (Group A: 15 horses, Table 1—horses 1–15 and Table 3), the crib-biters that did not crib-bite during the test (Group B: 7 horses, Table 1—horses 16–22 and Table 3) and the controls (Group C; 21 horses, Table 1—horses 23–43 and Table 3). Then, two-by-two comparisons between the three groups were carried out using LMMs including the same fixed and randomfactors as in the model carried out the three groups together. We applied a Tukey correction (function glht, package multcomp in R,Multiple comparisons of means) for these posthoc tests.

Using a standard model simplification procedure, we removed each non-significant interaction term or control factor, until the deletion did cause a reduction in goodness of fit (in this case, the term was left in the model). The residuals were checked graphically for normal distribution and homoscedasticity. To satisfy model assumptions, we used a log transformation for RMSSD1, rHR and rRMSSD. Because of a technical problem with a defective Bioharness unit, we only obtained HR and RMSSD measures on 9 crib-biters and 20 controls. Therefore, sample sizes vary between the analyses on the HPA and SAM axis responses (Table 3). Additionally, because of a technical problem with one of the videos, we scored movements on 21 crib-biters and 21 controls. The significance level of the factors was set at $\alpha = 0.05$. All means are given with standard errors.

RESULTS

HPA axis response

We analyzed the physiological responses of 43 horses to ACTH injections. We did not find any significant difference in Cortisol1 between groups (no effect of the group CB-C on Cortisol1, Table 3). However, there was an overall increase following ACTH injections (effect of the period on Icortisol for CB-C; Table 3; Fig. 2) Cortisol concentrations increased Significantly more strongly in crib-biting horses than in controls relative to Cortisol1 (effect of the group CB-C on Icortisol; Table 3; Fig. 2; see Supplementary material 1 for raw values). Stallions tended to have lower cortisol increases than geldings and mares (effect of sex on Icortisol for CB-C; Icortisol: stallions, 4.01 ± 2.49 ng/ml; geldings, 5.63 ± 3.0 ng/ml; mares, 5.5 ± 2.87 ng/ml; Table 3).

During the experiment, only 15 of 22 crib-biters displayed stereotypic behavior (crib-biting). We thus compared cortisol levels between controls (Group C) and crib-biters that crib-bit (Group A) and did not crib-bite (Group B) during the test. The three groups did not differ in Cortisol1 (no effect of the group C-A-B on Cortisol1; Table 3), but differed significantly in their cortisol increase (effect of the group C-A-B on Icortisol; Table 3; Fig. 3; see Supplementary material 1 for raw values). Again, in the same way as for the analyses testing differences between crib-biters and controls (comparison between groups CB-C), there was a significant cortisol increase following ACTH injections (effect of the period on Icortisol for C-A-B; Table 3; Fig. 3; see Supplementary material 1 for raw values), and sex tended to affect cortisol increase (effect of the sex on Icortisol for C-A-B; Table 3). Post-hoc comparisons showed that Group B had a significantly higher cortisol increase than Group C (Table 3; Fig. 3; Multiple comparisons of means $Z = -2.44$, $N = 28$, $p = 0.038$). However, we did not find any difference between Group A and Group C (Table 3; Fig. 3; Multiple comparisons of means $Z = -1.98$, $N = 36$, $p = 0.11$), nor between Groups A and B (Table 3; Fig. 3; Multiple comparisons of means $Z = 0.86$, $p = 0.66$). For the LMM carried out on Cortisol1, the age, sex and housing system were removed during model selection. For the LMM carried out on Icortisol, neither the interaction between group and period, nor the age and housing system significantly affected cortisol increase. These terms were thus removed during model selection.

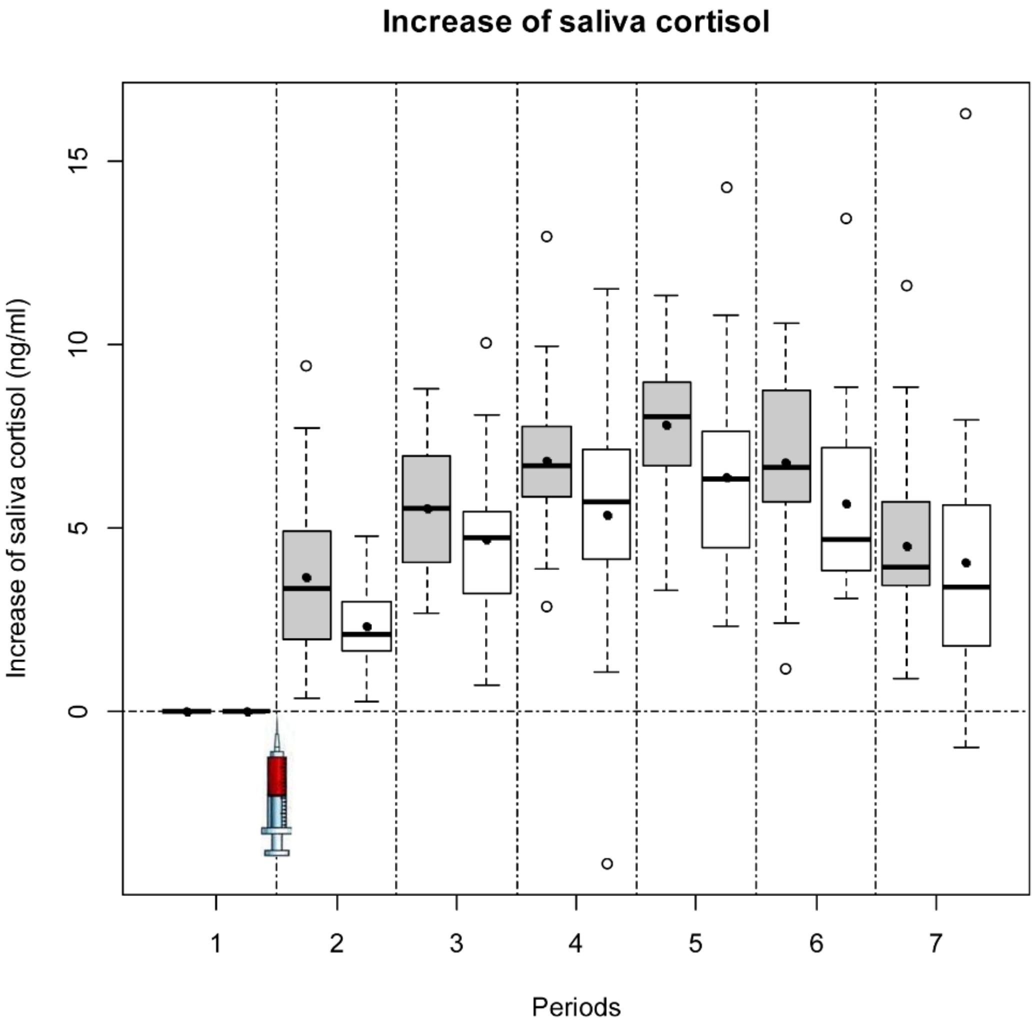

Fig. 2. Cortisol response (HPA axis) to the ACTH challenge test in crib-biters and control horses. Increase in saliva cortisol between each period (2–7) and the habituation (sample1 — period 1) for crib-biters (Group CB; N=22 horses, gray) and control horses (Group C; N=21 horses, white); box-and-whiskers plot (the horizontal line shows the median, the box extends from the lower to the upper quartile, and the whiskers to 1.5* the interquartile range above the upper quartile or below the lower quartile). The black dots indicate the means. The syringe indicates when the ACTH injection took place.

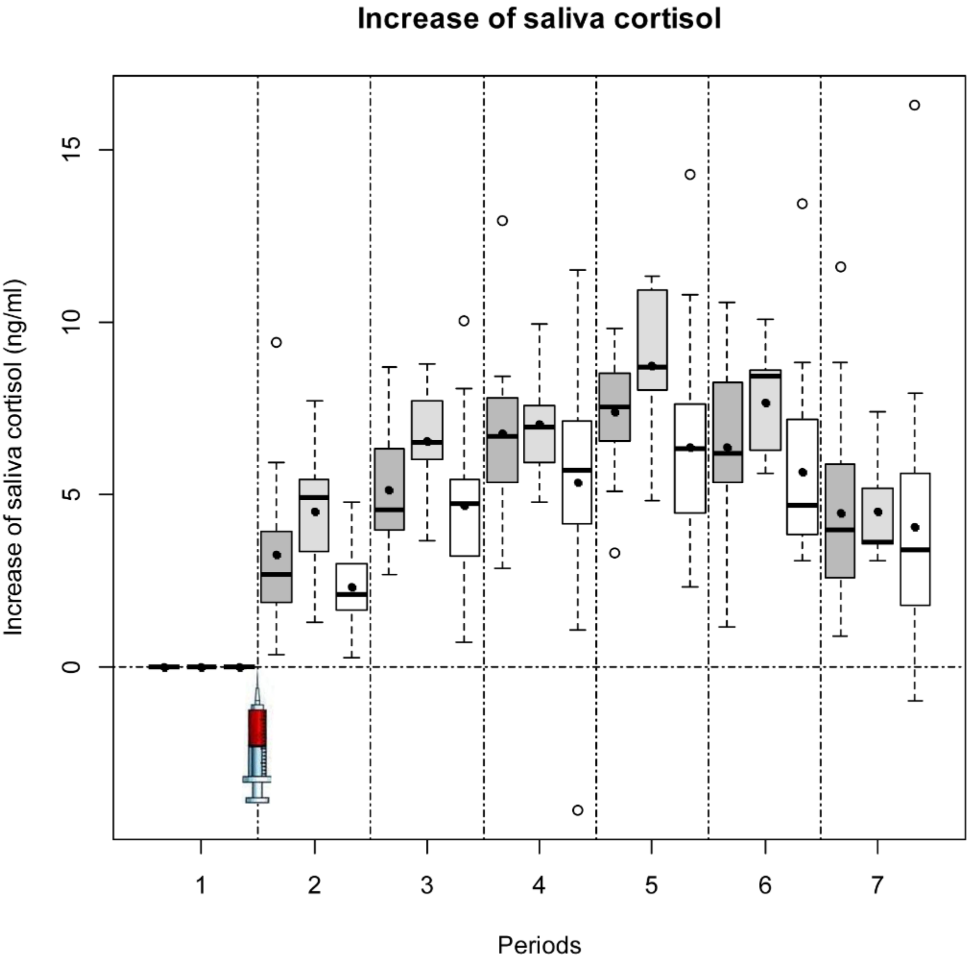

Fig. 3. Cortisol response (HPA axis) to the ACTH challenge test in crib-biters that did or did not crib-bite and control horses. Increase in saliva cortisol between each period (2–7) and the habituation (sample 1 — period 1) for crib-biters that did crib-bite during the test (Group A; N = 15 horses, dark gray) crib-biters that did not crib-bite during the test (Group B; N = 7 horses, light gray) and control horses (Group C; N = 21 horses, white); box-and-whiskers plot (the horizontal line shows the median, the box extends from the lower to the upper quartile, and the whiskers to 1.5* the interquartile range above the upper quartile or below the lower quartile). The black dots indicate the means. The syringe indicates when the ACTH injection took place.

Table 3. Mean and standard deviation for SAM and HPA axis measures, and results of the models investigating the effects of various factors on the HPA and SAM axis measures. Group (CB=crib-biters, C=non-crib-biters (controls), A=crib-biters that crib-bit, B=crib-biters that did not crib-bite during the ACTH challenge test); Mean, median and standard deviation (SD) for the following measures: Cortisol1=initial value of cortisol for the period 1 (sample 1, Fig. 1), Icortisol = average cortisol increase over periods 2–7, HR1

and RMSSD1=average initial values of HR and RMSSD for the period 1, rHR and rRMSSD=average HR and RMSSD ratio; Linear mixed effects models investigating the effects of the group, the period (1 or 2–7), the sex (gelding, mare or stallion), the age (3 to 24 years old) and the housing system on the physiological measures. Only the effects of the parameters kept after the model selection procedure are shown. Significant results are shown in bold.

Group com-parison	Response variables/ Measures	Group	*N*	Mean/ Median	SD	Fixed effect	*F* (df)	*P* value
CB-C	**Cortisol1 (ng/ml)**	C	21	1.03/ 0.56	1.60	Group	0.78 (1,23)	0.40
		CB	22	0.74/ 0.77	0.24			
	Icortisol (ng/ml)	C	21	4.76/ 4.34	3.04	Group	7.15 (1,21)	**0.014**
						Sex	3.20 (2,21)	0.060
		CB	22	5.84/ 5.8	2.62	Period	12.81(1,211)	**0.0004**
	HR1 (BPM)	C	20	40.25/ 39.7	4.27	Group	0.71 (1,12)	0.42
		CB	9	38.83/ 40.19	5.26			
	rHR	C	20	1.07/ 1.04	0.15	Group	0.90 (1,12)	0.36
		CB	9	1.10/ 1.08	0.16	Period	205.27 (1,144)	**<0.0001**
	RMSSD1 (ms)	C	20	46.63/ 44.20	15.46	Group	1.12 (1,9)	0.32
		CB	9	50.03/ 40.89	23.15	Sex	5.00 (3,9)	**0.030**
	rRMSSD	C	20	1.06/ 1.00	0.34	Group	0.004 (1,12)	0.95
		CB	9	1.07/ 0.95	0.42	Period	9.70 (1,144)	**0.002**
C-A-B	**Cortisol1 (ng/ml)**	C	21	1.03/ 0.56	1.60	Group	0.40 (2,22)	0.70
		A	15	0.74/ 0.76	0.26			
		B	7	0.72/ 0.77	0.21			
	Icortisol (ng/ml)	C	21	4.76/ 4.34	3.04	Group	3.87 (2,20)	**0.038**
		A	15	5.58/ 5.75	2.69	Sex	3.15 (2,20)	0.065
		B	7	6.44/ 6.14	2.38	Period	12.82 (1,211)	**0.0004**
	HR1 (BPM)	C	20	40.25/ 39.7	4.27	Group	0.34 (2,11)	0.72
		A	6	38.64/ 40.72	5.57			
		B	3	39.22/ 39.94	5.75			
	rHR	C	20	1.07 / 0.04	1.15	Group	2.12 (2,11)	0.17
		A	6	1.13/ 1.11	0.17	Period	205.27 (1,144)	**<0.0001**
		B	3	1.00/ 1.00	0.13			
	RMSSD1 (ms)	C	20	46.63/ 44.19	15.46	Group	0.74 (2,9)	0.51
		A	6	46.63/ 45.36	13.27			
		B	3	56.84/ 34.36	39.98	Sex	6.93 (2,9)	**0.020**
	rRMSSD	C	20	1.06/ 1.00	0.34	Group	0.38 (2,11)	0.70
		A	6	1.14/ 0.90	0.50			
		B	3	0.94/ 0.97	0.21	Period	9.70 (1,144)	**0.002**

SAM axis response

We analyzed the heart-rate responses of 29 horses during the test (periods 1–7). There was no difference between the HR1 of crib-biters and controls (no effect of the group CB-

C on HR1; Table 3). However, rHR (ratio between HR values at each period and the HR1 value) significantly varied between periods (effect of the period on rHR for CB-C; Table 3). When comparing rHR between controls (Group C) and crib-biters that did (Group A) or did not (Group B) show stereotypic behavior during the test, we found no significant differences between the three groups in their HR1 or rHR (no effect of the group C-A-B on HR1 or rHR; Table 3).

Finally, there was no difference in RMSSD1 between crib-biters and controls (no effect of the group CB-C on RMSSD1; Table 3). However, rRMSSD (ratio between RMSSD values at each period and the RMSSD1) significantly varied between periods (effect of the period on rRMSSD for CB-C; Table 3). Similarly as for rHR, rRMSSD did not differ between groups C, A and B (no effect of the group C-A-B on rRMSSD; Table 3). Stallions tended to have higher RMSSD1 than geldings and mares (effect of sex on RMSSD1 for CB-C and C-A-B; RMSSD1: stallions, 66.54±20.19 ms; geldings, 38.9±12.01 ms;mares, 41.52±13.65ms; Table 3). For all the LMMon the SAM axis response, neither the interaction between group and period, nor the age, sex (except for RMSSD1) and housing system, significantly affected HR1, rHR, RMSSD1 and rRMSSD. These terms were thus removed during model selection.

Behavioral measures

We analyzed the movements of 42 horses during the test (periods 1–7).We did not find any significant difference in movement between groups (CB versus C; LMM: F1,36 = 2.62, p = 0.11; comparison C-A-B; LMM: F2,35 = 1.48, p = 0.24), but we found a significant effect of the housing system on this parameter (effect of the housing system for CB-C; LMM: F4,36 = 6.11, p = 0.0007; for C-A-B; LMM: F4,35= 6, p = 0.0009). The interaction between group and period, the age and sex of the horses did not affect significantly the movements. These terms were thus removed during model selection.

DISCUSSION

Stereotypic behavior affects many domesticated species and other animals kept in captivity. The function of stereotypic behaviors is still largely unknown but it has been proposed to help affected individuals in dealing with stressful situations [18,41]. In this study, we addressed this stress-coping hypothesis in a large sample of horses, half of which showed crib-biting, one of the main stereotypies in domestic horses. We induced stress experimentally, by injecting synthetic ACTH. Because some of the stereotypic horses did not crib-bite during the test, we could then investigate differences in the physiological responses of three groups of animals (individuals identified as crib-biters, which responded by crib-biting or not to the test, and control horses). We collected measures related to the SAM and HPA stress axes. We did not find any group difference in terms of SAM axis measures (HR and RMSSD). However, we found significant differences in the HPA axis measures, with crib-biters showing higher cortisol responses than controls. More importantly, we also found that the difference between crib-biters and controls was mainly due to the seven crib-biters that did not crib-bite during the test, whereas crib-biters that showed stereotypic behavior during the test (N=15) had cortisol levels that were indistinguishable from control animals. Our results suggest that the presence of stereotypic behavior in horses is linked to differences in HPA axis response. These differences could be either inherited, caused by chronic stress or due to the long-term performance of the stereotypic behavior. Our results also suggest that crib-biting might be an effective coping strategy that helps stereotypic individuals to gain control over stressful situations, in order to reduce their cortisol levels. We conclude that preventing stereotypic horses from crib-biting could be counterproductive, because this behavior, once established, might have some beneficial effects for the animals.

HPA axis response of crib-biters and controls

Our results show that the HPA axis stress response differs between crib-biting and non-crib-biting horses. The activation of the HPA axis is an adaptive mechanism that helps to maintain physiological stability in response to stressful stimuli. Repeated or chronic exposure to stress can induce changes in HPA axis function [33]. Because of the suggestion that crib-biters are more proactive than controls [6], we had hypothesized that these horses would have a lower HPA axis response to the ATCH challenge test (i.e. lower cortisol values) than other horses. However, contrary to our hypothesis, crib-biters had a higher cortisol increase than controls. Increased or maintained HPA responses to novel stressors are often observed in chronically stressed animals compared to control animals [33]. A higher HPA axis response could result from a "facilitation process". This process results in an enhanced glucocorticoid (GC) response to a stressor in "acclimated" (i.e. animals that no longer respond in the same robust manner to chronic stressors) compared to "non-acclimated" animals [34]. Therefore, the high cortisol responses of crib-biters in our study could result from a "facilitation process" linked to chronic stress.

An alternative suggestion to the "facilitation process" hypothesis is that the changes that we observed in the HPA-axis response could be due to the long-term performance of the stereotypic behavior. In fact, corticosteroid hormones may have differential effects during the early and fully developed stages of a stereotypy [24]. It has been suggested that stress levels and high corticosteroids enhance the acquisition and expression of stereotypies, whereas an already-developed stereotypy may reduce corticosteroid levels [24]. It would be interesting, as suggested elsewhere [20,22,30,47], to perform a longitudinal study in order to establish whether the development of crib-biting leads to a reduction of cortisol levels from even higher original levels, and whether a transient peak in stress level occurs prior to the emergence of stereotypic behavior.

Reactive coping animals have a higher HPA axis reactivity and react with a higher cortisol response than proactive ones [4]. The higher cortisol response we observed in crib-biting horses therefore suggests that these individuals are, contrary to our hypothesis, more reactive than non-stereotypic horses. Hyperactivity of the HPA axis is also a characteristic of major depression; similar HPA axis modifications can be observed after repeated exposure to different stress procedures [42].

Finally, the observed difference in HPA axis stress response between crib-biters and controls might be related to genetic factors. Some studies have found genetic predispositions to crib-biting, which could explain why some horses but not others, develop this stereotypy after a similar period of chronic stress [15,43,44]. For instance, wind-sucking has been shown to occur more frequently in some pedigrees than others [44]. Vecchiotti and Galanti [15] reported an incidence rate of 7.4% of stereotypic behaviors in Italian thoroughbreds, and concluded that the genetic transmission of these behaviors is similar to some human mental disorders involving polygenic inheritance. Recently, Hemmann [43] found an unusually high prevalence of crib-biting in a small Finnhorse population, again suggesting that horses might inherit behavioral susceptibility to develop stereotypy. Other studies reported stress-induced alterations in the central nervous system (CNS) dopamine physiology in stereotypic animals [18,45,46]. This suggests that such alteration or sensitization in the CNS may be the result of chronic stress in combination with a genetic predisposition. Based on the literature and our own results, we could hypothesize that this inherited behavioral susceptibility consists in a higher sensitivity to stress in crib-biting horses compared to non stereotypic ones due to differences in HPA axis reactivity.

HPA axis response of crib-biters that did and did not crib-bite

Our results show that the differences in cortisol increase in response to the ACTH challenge test between crib-biters and controls were largely due to the crib-biters that did not perform the stereotypy during the test. Indeed, only the stereotypic horses that did not crib-bite during the test had a higher HPA axis reactivity than the controls. By contrast, there was no difference in cortisol response between the crib-biters that did crib-bite during the test and the controls. These promising results suggest that the stereotypic horses that did crib-bite during the test might have developed and installed a successful coping strategy that helped them to gain control and reduce cortisol levels during stressful situations. These results are in accordance with McBride and Cuddeford [18], who found a reduction in cortisol levels following bouts of crib-biting.

Previous studies that have investigated whether stereotypies lowered arousal and anxiety as well as corticosteroid levels, did not find consistent results [7,17–20]. If crib-biting is indeed a coping mechanism, animals should show signs of stress in situations in which they are prevented from crib-biting. McBride and Cuddeford [18] placed a collar, which prevented crib-biting, on crib-biters and controls. These authors showed differences in physiological stress responses between restricted and non-restricted horses, but could not conclude about the functionality of crib-biting, because the use of the collar also triggered a physiological stress response in the control horses. McGreevy and Nicol [20] found higher mean baseline levels of cortisol in crib-biters than in controls. However, this study did not find any significantly higher rise in cortisol levels in crib-biters transiently prevented from performing this stereotypy by removing a bar on which they could perform crib-biting, than in controls [20]. Indeed, an increase in plasma cortisol levels was found in both stereotypic and controls, when they were deprived of ad libitum hay and, for crib-biters, of the opportunity to crib-bite for 24 h.

The fact that the cortisol response in crib-biters that did show the behavior during the test was similar to the response of controls, whereas those that did not crib-bite had a higher response, suggests that preventing stereotypic horses from crib-biting, without reducing underlying motivation, could be counter-productive. Our results imply that crib-biting, once installed, has beneficial effects for the individual. Mason and Latham[8] discussed the link between welfare and stereotypies and concluded that in some cases, the performance of fully developed stereotypies could improve welfare. For example, stereotypies performed as "mantra effects", which help an individual to calm itself through repetition, decrease arousal. We could hypothesize that crib-biting has a similar effect on some horses. However, Mason and Latham [8] also warned against generalization. Indeed, stereotypies may have different underlying causes and mechanisms and are rarely comparable among species. For example "perseveration" is another process that could underlie stereotypies and in this case, it indicates altered behavioral control [8]. It is thus important, as Mason and Latham [8] suggested, to understand the mechanisms underlying stereotypies before implying a link with good, neutral or poor welfare of the animals.

Thirty-two percent of crib-biters did not crib-bite during the ACTH challenge test. The different responses between these horses and the crib-biters that did crib-bite could be explained by the fact that crib-biters might have been at different stages of the development of their stereotypies [8]. Horses that did crib-bite could have fully-developed stereotypies, while non-crib-biting individuals might be at an early stage of development [24]. However, the owners reported that the horses that did not crib-bite in the study had been crib-biting for 8 years on average (range = 2–15 years). Alternatively, horses could have developed more or less strong stereotypies. Indeed, the performance of stereotypic behavior varies between horses in term of the daily percentage of time occupied by the activity [13]. Thus, the fact that some horses did not crib-bite during our test could be explained by a generally lower propensity to crib-bite. We suggest that it may be necessary to not only take into account how long the behavior has been present in an animal, but also at what frequency the behavior is performed. We also suggest that the crib-biters that did crib-bite during our test were at the stage when full-blown stereotypies serve their coping function of reducing stress.

SAM axis response of crib-biters and controls

We did not find any difference in HR or RMSSD ratio (i.e. ratio between HR or RMSSD values at each period and the value before injection) in response to our ACTH challenge test between crib-biters and controls. One explanation could be the small sample of crib-biters for which we were able to measure HR (N = 9). Studies investigating the effect of crib-biting on the SAM axis reactivity found similar results for HR as we found for the HPA axis. For instance, HR was shown to decrease during bouts of crib-biting [17,19]. Differences between crib-biters and controls in heart rate variability have also been found in other studies [7]. In fact, crib-biting horses seem to have a reduced reactivity range of the autonomic nervous system. Crib-biting horses may therefore not be capable to react as efficiently as other horses to an external stimulus, suggesting that crib-biters are more stress-sensitive and less flexible when coping with stress [7]. By contrast, and in accordance with our results, other studies did not find any significant difference in the mean HR or in the HR variability between crib-biters and other horses [18,30,47,48].

Behavioral activity of crib-biters and controls

We did not find any difference in the physical activity (movements) during the ACTH challenge test between crib-biters and controls. Therefore, we can suggest that the difference in the HPA axis found between crib-biters and controls is not the result of a difference in activity during the test [27].

CONCLUSION

Our results suggest that crib-biters differ from controls in their HPA axis reactivity. Further experiments need to address whether this difference is a consequence of chronic stress, or if a genetic difference could predispose horses to develop such abnormal behavior. Indeed, many studies have mentioned the importance of longitudinal studies required to investigate the HPA axis function during the development of stereotypic behavior [20,22,30,47]. In fact, it is possible that horses develop stereotypies in order to cope with stressful situations, and the stereotypy itself could be part of a coping process. If stereotypic behavior is really a coping mechanism, then any attempt to prevent stereotypic horses to crib-bite would be counter-productive. In some cases, the performance of stereotypies, once developed, could even improve welfare. These hypotheses will require further testing taking into account the possibility that there may be important individual differences in whether and how stereotypic behavior can reduce stress.

ACKNOWLEDGMENTS

We are grateful to all the owners of the horses, who offered to participate in this study, and to Lorenz Gygax for statistical advice. EFB and ALM are funded by a Swiss National Science Foundation fellowship (grant number : PZ00P3_148200).

Appendix A. Supplementary data: Supplementary data to this article can be found online at http://dx. doi.org/10.1016/j.physbeh.2015.07.015.

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CHAPTER III

Based on:

Elevated sensitivity to tactile stimuli in stereotypic horses

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Submitted

ABSTRACT

Although stereotypic behaviours are a common problem in captive animals, why certain individuals are more prone to develop them remains elusive. In horses, individuals show considerable differences in how they perceive and react to external events, suggesting that this may partially account for the emergence of stereotypies in this species. In this study, we focussed on crib-biting, the most common stereotypy displayed by horses. We compared how established crib-biters (“CB”=19) and normal controls (“C” =18) differed in response to a standard ‘personality’ assessment test battery, i.e. reactivity to humans, tactile sensitivity, social reactivity, locomotor activity, and curiosity versus fearfulness (both in novel and suddenness situations). Our analyses showed that crib-biters only differed from control horses in their tactile sensitivity. Indeed, a greater proportion of crib-biters reacted to the tactile filaments compared to the control horses suggesting an elevated sensitivity to tactile stimuli. We suggest that this higher tactile sensitivity could be due to altered dopamine physiology, resulting from chronic stress exposition. We discuss these findings in relation to the hypothesis that there may be a genetic predisposition for stereotypic behaviour in horses, and in relation to current animal husbandry and management practises.

Keywords
Personality; crib-biting horse

INTRODUCTION

Stereotypies are defined as repetitive and invariant behaviours, which are thought to be a consequence of suboptimal environmental or housing conditions. Stereotypic behaviours are often described as abnormal and with no obvious goal or function (Mason and Latham, 2004), and are sometimes compared to human developmental, neurological or psychiatric disorders, such as autism, obsessive compulsive disorders or schizophrenia (McBride and Parker, 2015). In animals, stereotypies include locomotor (e.g. "pacing") and oral (e.g. "sham chewing"; "crib-biting") behavioural abnormalities, which can be debilitating for individuals, especially if they are expressed extensively.

The causal factors and neurobiological mechanisms underlying stereotypic behaviours are only partially understood (McBride and Parker, 2015). A recurrent hypothesis is that sustained 'stress' or chronic stress, mainly in the form of restricted and suboptimal living conditions, can lead to the development of stereotypic behaviours in animals (McBride and Parker, 2015). At the neurobiological level, the idea is that if animals are prevented from executing some behaviours, then this can facilitate the development of alternative behaviours such as stereotypies, via sensitisation of the underlying neural systems involved (Spruijt et al., 2001). The *basal ganglia* are thought to constitute the location where neural alterations might take place, and hence to play a key role in the development of stereotypies, particularly within the dopaminergic system (McBride and Hemmings, 2005; Parker et al., 2009). Yet, it is still largely unclear why only certain individuals develop stereotypic behaviours, while others remain unaffected (Ijichi et al., 2013).

One possible explanation for the susceptibility of some individuals but not others to develop stereotypies despite being exposed to similar environments is the existence of individual differences, or personality. Personality and its various sub-traits, such as temperament, can be defined as behavioural attitudes that are consistent over time and across situations (Reale et al., 2007; Borstel, 2013; Finkemeier et al., 2018). As mentioned in Reale et al. (2007), the term "consistent" refers to differences between individuals that are largely maintained, and does not mean that behavioural attitudes cannot change with age or environment. Individual differences (i.e. personality) are thought to result from a combination of nature and nurture influences, that is, from an interaction between neural, genetically inherited system (i.e. temperament) and specific environmental influences during ontogeny (Seaman et al., 2002; Borstel, 2013). Quantifying individual differences (called phenotypes) is usually done via multivariate analyses, which allow behavioural traits to be grouped into larger categories (e.g. 'fearfulness' is defined by a range of behavioural reactions to different fear-inducing situations) (Borstel, 2013). The more general goal of personality assessments is thus to establish categories that reflect how these animals behave, perceive and react to the world beyond individual stimuli or specific situations.

One aspect of personality that might affect predispositions to develop stereotypies are individual differences in motivation to perform specific behaviours. Indeed, stereotypies often develop following the prevention of highly motivated behaviours, such as consummatory acts (Toates, 2004). In captivity, the performance of some highly motivated consummatory behaviours may be impossible. This can result in frustration-related stress and, if sustained or repeated, in stereotypies (Mason and Rushen, 2006). A classic example is carnivores, which are highly motivated to hunt. In captivity, however, individuals are usually prevented from hunting and, according to the stress-by-frustration hypothesis, are thus prone to develop locomotor stereotypies (Clubb and Mason, 2003). Another classic example is ungulates, which are highly motivated to engage in food processing over long periods of time. Since this is usually not possible in captivity, it frequently results in oral stereotypies (Sambraus, 1985; McGreevy et al., 1995). Similarly, within each of these systems, individual differences in the propensity to develop certain types of stereotypies could exist. Specifically, in carnivores, more active individuals could be more prone to develop locomotor stereotypies, while in ungulates, more explorative individuals could be more prone to develop food-related stereotypies (Vandeleest et al., 2011; Gottlieb et al., 2013).

Chronic stress, which can occur when animals face aversive situations over prolonged periods of time (Koolhaas, 2011), is another potential precursor of stereotypies. Individual differences in response to chronic stress could thus also affect propensities to develop stereotypies (Joshi and Pillay, 2016). In particular, a distinction has been made between animals responding proactively or reactively when facing an aversive stimulus (Koolhaas et al., 2010). Proactively coping individuals tend to escape from or remove aversive stimuli (*fight-or-flight*), whereas reactively coping individuals show no obvious reactions in similar situations (*conservation – withdrawal)* (Seaman et al., 2002). In addition, proactive individuals are generally characterised by higher levels of mobility, aggression, exploration and persistence than reactive individuals (Koolhaas et al., 1999). As a consequence, proactive individuals tend to have more difficulties than reactive individuals with variable environments they cannot predict, a difference which may have fitness consequences (Koolhaas et al., 2010).

These individual differences in coping styles are also frequently related to underlying physiology (Koolhaas et al., 2010). Proactive individuals tend to have a lower reactivity of the hypothalamo-pituitary-adrenocortical axis (HPA) but a higher reactivity of the sympatho-adreno-medullary (SAM) axis compared to reactive individuals (Koolhaas et al., 2010). However, based on the outcome of several studies, the differential neuroendocrine characteristics seem to be mainly a consequence rather that the cause of these differences (Koolhaas et al., 2010). Because proactive animals act to exert control over their environment, they might be more prone to form routines and, by extension, to fall into stereotypies (Ijichi et al., 2013). This hypothesis, however, has not been supported by the physiological results of our previous study, which revealed higher HPA-axis reactivity in stereotyped (crib-biters) compared to control horses, which is more characteristic of reactive individuals than proactive ones (Briefer Freymond et al., 2015). In sum, the issue of whether stereotypic behaviour can be linked to individual differences, and particularly coping styles in response to chronic stress, has not been resolved.

The most common form of stereotypy in horses is crib-biting behaviour (Luescher et al., 1991). Crib-biting consists in grasping a fixed object with the incisors, pulling back and drawing air into oesophagus. Crib-biting is related to another common stereotypic behaviour, windsucking, which consists of the same behavioural elements, but without grasping an object. Factors such as sex, age and breed have been associated with the development of these two stereotypies (Luescher et al., 1991; Bachmann et al., 2003). The evidence so far seems to suggest that crib-biters are less anxious and show no difference in trainability compared to non-crib biters (Nagy et al., 2010). However, this low level of anxiety in crib-biting horses might be preceded by an initial increase in anxiety, as revealed by an elevation of dopamine, until the stereotypy is established or fully-blown (Roberts et al., 2016). Overall, a more thorough investigation of crib-biters' personality is required to understand the causal relationship between horse personality and the development of crib-biting.

The aim of this study was to investigate if certain personality traits could predispose horses to the development of crib-biting behaviour. Our current knowledge of crib-biters' personality is limited to the use of questionnaires (Nagy et al., 2010; Roberts et al., 2016). Here, we aimed to obtain more objective measures, by comparing crib-biting and control horses along five "personality" traits following a previously validated model of tests, relevant for equitation practice (reactivity to humans, tactile sensitivity, social reactivity, locomotor activity and curiosity versus fearfulness (both in novel and suddenness situations)) (Lansade, 2005; Borstel, 2013). These traits have been shown to appear early in life and remain relatively stable across time and situations (Lansade and Bouissou, 2008; Lansade et al., 2008b; a; Lansade et al., 2008c). Any possible links between the five traits and crib-biting behaviour could, in the long term, help to rapidly identify horses that are more prone to develop stereotypic behaviour. According to our previous results on crib-biters' physiology (Briefer Freymond et al., 2015), we predicted these horses to show behavioural characteristics of reactive coping individuals, and hence to be generally less anxious (Nagy et al., 2010; Roberts et al., 2016), or less prone to express their emotions (Koolhaas et al., 2010), compared to control horses. We therefore also expected them to interact less with unfamiliar humans (i.e. less bold), to show less locomotor behaviour and less exploratory behaviour (Górecka-Bruzda et al., 2011; Finkemeier et al., 2018). Regarding social reactivity, it has been shown in pigs that reactive coping individuals are more social (Reimert et al., 2014). We thus expected, if the same applies to horses, that crib-biters would show more social reactions. Regarding tactile sensitivity, we were not able to make any predictions (Koolhaas et al., 2010). This personality trait, despite being interesting to measure in ridden horses, has not yet been assessed in other species (Lansade et al., 2008c; Borstel, 2013; Finkemeier et al., 2018).

METHODS

Subject and management conditions

The present study was carried out on 19 crib-biters and 18 control horses (total = 37 horses) of various breeds, sex (mares, geldings and stallions) and ages (4 to 24 years old; Table 1), housed in 19 different farms in Switzerland between September 2013 and February 2014. Except for one control, all horses were tested previously in another study (Chapter II of the PhD, Annex II of the PhD). Twenty-six horses were privately owned, and 11 were obtained from the Swiss National Stud Farm. All the horses had been at their respective farms for at least one year. To be eligible for inclusion in the study, crib-biters were required to have demonstrated crib-biting behaviour, according to the horse owners, for a minimum of one year. The control group was made up of horses that had never been observed crib-biting or performing other stereotypies. For each crib-biting horse, we tried to find a control horse that was of similar breed, sex and age, and that was housed in the same conditions (i.e., if possible, on the same farm, Table 1). Horses were housed, either individually or in groups, in single boxes or in boxes with paddocks (Table 1). Routine care of the study animals was provided by the farm/horse owners. The study was approved by the Swiss Federal Veterinary Office (approval number VD 2677 bis; Switzerland).

Table 1. Characteristics of the horses used in the experiment. Sex (M = mare; G = gelding, S = stallion), group (CB = crib-biters; C= controls), age, breed, housing system (loose housing, paddock, box), housed alone or in group, and place (each letter refers to a given farm).

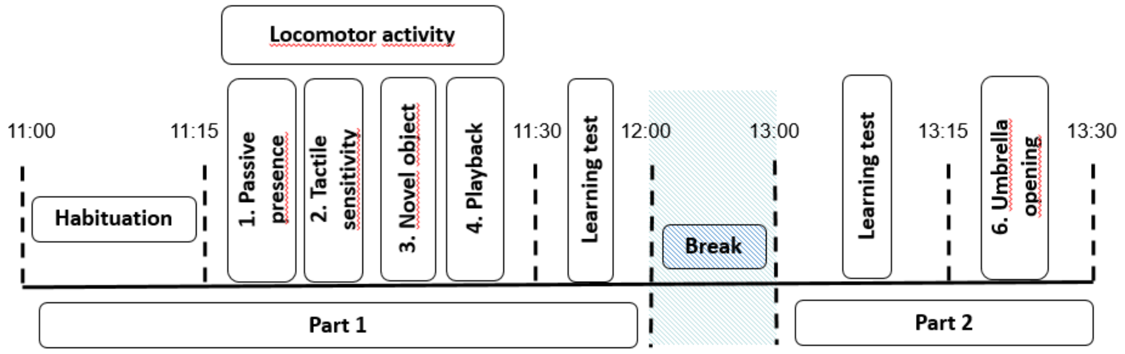
horses	sex	crib-biter or contro	age (years)	breed	housing system	alone/	place
1	M	CB	6	Warmblood	box paddock	alone	c
2	M	CB	22	Criollo	box	alone	g
3	M	CB	16	Franches-Montagnes	box	alone	y
4	M	CB	9	Hispano-Arabian	box paddock	alone	b
5	M	CB	5	Quarter horse	box	alone	s
6	M	CB	9	Paint horse	box	alone	r
7	M	CB	5	Paint horse	box paddock	alone	k
8	G	CB	9	Franches-Montagnes	box	alone	d
9	G	CB	11	Warmblood	box	alone	g
10	G	CB	23	Franches-Montagnes	box paddock	group	n
11	G	CB	11	Franches-Montagnes	box	alone	bo
12	S	CB	17	Franches-Montagnes	box	alone	h
13	S	CB	15	Franches-Montagnes	box	alone	h
14	M	CB	5	Franches-Montagnes	box paddock	group	m
15	G	CB	19	Haflinger	box paddock	group	se
16	G	CB	18	Warmblood	box	alone	a
17	G	CB	7	Unknown origin	box paddock	alone	v
18	G	CB	10	English thoroughbred	paddock	group	d
19	S	CB	11	Franches-Montagnes	box	alone	h
20	M	C	7	Quarter horse	box paddock	alone	s
21	M	C	20	Franches-Montagnes	box	alone	y
22	M	C	14	Warmblood	loose housing	group	h
23	M	C	18	Camargue	box paddock	alone	b
24	M	C	14	Warmblood	loose housing	group	h
25	M	C	16	Trotter	box	alone	h
26	M	C	18	Franches-Montagnes	loose housing	group	h
27	M	C	10	Warmblood	box	alone	g
28	G	C	4	Friso-Arabian	box paddock	group	n
29	G	C	24	Unknown origin	box paddock	alone	v
30	G	C	22	English thoroughbred	paddock	group	d
31	G	C	7	Quarter horse	loose housing	group	k
32	G	C	6	Franches-Montagnes	box paddock	alone	di
33	G	C	8	Franches-Montagnes	box	alone	d
34	G	C	15	Warmblood	loose housing	group	h
35	G	C	11	Warmblood	box	alone	h
36	S	C	17	Franches-Montagnes	box	alone	h
37	S	C	7	Franches-Montagnes	box	alone	h

Experimental procedure

The content of this paper is the first part of a study. The other part, aimed at characterising the learning capabilities of crib-biters, is being prepared for submission (Briefer Freymond et al., in preparation, Chapter IV of the PhD).

Horses were tested at their home farm in a standardised way. Each horse was subjected to a total of 5 "personality" tests. The tests were divided in two sessions of 1h-1h15. Between the two sessions, the horses were returned to their home pen for a break of about 1 hour. During this time, in the farms where two horses were tested, the second horse took part in the experiment (Table 1). The procedure was as follows; at the start of the experiment, the subject was led by one experimenter (experimenter 1) to a 8x10m delimited arena that was familiar and at the same time, a second experimenter (experimenter 2) started to record the behaviour with a camera, Sony Handycam HDR-CX700. After 15 min of habituation to the recording material, the first session started and the horse was subjected to 4 personality tests (about 15 min duration in total, Figure 1, Table 2). Directly after these tests, another set of learning tests were carried out (Briefer Freymond et al., in preparation, Chapter IV of the PhD). A final personality test (about 3 min duration) was performed at the end of the second part (after the learning tests, Figure 1). During the experiment, experimenter 1 recorded the tests with the video and was in charge of preparing the arena for the next tests; whereas experimenter 2 was performing the tests (see details below, in "Personality tests").

Figure 1. Experimental procedure for the personality tests. The black dotted lines indicate the time at which each period started and ended. The personality tests are indicated (1–5). The different tests are Test 1, *passive presence test (i.e reactivity to human);* Test 2, *tactile sensitivity test (i.e. tactile sensitivity);* Test 3, *novel object test (i.e. curiosity/ fearfulness)*; Test 4, *playback test (i.e. social reactivity)*; Test 5, *umbrella opening test (i.e. curiosity/ fearfulness).* The trait locomotor activity of the horses, was score as the propensity to demonstrate locomotor activity during the *passive human test*, the *social motivation test*, and the *novel object test.* The learning tests that are indicated are part of another study (Briefer Freymond et al., in preparation, Chapter IV of the PhD).



"Personality" tests

The horses performed 5 "personality" tests adapted from (Lansade and Bouissou, 2008; Lansade et al., 2008b; a; Lansade et al., 2008c) (Figure 1, Figure 2). The tests were always conducted in the same order. They are presented in the order in which they were conducted. The behavioural measures, which were scored from a video later on, are detailed for each test.

Test 1: Passive presence test

This test assesses the propensity of a horse to react to a passive human, i.e. "reactivity to humans". An unknown experimenter (always the same person; experimenter 2) entered the test pen and settled motionless in the middle of the arena. The horse was then released and had the possibility to interact with the motionless person for three minutes (Figure 2). We scored the following behaviours related to the trait "reactivity to humans"; the time the horses spent interacting with the unknown experimenter ("Conth"), the time spent attentive ("Att"), the time spent moving ("Mov") and the time spent close (0-1m, "CAT1") or far (> 1m, "CAT2") from the unknown person (Table 2).

Test 2: Tactile sensitivity test

This test assesses the propensity of a horse to react to a greater or lesser extent to tactile stimuli, i.e. "tactile sensitivity". Experimenter 2 held the horse and applied a "Filament von Frey" on its skin (Figure 2). These filaments consist of a hard plastic body connected to a nylon thread, and are calibrated to exert a specific magnitude of force on the skin, ranging from 0.008-300g. Five different forces were applied, always in the same random arrangement (300g, 0.6g, 0.02g, 0.008g, 1g, perpendicular to the animal's skin at wither's height, until the nylon thread started to bend). The interval between the application of each filament was about 30 s. Experimenter 1 recorded directly in binary form the following behaviour related to the trait "tactile sensitivity"; trembling of plasma muscle (trembling or not, "React"; Table 2).

Test 3: Novel object test

This test assesses the propensity of a horse to react with fear or curiosity when exposed to a novel situation, i.e. "curiosity/fearfulness". A novel object (i.e. transparent hose fixed with colourful string) was placed in the middle of the arena, in front of the horse held by experimenter 2. The horse was then released for a duration of three min, during which it had the possibility to explore the object (Figure 2). We analysed the following behaviours related to the trait "curiosity/fearfulness"; the time the horse spent interacting with the novel object ("Conto"), the time spent attentive ("Att"), the time spent moving ("Mov") and the time spent close (0-1m, "CAT1") or far (> 1m, "CAT2") from the novel object (Table 2).

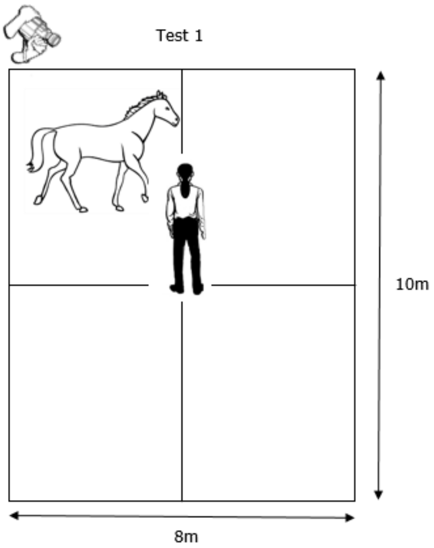
Test 4: Playback test

This test assesses the propensity of a horse to react to a conspecific, i.e. "social reactivity". This test was adapted from a study including a playback procedure (Briefer et al., 2017). It consisted in measuring the reaction of the subjects to the vocalisations of conspecifics. We used a loudspeaker located next to the camera, on one side of the arena, and played one 2-s whinny from an unknown mare, one 3-s whinny from an unknown gelding, and a control sound (15 s of skylark song, *Alauda arvensis*) (Figure 2). All the sounds were played at similar amplitude, estimated to be normal for the horses. All the horses received the same three sounds, played in a random order, with 10-s silence interval. We analysed the following behaviours related to the trait "social reactivity"; the vocal response of the horses to each sound ("Whin"), the time spent moving ("Mov") and the time spent attentive ("Att") (Table 2).

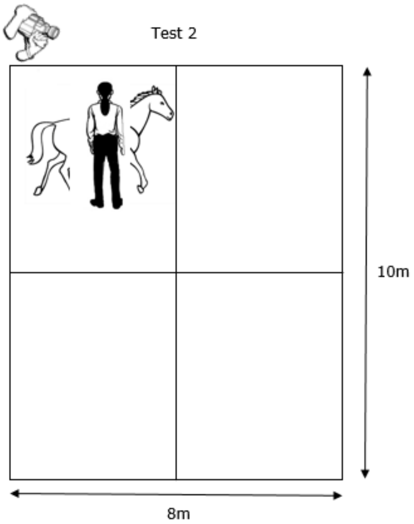
Test 5: Umbrella opening test

This test was carried out after a battery of learning tests (Briefer Freymond et al., in preparation, Chapter IV of the PhD). It assesses the propensity of a horse to react with fear or curiosity to a sudden situation (umbrella opening), i.e. "curiosity/fearfulness". This test, in a similar way to Test 3 (*novel object test*), measures fear reactions but this time in situations involving suddenness. A bucket of pellets was placed next to the entrance, with a closed umbrella held at one meter from the bucket and one meter above the ground by experimenter 2. Experimenter 1 released the horse and it was free to go eat in the bucket. When the animal was eating with its head in the bucket for more than three second, experimenter 2 suddenly opened the umbrella and the chronometer started (Figure 2). The test stopped when the horse resumed eating. The time it took for the horses to come back to eat in the bucket after the umbrella opened was directly recorded ("Time"). A maximum of 300 s was allocated. In case the horse did not come back to eat, the time was fixed at 300 s (Time = 300 s). We scored the following behaviours related to the trait "fearfulness"; the intensity of the reaction using a scale ("React") (Table 2) and the estimated flight distance of the horses after opening the umbrella ("Flight") (Table 2). Because of a technical problem with the cameras, we were not able to score the behaviours React and Flight of 2 control horses in this test.

Test 1



Test 2



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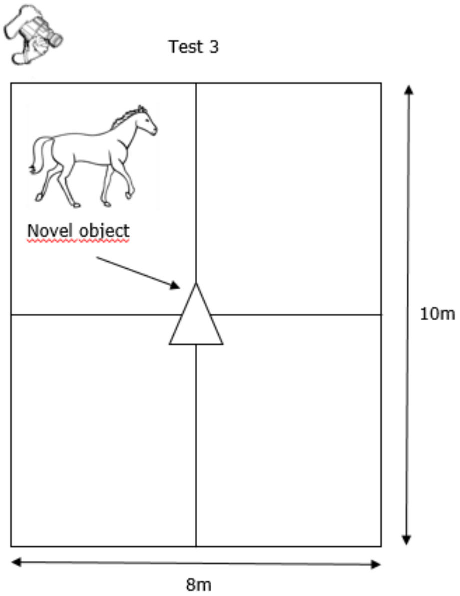
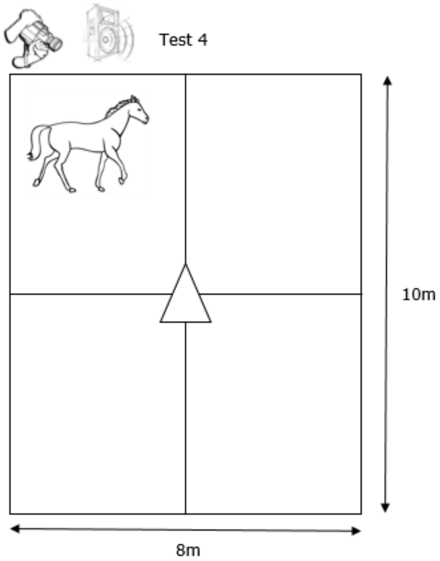


Test 4



Test 5

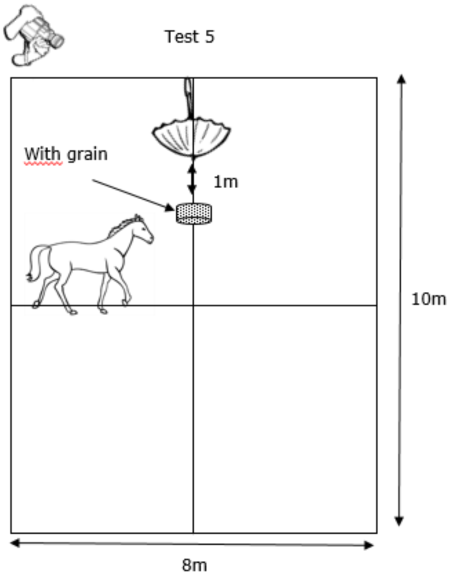


Figure 2. Scheme of the different "personality" tests. The camera is represented at top left. In Test 1 (*passive presence test*), Experimenter 2 is in the middle of the arena. In Test 2 (*tactile sensitivity test),* the Experimenter 2 holds the horse and applies the different filament von Frey. The white triangle in Test 3, (*novel object test)*, designates the unknown object. In Test 4 (*playback test*), the loudspeaker is represented at the top left, next to the camera. In Test 5 (*umbrella opening test*), the dotted box designates the bucket with food. The black line in the arena designates the arena divided into four sectors to assess locomotor activity (*See Behavioural analyses*).

Behavioural analyses

All tests were video recorded by experimenter 1, who was located outside of the arena (Figure 2), using a Sony Handycam HDR-CX700. From the video of the tests, two different observers (one who was blind to the group of the horses and experimenter 2) scored each test (i.e. all the videos) continuously, as occurrence (for discrete behaviours; "Point Events"), or as duration (for behaviours lasting in time; "State Events") using the Observer software XT v.11 (Noldus). We then calculated the frequency of occurrence per minute for the Point Events, and the proportion of the total time spent performing the behaviour for State Events (Table 2). The last personality trait assessed in the study, locomotor activity of the horses, was scored as the propensity to demonstrate locomotor activity during the *passive human test*, the *social motivation test*, and the *novel object test*. In order to record this personality trait, the arena was divided into four sectors of equal size using tracks made in the sand beforehand (Figure 2). To assess the locomotor activity, experimenter 1 recorded directly the number of times the horses changed sectors ("Locom") (Table 2). Because of a problem, we were not able to score the activity level of three control horses in this test.

Table 2. Parameters and definitions of the behaviours recorded during the different personality tests (1-5) and to measure the locomotor activity. The crosses indicate which parameters or response variables were recorded for each personality trait. The different tests are Test 1, *passive presence test (i.e reactivity to human);* Test 2, *tactile sensitivity test (i.e. tactile sensitivity);* Test 3, *novel object test (i.e. curiosity/ fearfulness)*; Test 4, *playback test (i.e. social reactivity)*; Test 5, *umbrella opening test (i.e. curiosity/ fearfulness); Locomotor activity ("Locom")*.

parameters abbreviation	**definition**	**Tests / personality trait**
"Att"	Proportion of time spent looking outside; horse stands still with raised neck and ears pointing toward stimuli situated outside of the arena (experimenter, object or camera)	Test 1, 3, 4
"Mov"	Proportion of time spent walking toward outside; horse walks with raised neck and ears pointing or low neck exploring toward stimuli situated outside of the arena (experimenter, object or camera)	Test 1, 3, 4
Contact with human, "Conth" or object, "Conto"	Proportion of time spent sniffing, licking or nibbling the experimenter or object, moving the object with the foreleg or the mouth	Test 1, 2
"Whin"	Number of whinnies produced; longest, loudest and most common horse vocalisation	Test 4
"Sound"	Sound treatments played to the horses; whinny from a mare, whinny from a gelding, and a control sound (skylark song)	Test 4
"Order"	Order in which the sound treatments were played to the horses	Test 4
"CAT1" (0-1m), "CAT2" (>1m)	Proportion of time spent at (0-1m) and (>1m), respectively; estimated distance between the horse and the object or human	Test 1, 3
"React"	Reaction to the tactile filament - trembling or not	Test 2
"Force"	5 different filament forces ("F") applied to the skin ("F1"=300 g, "F2"=0.6 g, "F3"=0.02 g, "F4"=0.008 g, "F5"=3 g)	Test 2
"Locom"	Sectors entered; number of sectors entered during the Tests 1, 3 and 4	Locom
"Reacum"	Intensity of the reaction to the opened umbrella; nothing ("A"), raises head ("B"), steps back ("C"), jumps back and looks at the umbrella ("D"), jumps back and looks outside ("E"), jumps back and canter ("F")	Test 5
"Flight"	Flight distance from the opened umbrella; how far the horse moves away from the food bucket ("A" = 0 m; "B" = 0-1 m, "C" = 1-2 m, "D" ≥ 2 m)	Test 5
"Time"	Time until eating in seconds; time to resume eating in the bucket after the umbrella was opened	Test 5

Statistical analysis

Inter-observer reliability (ICC)

Inter-observer reliability between the two observers scoring the videos continuously was assessed by intraclass correlation coefficients (ICC). ICC were calculated using a two-way mixed design to assess the absolute agreement between the scores of the two observers (Shrout and Fleiss, 1979; Landers, 2015). ICCs range from 0 to 1, with 0 indicating no agreement and 1 indicating full agreement. Generally, ICCs ≤ 0.40 are considered as poor, those between 0.40 to 0.59 as fair, those between 0.60 to 0.74 as good, and those between 0.75 to 1.00 as excellent (Cicchetti, 1994). We kept for the analysis only the behaviours for which ICCs revealed fair to excellent agreements between the scores of the two observers.

Behavioural measures

The statistical analyses were carried out on the behavioural parameters expressed by at least 40% of the horses, and for which the inter-observer reliability (ICC) between the two observers was fair to excellent (ICC: mean ± SD= 0.84 ± 0.14; range = 0.55-0.98). The behaviour of the crib-biters (CB) was compared to the behaviour of the control horses (C) for each test separately (Table2, Table 3), using linear mixed-effects models (LMM; lmer function, lme4 library), generalised linear mixed models (GLMM; glmer function; lme4 library; multcomp library; (Pinheiro and Bates, 2000), or cumulative link mixed models (CLMM, clmm function) in R 3.0.2 (R Development Core Team, 2013). The different models included as a response variable the behavioural parameters scored (Tables 2 and 3). The fixed and control factors are described in Table 2 and 3. To control for repeated measurements of the same subjects, the identity of the horses nested within the farms where they were housed ("Farms") was included as a random factor for Tests 2 and 4. For Test 1, Test 5 and for the locomotor activity, only Farms was included as a random factor, as there was only one behavioural value for each horse. When significant interaction effects between fixed and/or control factors were found, further post-hoc analysis were carried out using further LMMs and GLMMs. Bonferroni corrections were applied to these post-hoc tests accordingly.

Table 3. Response variables, as well as fixed and control factors used in the different model (LMM and GLMM). The abbreviations are described in Table 2. The crosses indicate which response variables or factors were recorded in the different personality tests (1 - 5) and to assess the locomotor activity "Locom". The different tests are Test 1, *passive presence test;* Test 2, *tactile sensitivity test;* Test 3, *novel object test*; Test 4, *playback test*; Test 5, *umbrella opening test.* The fixed parameters are the "Group"

("CB"=crib-biters and "C"=controls), the filament forces "Force" (Test 2), the interaction term between the Force and Group CB-C (Test 2), the interaction term between sound treatment "Sound" and Group CB-C and between the order of the sound "Order" and Group CB-C (Test 4). The other parameters are control parameters.

Response variables	**Test 1**	**Test 2**	**Test 3**	**Test 4**	**Test 5**	**Locom**
Att	x		x	x		
Mov	x			x		
Conth	x					
Conto			x			
Whin				x		
CAT1	x		x			
CAT2	x		x			
React		x				
Locom						x
Reacum					x	
Flight					x	
Time					x	
Control and Fixed factors	**Test 1**	**Test 2**	**Test 3**	**Test 4**	**Test 5**	**Locom**
Group	x	x	x	x	x	x
Force		x				
Force * Group		x				
Sound * Group				x		
Order * Group				x		
Sex	x	x	x	x	x	x
Age	x	x	x	x	x	x
Arena	x	x	x	x	x	x
Farm	x	x	x	x	x	x
Order				x		
Sound				x		

The residuals were checked graphically for normal distribution and homoscedasticity. To satisfy the model assumptions, we used log transformation for "Att", in the *presence passive test* and for "Time" in the *umbrella test (*Table 2)*.* All the parameters satisfying model assumptions were then input into LMMs (lmer function). Some parameters did not meet the statistical assumptions despite transformation. They were thus transformed to binomial data as follows; behaviour occured = 1 or did not occur = 0 for "CAT1" in *novel object test,* and "Mov" in *presence passive test* and *playback test* (Table 2); and value equal or higher than median = 1 or value lower than median = 0, for "Att", "Conth", "CAT2" in *novel object test,* "Att" in *playback test* and "Locom" for *locomotor activity* (Table 2)*.* The parameters scored as binomial ("React" and "Whin"), as well as parameters transformed to binomial data, were input into GLMMs fit with binomial family distribution and logit link function (glmer function). In the *umbrella opening test,* in order to compare the intensity of the reaction after opening the umbrella ("Reacum") and the distance of flight from the open umbrella ("Flight") (Table 2), we used CLMM (clmm function) (Haubo and Christensen, 2015). To this aim, Reacum was transformed in 6 distinct categories and Flight in four categories (Table 2).

For the LMMs and GLMMs, a standard model simplification procedure was used to remove each non-significant term until the deletion caused a reduction in goodness of fit (in this case, the term was left in the model). P-values were calculated based on Satterthwaite's approximations (anova function, lmerTest package in R). The significance level was set at $\alpha = 0.05$.

RESULTS

Passive presence test

There was no difference between groups CB and C in their time to interact with the person ("Conth") and their time spent attentive ("Att") (LMM: effect of Group CB-C on Conth, X_1^2 = 1.71, p = 0.19; effect of Group CB-C on Att, X_1^2= 1.66, p = 0.20). There was no group differences, neither on the proportion of time spent moving ("Mov"), nor on the time spent close ("CAT1") or far ("CAT2") from the unknown person (GLMM: effect of Group CB-C on Mov, X_1^2= 0.11, p = 0.74; LMM: effect of Group CB-C on CAT1, X_1^2 = 0.89, p = 0.35; LMM: effect of Group CB-C on CAT2, X_1^2 = 0.89, p = 0.35).

Tactile sensitivity test

A greater proportion of crib-biters reacted to the filaments von Frey ("React") than the control horses (GLMM: effect of Group CB-C on React, X_1^2 = 8.14, p=0.004, Figure 3). In this test, the strength of the filaments ("Force") also had an effect on React (GLMM: effect of Force on React, X_4^2 = 13.78, p = 0.008).

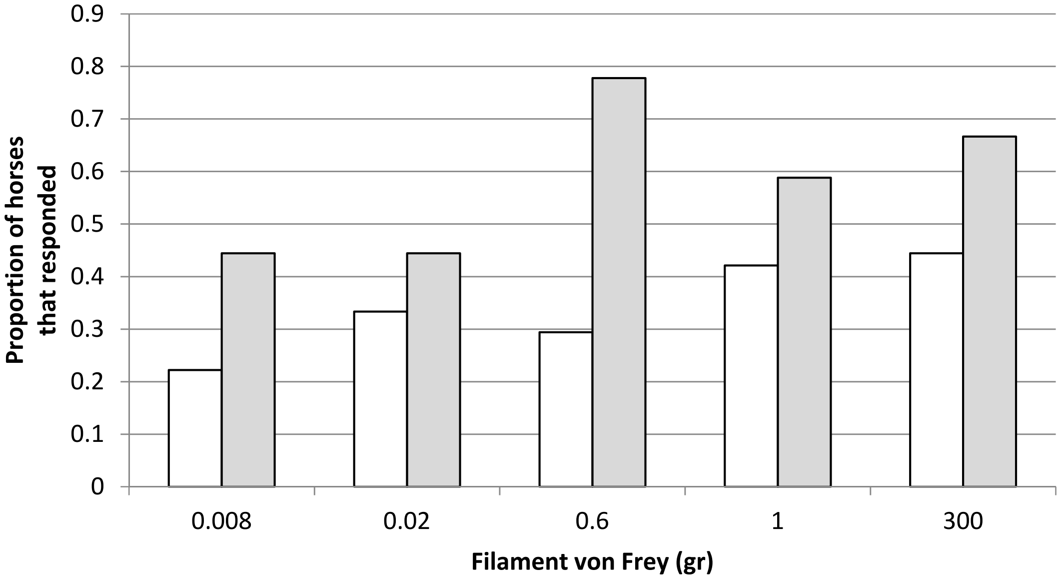


Figure 3. Responses to the different filaments von Frey. Proportion of controls (C, white, *N*= 18) and crib-biters (CB, grey, *N* =19), respectively, on y axis that responded to each Filament von Frey, on x axis (300, 0.6, 0.02, 0.008, 1 gr.).

Novel object test

There was no difference between groups CB and C in the time to interact with the unknown object ("Conto") and the time spent attentive ("Att") (GLMM: effect of Group CB-C on Conto, X_1^2 = 0.30, p = 0.58; effect of Group CB-C on Att, X_1^2 = 0.67, p = 0.41). There was also no group effect on the time spent very close ("CAT1") or very far ("CAT2") from the object (GLMM: effect of Group CB-C on CAT 1, X_1^2 = 32.80, p = 0.94; effect of Group CB-C on CAT 2, X_1^2 = 0.02, p = 0.89).

Playback test

There was no difference between groups CB and C in the vocal responses to the playbacks ("Whin") (GLMM: effect of the Group CB-C on Whin, X_1^2 = 2.77, p = 0.10). There was also no group effect on the proportion of movements ("Mov") during this test (GLMM: effect of the Group CB-C on Mov, X_1^2 = 0.43, p = 0.83). There was a marginally significant interaction effect between Group CB-C and Order on the time spent attentive ("Att") (GLMM: interaction effect between Group CB-C and Order on Att, X_1^2 = 3.81, p = 0.051). However, further post-hoc analyses conducted on each group separately did not show any significant effect of the order in which the sound treatments were played on Att (GLMMs: p≥ 0.19 for all).

Locomotor activity

There was no difference between groups CB and C on the number of sectors entered, which reflects locomotor activity ("Locom") (GLMM: effect of the group CB-C on Locom, X_1^2 = 1.74, p = 0.18).

Umbrella test

There was no difference between groups CB and C in the time taken to resume eating in the bucket after the umbrella was suddenly opened ("Time") (LMM: effect of the Group CB-C on Time, X_1^2 = 0.69, p = 0.41). There was also no difference between groups in the intensity of the horses' reaction after the umbrella was opened ("Reacum") (CLMM: effect of the Group CB-C on Reacum, X_1^2 = 0.366, p = 0.55). Finally, there was no group effect on the flight distance ("Flight") (CLMM: effect of the Group CB-C on Flight, X_1^2 = 1.065, p = 0.30).

Control factors

The type of arena (indoor or outdoor, "Arena") had a significant effect on Reacum in the *umbrella test*, (CLMM: effect of Arena on Reacum, X_1^2 = 4.2, p = 0.04), and on React in the *tactile sensitivity test* (GLMM: effect of Arena on React, X_1^2 = 5.36, p = 0.02). The age of the horses ("Age") had a significant effect in the *passive presence test* on CAT1 and CAT2 (GLMM: effect of Age on the CAT1, X_1^2 = 6.09, p = 0.01; effect of Age on CAT2, X_1^2 = 6.09, p = 0.01) and on Att (LMM: effect of Age on Att, X_1^2 = 9.15, p = 0.002). In the *Novel object* test, Age had an effect on CAT2 (GLMM: effect of Age on CAT2, X_1^2 = 9.90, p = 0.002). The sex of the horses ("Sex") had a significant effect in the *playback test* on Att (GLMM: effect of Sex on the Att, X_2^2 = 3.40, p = 0.14). The order in which the sounds were played *in the playback test* ("Order") had an effect on Mov (GLMM: effect of Order on Mov, X_1^2 = 7.13, p = 0.008).

The control factors not mentioned above were not significant and were thus removed from the models during model selection.

The means and standard deviations of parameters recorded for crib-biters and control horses for which there were no differences between the groups are summarised in Annex III of the PhD.

DISCUSSION

Environmental causes responsible for the development of stereotypies are partially known (Nagy et al., 2009), but little is still known about why some individuals develop stereotypies and others do not, despite being exposed to the same environmental conditions. Here, we tested the hypothesis that predispositions to stereotypies might be linked to individual differences in behaviour ("personalities"), which are in part genetically determined. To this aim, we compared how stereotypic and non-stereotypic horses (controls), responded to a standardised test battery commonly used to assess individual differences in horses (Lansade and Bouissou, 2008; Lansade et al., 2008b; a; Lansade et al., 2008c). Based on previous findings (Koolhaas et al., 2010; Nagy et al., 2010; Górecka-Bruzda et al., 2011; Briefer Freymond et al., 2015; Finkemeier et al., 2018), we expected crib-biters to show behavioural characteristics of reactive coping individuals, namely to be less anxious (e.g. fearful), to interact less with unfamiliar humans, to be less active, to show fewer exploratory behaviours, and to be more social compared to control horses. However, contrary to our expectations, we did not find any differences in these traits between stereotypic and control horses. Since reactive coping strategies are characterised by freeze responses and unresponsiveness, it might be more difficult to detect fear in these animals (Squibb et al., 2018). Surprisingly, however, we found that a greater proportion of crib-biters reacted to the tactile filaments compared with the control horses suggesting a higher tactile sensitivity, which to our knowledge has never been reported before. Stereotypic animals are often compared to human autistic patients (Lewis et al., 2007), and especially to those with Asperger syndrome. Interestingly, autistic people are known to be highly sensitive to tactile stimulation (Blakemore et al., 2006), in the same way as crib-biters in our study. We conclude that it might be valuable to conduct further investigations to assess the personality of stereotypic horses, as it could help to identify genetic loci associated with stereotypies.

Reactivity to humans

We did not find any difference between crib-biters and controls in their propensity to react to a passive human. Because this trait has been previously related to boldness, a characteristic of proactive individuals (Górecka-Bruzda et al., 2011; Finkemeier et al., 2018), we expected crib-biters, as potential reactive individuals, to interact less with unfamiliar humans. Since how animals react to humans is known to be heavily influenced by the environment (e.g. previous human handling) (Hausberger and Müller, 2002), genetic predisposition acting on this trait might be difficult to detect. We thus suggest that environmental components could have influenced previously existing differences between stereotyped and control horses in our study.

Tactile sensitivity

In our *tactile sensitivity test,* a greater percentage of crib-biters reacted to the tactile filaments compared with the control horses. This suggests that crib-biters might be highly sensitive to tactile stimulation. Because some people affected by human developmental disorders, such as autism may also develop stereotypic behaviours, a comparison could be made with autistic people, especially those with Asperger syndrome, also known to be highly sensitive to tactile stimulation (Blakemore et al., 2006). They are indeed often described as being disturbed by their environment because they perceive external stimuli with higher intensities than other people. Some studies also report hypersensitivity to senses, such as touch, smell and taste (Talay-Ongan and Wood, 2000). We could thus suggest that crib-biters, because of their low threshold for responding to tactile sensory stimuli may be, in the same way as autistic people, easily irritated by tactile stimulations (Dunn, 2001). This higher tactile sensitivity could be also one of the underlying causes of their higher stress sensitivity (Briefer Freymond et al., 2015), which might result in the development of stereotypic behaviour in these individuals. It would be interesting to conduct further experiments testing the sensitivity of crib-biters within other senses (e.g. gustato-olfactory, auditory and visual sensitivity; (Lansade et al., 2008c)

The hypersensitivity that we found in crib-biters could also be explained by neural differences between these horses and controls. Tactile stimuli stimulate skin receptors. This information is then transmitted via the spinal cord to the thalamus and on to cortical sensory areas. Tactile information is mapped onto the primary and secondary *somatosensory cortex*. This cortex shows a somatotopic organisation, with the most sensitive parts of the body occupying the most cortical territory (Blakemore et al., 2006). A distinction can be made between sensory processing and sensory sensitivity; individual can perceive stimuli and not respond to it. Existing theories and hypersensitivity in autistic spectrum disorder suggest enhanced processing of stimuli details in the secondary somatosensory cortex, or impairment of top-down modulation of incoming stimuli (Plaisted et al., 1998; Frith, 2003). Additionally, we could suggest that exposition to chronic stress may cause alteration of dopaminergic systems, not only in the *mesoaccumbens* dopamine system as reported in stereotypic animals (Cabib and Puglisi-Allegra, 2012; McBride and Parker, 2015) but also in dopaminergic nerve cells implicated in sensory sensitivity (Reinig et al., 2017). Therefore, the dopaminergic modulation impairment that crib-biters potentially suffer from could also be implicated in their sensory hypersensitivity (McBride and Hemmings, 2009). We could thus suggest that the hypersensitivity that we found in crib-biters is explained by differences between stereotypic and non-stereotypic horses in their neural processing of tactile stimuli or in dopamine modulation (McBride and Hemmings, 2005; Roberts et al., 2016).

Social reactivity

We did not find any difference between crib-biters and controls in their social reactivity, suggesting that crib-biters are not more aroused than control horses when hearing unknown horses, according to our hypothesis. If crib-biters indeed display a reactive coping strategy, these results might contradict findings in pigs, which showed that reactive pigs could be more social (Reimert et al., 2014).

Locomotor activity

The data used to score the "locomotor activity" in the *passive human test*, the *social motivation test*, and the *novel object test* did not reveal any difference between crib-biters and controls in this trait. According to previous studies, we expected crib-biters, if they indeed behave as reactive individuals, to show less locomotor behaviour than control horses (Koolhaas et al., 2010; Finkemeier et al., 2018). On the other hand, in stereotypic animals of other species (e.g. mice, (Joshi and Pillay, 2016); (Würbel and Stauffacher, 1994); and rhesus macaques, (Gottlieb et al., 2013)), higher incidence of stereotypy development have been associated with more activity. Yet, discrepancies between these studies and ours could be explained by differences in the type of stereotypy displayed (locomotor stereotypies versus oral stereotypy in our case), by species or experimental protocol differences. Indeed, the measured phenotype of individuals will depend on the initial definition and use of each trait and on the terminology used to define personality, which varies widely between studies (Borstel, 2013; Finkemeier et al., 2018).

Curiosity/fearfulness

According to our previous results showing that crib-biters display physiological characteristics of reactive individuals, i.e. high HPA-axis reactivity (Briefer Freymond et al., 2015) and to the results of Nagy et al. (2010), we expected stereotypic horses to also display behavioural characteristics of reactive individuals, such as being less fearful (or anxious) than control horses. However, we did not find any difference between our two groups in their fear reaction to the sudden opening of the umbrella. Previous studies showed that crib-biters seem to be less reactive while restrained with a lip-twitch, but to react more strongly to a rapidly inflating balloon compared to non-stereotypic horses (Minero et al., 1999). Our results did not confirm these results. Discrepancies between

these studies and ours could be explained, once more, by experimental protocol differences (Ladewig, 1987).
We could also suggest that there might exist a difference in fearfulness between crib-biters and non-stereotypic horses, but that the behavioural indicators that are generally used to assess fearfulness are not appropriate to detect such differences between reactive and proactive individuals. Indeed, behavioural reactions to fear-induced reactions might be less strongly expressed (e.g. characterized by freeze responses and unresponsiveness) in those individuals compared to proactive ones (Koolhaas et al., 2010; Squibb et al., 2018). Therefore, if crib-biters are really more reactive than other horses, it is possible that, despite a stronger physiological reaction to the opening of the umbrella, their behaviour did not change (Squibb et al., 2018). It would thus be useful, in further studies investigating differences in fearfulness between proactive and reactive animals, to measure other types of fear indicators (e.g. physiological responses, Equine Facial Action Coding Systems (FACS)) (Wathan et al., 2015) in addition, in order to increase the accuracy of fear assessments.

In the same way as for the reaction to the opening of the umbrella, we did not find any difference in reaction towards the novel object between crib-biters and controls. Links between reactions to novel objects and stereotypies has also been tested in other species than horses. Unlike in our study, stereotypic mice show greater reactivity, quicker time to approach novel objects and increased object manipulation, suggesting less fearfulness or higher levels of curiosity compared to control mice (Joshi and Pillay, 2016). Similarly, rhesus macaques that express a higher rate of motor stereotypic behaviour in captivity are characterised by more frequent contacts with a novel object, indicating higher levels of curiosity than other monkeys (Gottlieb et al., 2013). Differences between these studies and ours might be due to the type of stereotypies investigated in mice and rhesus macaques, which was, unlike in ours, locomotor (Gottlieb et al., 2013; Joshi and Pillay, 2016). We suggest that some similarities with these studies could be found in weaving more than crib-biting horses.

CONCLUSION

Our results suggest that crib-biters are more sensitive to tactile stimulation than non-stereotypic horses, a specificity also found in human autistic patients (Blakemore et al., 2006). This suggest that this higher tactile sensitivity could be also one of the underlying causes of their higher stress sensitivity (Briefer Freymond et al., 2015), which might result in the development of stereotypic behaviour in these individuals. We also suggested that this higher tactile sensitivity could be due to altered dopamine physiology, resulting from chronic stress exposition. On the other hand, we did not find any personality traits that are characteristic of reactive coping individuals in crib-biters, as we had expected (i.e. to be less fearful, to interact less with unfamiliar human, to be less active, to show less exploratory behaviours and to be more social). We suggest that further studies investigating differences in fearfulness between proactive and reactive animals, which in our case were expected to reflect differences between control and stereotyped horses, should include further behavioural and particularly physiological measures. Indeed, this might to help detect differences between proactive and reactive coping strategies, since fear-induced behavioural reactions might be less strongly expressed in reactive individuals compared to proactive ones (Koolhaas et al., 2010), as recently suggested by Squibb et al. (2018). We conclude that further investigations are required to fully characterise the personality of stereotypic horses. This could allow an early detection of individuals prone to develop stereotypies, and hence might help to prevent them to develop this abnormal behaviour.

COMPETING INTEREST

We have no competing interests to report.

ACKNOWLEDGMENTS

We are grateful to all the owners of the horses, who offered to participate in this study.

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CHAPTER IV

Based on:

Stereotypic behaviour in horses may influence stress and expression of frustration during learning tasks

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In preparation

ABSTRACT

Stereotypies are a common phenomenon in captive animals but it remains unclear whether such behaviours are pathological by-products of captive conditions and/or whether they have an adaptive function. Here, we address this problem with a study on crib-biting, a common type of stereotypic behaviour in domestic horses, thought to emerge as an interaction between chronic stress and genetic predispositions. Since chronic stress is known to affect learning abilities via dopaminergic modulation in the basal ganglia, we predicted that stereotypic horses would perform learning tasks using different strategies than healthy controls. We tested 19 crib-biters and 18 control horses in 6 challenging spatial tasks, while measuring behavioural and physiological data (heart rate and salivary cortisol) to assess learning performance and stress levels. We found that, during the learning tests, 10 crib-biters executed stereotypic behaviour while 9 crib-biters and the control horses did not. We found that the stereotyping horses had lower salivary cortisol values compared to non-stereotyping crib-biters and control horses after the first part of the experiment, and displayed lower levels of frustration behaviour during one of the test. However, we found no difference between the three groups in their learning capacities. Our results therefore suggest that crib-biting might be adaptive as it reduces stress and frustration, however, they do not allow us to draw conclusion on learning alterations.

INTRODUCTION

Stereotypies in animals have been defined as repetitive, relatively invariant patterns of behaviour, with no apparent goal or function (Mason and Latham, 2004). Stereotypies also exist in humans, can be either psychologically or environmentally induced, and are often associated with developmental disorders, such as autism, neurological disorders, obsessive compulsive disorder (OCD), Tourette's syndrome and severe psychiatric disturbances (e.g. schizophrenia) (McBride and Parker, 2015). In animals, they can appear as stereotypic locomotor patterns (e.g., compulsive digging and looping in gerbils and mice (Würbel and Stauffacher, 1994)) or stereotypic oral movements (e.g. bar-biting and sham-chewing by sows, tongue rolling by cows (Sambraus, 1985)). In contrast to comparable disorders in humans, stereotypies in animals appear to be mainly induced by restricted environments, suboptimal housing conditions or management problems (McBride and Parker, 2015).

Stereotypies have been reported to result from an interaction between a genetic predisposition and stressful or frustrating environments (Luescher et al., 1991; McBride and Parker, 2015). Internal or external stressors normally induce necessary and adaptive physiological reactions (von Borell, 2001). These reactions help individuals to cope with a situation and regain homeostasis (or a steady state). However, stressors differ qualitatively (physical or psychological) and quantitatively (chronic, acute, intermittent) (McBride and Parker, 2015). When stressors such as unpredictable, repetitive situations, are sustained for an extended period, "stress" or a chronic stress state can occur (Koolhaas, 2011), and this can have deleterious effects on animals, including the development of stereotypies. Physiologically, two pathway are activated during a stress response; the hormonal hypothalamo-pituitary-adrenocortical (HPA) axis and the neural sympatho-adreno-medullary (SAM) axis. Chronically stressed animals can develop, for example, an increased or maintained HPA responses to a novel stressor (Bhatnagar and Vining, 2003).

Following long-term chronic stress exposure, the interaction among stress hormones can affect the neurobiological system of stereotypic animals (Cabib and Puglisi-Allegra, 2012). More specifically, the functioning of the *basal ganglia* can be modified through alterations in the dopaminergic system (Saka et al., 2004; McBride and Hemmings, 2005). This has been described for crib-biter horses, which have been reported to have structural alterations in the *striatum* of the *basal ganglia*, due to higher numbers of dopamine receptors in the *ventral* and lower numbers in the *dorsomedial striatum*, resulting in a sensitisation to dopamine release (McBride and Hemmings, 2005). Similar neurophysiological differences within the *basal ganglia* have been identified in stereotyping mice (Cabib et al., 1998; McBride and Parker, 2015). Dopaminergic dysfunction, in turn, is thought to have cognitive implications, such as excessive desire for food rewards or lack of flexibility (e.g. perseveration), which is likely to impact on learning performance and predisposition to frustration (Ijichi et al., 2013). In addition, short-term acute stress can have a negative impact on learning performances and memory (Valenchon et al., 2013), mediated by HPA-released glucocorticoids and SAM-released catecholamines (Schwabe and Wolf, 2010).

Support for a role of *basal ganglia* dysfunction in learning has been shown in autistic people, which show poor abilities to suppress behaviours (perseveration), although high stereotypic levels do not always correlate with higher perseveration (Lopez et al., 2005; Boyd et al., 2009). In schizophrenic and autist patients, a correlation between inappropriately repeat response, "recurrent perseveration" and intensity of stereotypy was shown in two-choice guessing tasks (Frith and Done, 1983; Turner, 1997). In animals, stereotypy levels (frequency of stereotypy performance) have been also shown to constitute strong predictors of the latency to extinguish conditioned responses or of the tendency to inappropriately repeat responses, which are both indirect measures of *basal ganglia* dysfunction in several species such as bears (Vickery and Mason, 2005), Orange-Wing Amazon Parrots, (Garner et al., 2003) and bank voles (Garner and Mason, 2002). This further suggests a relationship between dopaminergic dysfunction and lack of cognitive flexibility (Garner and Mason, 2002; McBride and Parker, 2015). Since the extinction of a previously learned response when it no longer leads to the desired outcome is a cognitive process that is essential in response to a changing environment, this implies that stereotypies constitute maladaptations in animals (Cooper and Mason, 1998; Valenchon et al., 2013).

In domesticated horses, oral (crib-biting or windsucking) and locomotor stereotypies (weaving and box-walking) are the most common repetitive abnormal behaviours (Mason and Rushen, 2006). For instance, crib-biting has a prevalence of 2.4–8.4% (Vecchiotti and Roberto, 1986; Luescher et al., 1991). Similar to stereotypic bears, crib-biting horses persist longer during the extinction phase of operant learning tasks than controls (Hemmings et al., 2007; Roberts et al., 2015). However, crib-biters are also faster at spatial learning than controls and therefore are suggested to be more prone to habit formation after overtraining (Parker et al., 2008; Parker et al., 2009). Overall, the current consensus is that stereotypies favour habit formation and thus a loss in cognitive flexibility (Schwabe and Wolf, 2011). On the other hand, a recent study did not show evidence of learning impairment between crib-biters and control horses in a visual reversal learning task, considered as an indirect behavioural measure of flexibility of learning and attention (Briefer Freymond et al., 2018). However, most of these results are based on limited sample sizes. In addition, locomotor detour tasks, which are generally considered as disinhibition of prepotent responses to reward cues, a form of impulsivity (Dallaire et al., 2011; Kabadayi et al., 2017), have, to our knowledge, never been tested in stereotypic horses.

In this study, we compared the performance of a relatively large number (*N* = 19) of horses affected by stereotypic behaviour (crib-biters) and a similar number of non-stereotypic control individuals in frustration-causing spatial learning tasks, i.e., reversal, detour learning. Frustration in learning tasks can occur when previously learned expectations are no longer met, or when sudden barriers prevent access to a reward (Psyrdellis et al., 2016). Based on previous results, we predicted that crib-biters would be less flexible than controls, with a stronger dependency on rewards and use different strategies due to alterations in their dopaminergic system (McBride and Hemmings, 2005; Hemmings et al., 2007; Parker et al., 2008; Parker et al., 2009; Ijichi et al., 2013; Roberts et al., 2015). Additionally, according to our previous results (Briefer Freymond et al., 2015), we expected crib-biters to show higher physiological stress responses during the experiment than controls, but that executing stereotypic behaviours would have a beneficial effect on reducing stress and potentially on learning capacities.

METHODS

Subjects and management conditions

The study was carried out from September 2013 to February 2014 on 19 crib-biters and 18 control horses of various breeds, sexes (mares, geldings and stallions) and ages (3 to 24 years old; table 1), housed in 19 different farms in Switzerland. Except for one control, all horses participated in a previous study (Chapter II of the PhD, Annexe II). Twenty-six horses were privately owned, 11 belonged to the Swiss National Stud Farm (https://www.agroscope.admin.ch/agroscope/en/home/about-us/snsf.html). All subjects had lived on their premises for at least one year prior to testing. To qualify as a crib-biter, a subject must have shown crib-biting behaviour for a minimum of 1 year, according to its owner. Controls were horses that had never been observed crib-biting or other stereotypies. We matched each crib-biting horse with a control horse of similar breed, sex and age, housed in similar conditions and if possible on the same farm (table 1). Housing was either individually or in groups, in single boxes or paddocks (table 1). Routine care was provided by the owners. The study was approved by the Federal Veterinary Office (approval number VD 26777 bis; Switzerland).

Table 1. Characteristics of the horses used in the experiment. Sex (M = mare; G = gelding, S = stallion), Group (CB = crib-biters; C = controls), part 1 and 2 of the learning tests, age, breed (FM = Franches-Montagnes; ET = English Thoroughbred), and farm (each letter refers to a given farm). Group A-B-C indicates which sub-group the horses were part of (Group A corresponds to the crib-biters that crib-bit during part 1 and/or during part 2 of the learning test, Group B to the crib-biters that did not crib-bite at all, and Group C to the controls) during part 1 and part 2 of the experiment.

Horses	Sex	Group CB-C	Group A-B-C Part 1 /Part 2	Age (years)	Breed	Place
1	M	CB	B - B	6	Swiss Warmblood	c
2	M	CB	B - A	22	Criollo	g
3	M	CB	A - A	16	FM	y
4	M	CB	A – A	9	Hispano-Arabian	b
5	M	CB	B – B	5	Quarter Horse	s
6	M	CB	A – A	9	Paint Horse	r
7	M	CB	A – A	5	Paint Horse	k
8	G	CB	A – B	9	FM	d
9	G	CB	A – B	11	Swiss Warmblood	g
10	G	CB	B – A	23	FM	n
11	G	CB	A – A	11	FM	bo
12	S	CB	B – B	17	FM	h
13	S	CB	B – B	15	FM	h
14	M	CB	A – A	5	FM	m
15	G	CB	B – A	19	Haflinger	se
16	G	CB	A – A	18	Swiss Warmblood	a
17	G	CB	B – A	7	Unknown origin	v
18	G	CB	A – A	10	ET	d
19	S	CB	B – B	11	FM	h
20	M	C	C	7	Quarter Horse	s
21	M	C	C	20	FM	y
22	M	C	C	14	Swiss Warmblood	h
23	M	C	C	18	Appaloosa	b
24	M	C	C	14	Swiss Warmblood	h
25	M	C	C	16	Trotter	h
26	M	C	C	18	FM	h
27	M	C	C	10	Swiss Warmblood	g
28	G	C	C	4	Friso-Arabian	n
29	G	C	C	24	Unknown origin	v
30	G	C	C	22	ET	d
31	G	C	C	7	Quarter Horse	k
32	G	C	C	6	FM	di
33	G	C	C	8	FM	d
34	G	C	C	15	Swiss Warmblood	h
35	G	C	C	11	Swiss Warmblood	h
36	S	C	C	17	FM	h
37	S	C	C	7	FM	h

Experimental procedure

Two horses per farm in most cases (crib-biter, control; table 1) were tested, successively, at their home farms at around 11am, as follows. First, a wireless heart-rate monitor was fixed to a surcingle strapped around the subject's heart girth, and a saliva sample taken. The horse was then led to an 8x10m familiar test arena for a 15 min habituation period before subjected to six learning tests (figure 1, figure 2). A daily session began with three learning tests (part 1, figure 2), delivered successively over a period of 60-75min, after which the subject was returned to its home pen for a second saliva sample and a pause of 60-75min. During this time, the second horse was tested in the same way, after which the remaining three tests (part 2, figure 2) were administered, before being returned to its home pen for a third saliva sample. The second subject then also completed the remaining three tests. Tests were always conducted in the same order. At the beginning of each trial, the experimenter led the subject to the start, said "top", and released the horse from its lead. All the tests were video recorded by a second experimenter outside the arena with a camera (Sony Handycam HDR-CX700).

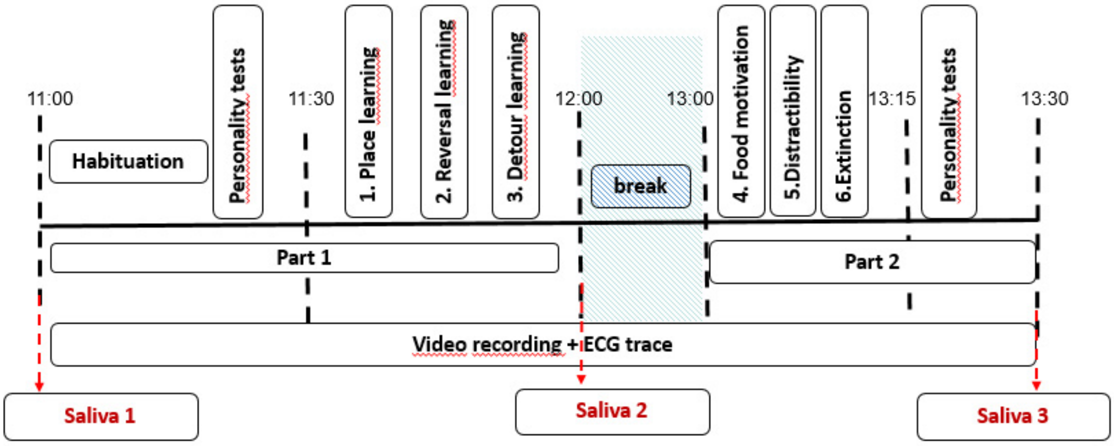


Figure 1. Experimental procedure for the learning tests. The black dotted lines indicate the time at which each period started and ended. The learning tests are indicated with number (1–6). "Saliva" indicate when the saliva samples for the cortisol measurements were collected (1–3). Video and ECG traces were recorded continuously. Learning tests were encased by two sets of personality tests, which were part of another study (Briefer Freymond et al, in preparation, Chapter III of the PhD).

Test 1: Place learning

Test 1 consisted of eight trials during which a subject could learn that food was always situated on one side of the arena. To this end, a solid fence was placed to divide the arena into equal parts. Then, two identical buckets were placed on each side of the fence, one filled with grain (*Hypona 788-2*), the other empty (figure 2), randomly assigned. The experimenter then positioned the horse in front of the fence before releasing it alternatively either to her left or right side. The horse was then allowed to explore the arena for up to three minutes. The goal was defined as reaching the baited bucket. If the horse found the baited bucket within a maximum of three minutes, it was allowed to eat for a while;

otherwise it was returned to the starting position by the experimenter for the next trial. For each trial, we recorded directly the time required to find the baited bucket, the side of the fence selected by the horse from the start zone, and whether or not it inspected the empty bucket. We predicted that both groups would learn quickly. This test was a requirement for the next test (Test 2).

Test 2: Reversal learning

The second test consisted of eight trials during which the horses could learn that food was no longer available on the previously established side, but instead on the opposite side (figure 2). The experimental procedure and data collection was the same as in test 1. Test 2 was performed because crib-biters, due to their potential propensity for habit formation, were expected to perform poorly on reversal learning compared to non-stereotypic horses (Hemmings et al., 2007; Parker et al., 2008; Parker et al., 2009; Ijichi et al., 2013; Roberts et al., 2015).

Test 3: Detour learning

The third test consisted of five trials during which a subject could learn that it needed to move around a fence to reach a bucket with food (Wolff and Hausberger, 1996; Kabadayi et al., 2017). The bucket was placed at one metre from the fence, opposite to the side from which the horse was released, randomly assigned across subjects (figure 2). The experimenter then positioned herself with the horse at one metre perpendicular to the fence before releasing the horse alternatively from her left or right side. The horse was then allowed to explore the arena for up to 3 min. If it reached the bucket within this time it was allowed to eat for a while, before it was returned to the start position for the next trial. We scored directly the time required to reach the bucket. We predicted that crib-biters would show more difficulties to perform this detour task compared to normal horses, due to their lower frustration tolerance and increased stress sensitivity (Ijichi et al., 2013; Briefer Freymond et al., 2015).

Following this first set of tests, horses were returned to their home box for a 60-75 min pause. Then, we carried out three more spatial tests after an initial 5 min habituation phase to the recording material. The learning tests lasted 10-15 min.

Test 4: Food motivation

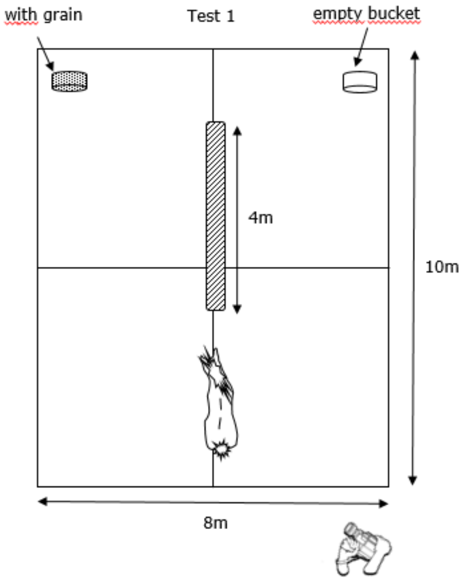
This test consisted of three trials during which subjects had to learn that one bucket with food was situated at the end of a corridor (established by a fence erected in the arena; figure 2). After being released by the experimenter (alternatively from her left or right side), subjects were allowed to explore the arena for three minutes, after which they were returned to the start zone for the next trial. Across trials, we recorded directly the time to reach the bucket, predicting that both groups would learn quickly (Parker et al., 2009). This test was performed in order to assess if one group was more motivated to eat than the other group (Ijichi et al., 2013).

Test 5: Distractibility

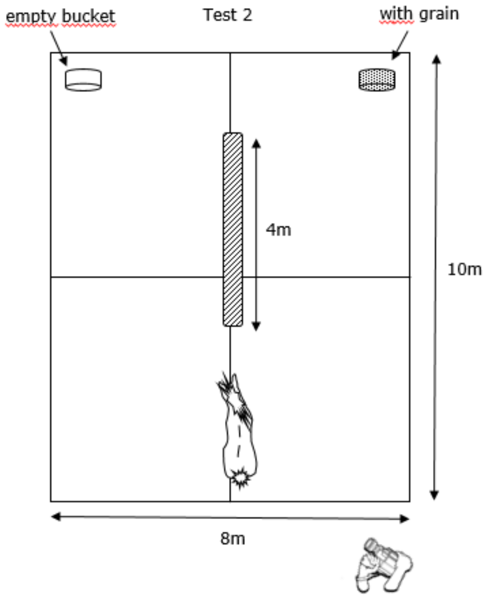
This test was a continuation of test 4 and consisted of one single trial during which subjects had to reach a food bucket. However, this time a piece of wood was placed on one side of the corridor (figure 2). After release, subjects were allowed 30s to explore the arena. We recorded directly the time to reach the bucket and whether subjects looked at or touched the piece of wood, as a measure of distractibility. We predicted that crib-biters, after having established a response strategy in the previous test, would show limited exploration, i.e., devoting less attention to the piece of wood than control individuals (Parker et al., 2009; Ijichi et al., 2013).

Test 6: Extinction

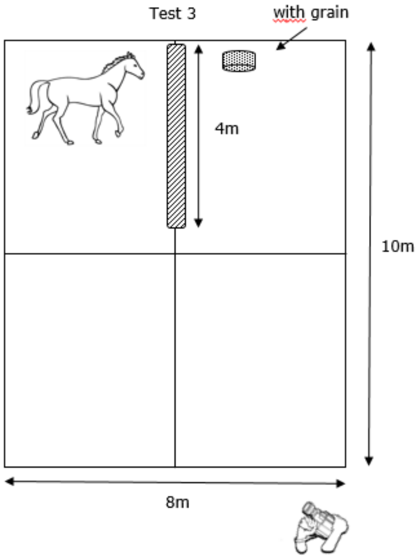
This test consisted of eight trials, during which subjects could learn that there was no more food in the bucket situated at the end of the corridor. After release, alternatively either from the experimenter’s left or right side, subjects were allowed to explore the arena for 30s, before being returned to the start for the next trial. We recorded directly the time during which subjects waited at the entrance of the corridor and whether or not subjects went to look into the bucket. We predicted that crib-biters would show more perseverance by visiting the now empty buckets more often and longer than controls, due to their potential habitual responding patterns and their stronger dependency on rewards (Hemmings et al., 2007; Ijichi et al., 2013; Roberts et al., 2015).



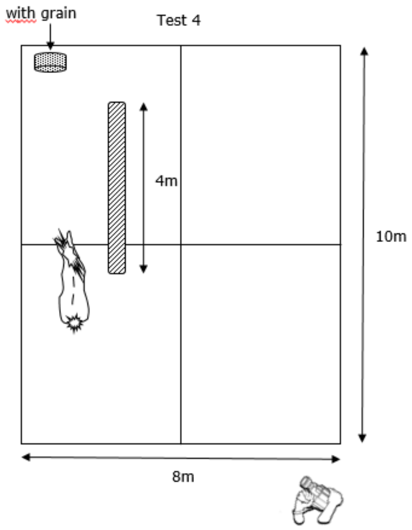
Test 1



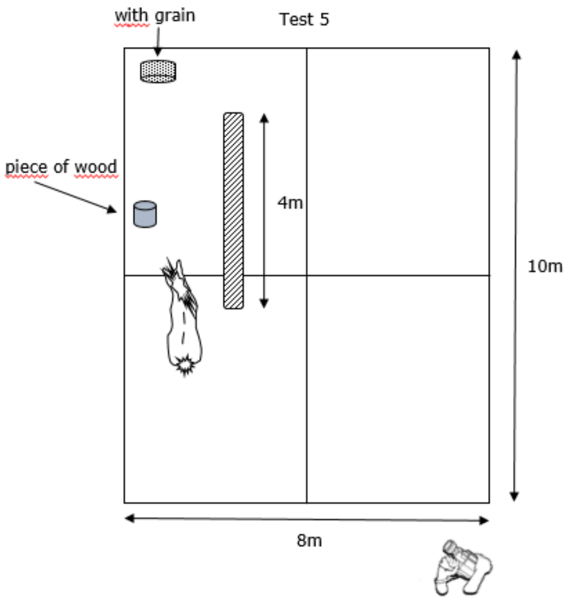
Test 2



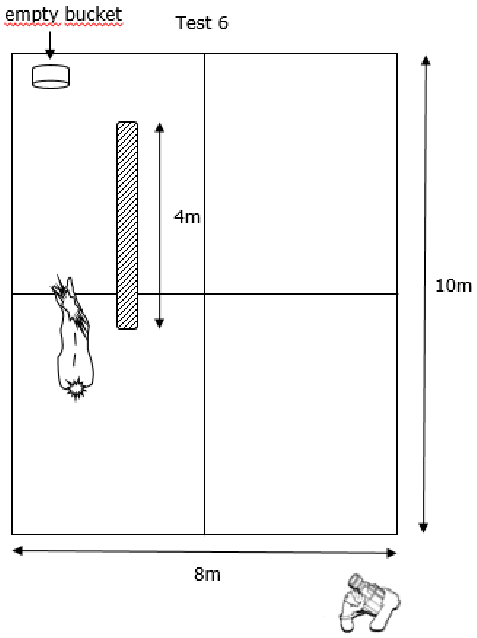
Test 3



Test 4



Test 5



Test 6

Figure 2. Schematic representations of the six tests. The camera was positioned at the bottom right; performance was documented continuously on video. The test arena was surrounded by a fence (large rectangle). During *Tests 1* and *2*, subjects were released in front of the fence. In *Test 3*, subjects were released one metre perpendicular to the fence and in *Tests 4-6*, at the start of the corridor (*Test 4-6*).

Response measures

Stereotypic behaviours

We noted directly all instances of stereotypic behaviour (crib-biting, windsucking) on an all occurrence basis (table 2). Crib-biting instances were then verified by an independent observer, blind to the groups, based on video. Crib-biting was defined as a subject grasping the solid fence with its incisors, pulling back, contracting the neck muscles and drawing air into its oesophagus with an audible grunt (McGreevy et al., 1995). Windsucking was defined in the same way, but without grasping an object (McGreevy et al., 1995).

Table 2. Crib-biting events per horse in the different learning tests. The number of the horses corresponds to those shown in Table 1. In the two last rows, the sum of crib-biting event per test ("CBnb") for all the crib-biters, and the number of horses per test that crib-bit ("Nb") are indicated.

Crib-biters	**Test1**	**Test2**	**Test 3**	**Test 4**	**Test 5**	**Test 6**
1	0	0	0	0	0	0
2	0	0	0	0	0	**1**
3	**1**	0	0	0	0	**8**
4	0	0	**128**	**3**	**4**	**53**
5	0	0	0	0	0	0
6	**12**	0	**1**	0	0	**2**
7	0	0	**11**	0	0	**11**
8	0	**2**	0	0	0	0
9	0	**2**	0	0	0	0
10	0	0	0	0	0	**7**
11	0	**2**	**6**	0	0	**7**
12	0	0	0	0	0	0
13	0	0	0	0	0	0
14	**2**	**1**	0	0	0	**2**
15	0	0	0	0	0	**2**
16	**9**	**11**	0	0	0	**1**
17	0	0	0	0	0	**30**
18	0	0	**7**	0	**1**	**2**
19	0	0	0	0	0	0
CBnb	24	18	153	3	5	125
Nb	4	5	5	1	2	12

Behaviours (scored from the videos)

All tests were video recorded for further behavioural analyses (figure 1). Duration of behaviours were measured using the software Observer XT v.11 (Noldus) as "State Events" (table 3). For each behaviour, we calculated the duration relative to the total time available. 'Frustration' and 'alert' were scored by an observer who was blind to categories (crib-biters or controls) according to behaviours listed in table 3 as "State Events" (table 3). Frustration and alert states are known to indicate stress potentially be caused by the tests (Valenchon et al., 2013; Psyrdellis et al., 2016).

Physiological measures

We assessed the activity of the hypothalamic-pituitary-adrenal stress pathway (HPA) by collecting salivary cortisol at three time points during the experiment (figure 1). Saliva was collected with Salivette cotton rolls placed loosely onto the tongue of the horse for 1 min using forceps. At the end of the test, the Salivettes were centrifuged for 6 minutes at 5000 rpm with a Hettich EBA 20 centrifuge. Samples were stored at −20°C before being sent for analyses (Salimetrics, USA). Concentrations of cortisol were determined with the Salimetrics® High Sensitivity Salivary Cortisol Enzyme Immunoassay kit, a direct enzyme immunoassay without extraction that has been validated for equine saliva (Schmidt et al., 2010).

Concerning the sympathomedullary stress pathway SAM axis, we assessed two physiological measures, heart rate (HR) and root mean square of successive inter-beat interval differences (RMSSD) (von Borell et al., 2007). The ECG trace was collected continuously using a wireless, non-invasive monitor (MLE120X Bioharness Telemetry System, Zephyr) fixed to a surcingle placed around the horse‘s heart girth, which produces more reliable heart rate (HR) measures compared to alternative methods (Parker et al., 2010). The data were transmitted in real time to a laptop using LabChart software v.7.2 (ADInstrument) for analyses. This allowed to enter comments in the software during the experiment to indicate when each learning test started (figure 1). We extracted HR and RMSSD from good-quality sections with clearly visible heartbeats on the ECG trace. When possible, we selected, due to the different duration of the tests, five periods of short selections of about 10 s each for *Tests 1–3* and *6*, three periods of 10 s for *Test 4* and one period of 10 s for *Test 5* (Reefmann et al., 2009; Briefer et al., 2015). These short selections allowed us to identify even short-term changes in physiology linked to each behaviour, as these measures rapidly return to baseline after transient perturbations (von Borell et al., 2007). We checked visually that the software tracked the heartbeats properly and extracted HR and inter-heartbeat (RR) intervals (in ms). RR intervals were then used to calculate RMSSD (ms). If atrioventricular blocks were observed in the signal, we excluded the respective sections of the ECG trace (Schmidt et al., 2010). For one crib-biter and one control, we were unable to obtain HR and RMSSD data due to the bad signal quality.

Statistical analysis

Behaviours (directly scored)

To compare the behaviour of crib-biters (“CB”) (*N* = 19) and controls (“C”) (*N* = 17) during the learning tests, we investigated group differences in the time needed to reach the bucket (*Tests 1-4*) or in the time spent waiting in front of the corridor (*Test 6*) (“Time”) using linear mixed-effects models (LMM; lmer function, lme4 library) and generalised linear mixed models fit with binomial family distribution and logit link function (GLMM; glmer function; lme4 library; multcomp library; (Pinheiro and Bates, 2000) in R 3.0.2 (table 4)). We also investigated group differences in looking or not at the empty bucket (“Bucket”) (*Tests 2, 6*), in looking or not at the piece of wood (“Wood”) and touching or not the piece of wood (“Touch”) (*Test 5*) (binary responses) (tables 3 and 4). The LMMs included “Time” as a response variable. The GLMMs included “Time” transformed as a binary variable (*Tests* 4), “Bucket” (*Tests 2, 6*), “Wood” or “Touch” (*Test 5*) as response variables (one model per variable) (tables 3 and 4). The fixed, control factors and interactions terms are described in tables (3 - 4). To control for repeated measurements of the same subjects, the identity of the horses nested within the farms ("Farms") where they were housed was included as a random factors. For *Test 5*, only Farms were included as a random factor, as there was only one value for each horse. All parameter abbreviations are defined and summarised in table 3.

Table 3. Abbreviations of the behavioural elements measured during the different tests and of the other parameters used in the different analysis.

Abbreviations	Definition	Test
Behaviours (directly scored)		
"Time" (s)	Time to go to the bucket filled with grain	Tests 1-5
	Time spent waiting in front of the corridor	Test 6
"CBnb" (n)	The number of crib-biting events per test	Tests 1-6
"Side" (b)	The side of the fence the horses took to go to the bucket, i.e. left or right	Tests 1-2
"Bucket" (b)	Whether or not the horse looked into the empty bucket	Tests 1-2, 6
"Touch" (b)	Touching or not the piece of wood	Test 5
"Wood" (b)	Looking or not at the piece of wood	Test 5
Behaviours (scored from the videos)		
"Frustr" (p)	Pushing the solid fence with the nose or the body, turning the empty bucket over with the nose or foreleg, shaking the head	Test 1
	Scratching the ground with the foreleg, pushing the solid fence with the nose or the body, turning the empty bucket over with the nose or foreleg	Test 2
	Scratching the ground with the foreleg, pushing the fence with the nose or the body	Test 3
	Scratching the ground with the foreleg, turning the empty bucket over with the leg or the nose	Test 6
"Att" (p)	Standing alert, without moving, with the head and neck up and the ears pointing forward	Tests 1-3, 6

Other parameters		
Group ("CB" or "C") (c)	Crib-biting and control group	Tests 1-6
Group ("A", "B", "C") (c)	A= Crib-biting individual that showed crib-biting during a test; B = Crib-biting individual that did not show crib-biting during a test	Tests 1-6
"Trial" (n)	The trial number for each test. 1–8 for *Tests 1, 2, 6*; 1–5, for *Test 3*; 1–3, for *Test 4*	Tests 1-4, 6
"Sideb" (b)	The side of the bucket that contained food, i.e. left or right	Tests 1-2
"HR"(a)	Heart rate, average value per test	Tests 1-6
"RMSSD" (a)	Root mean square of successive inter-beat interval difference, average value per test	Tests 1-6
"Part" (c)	Part 1	Tests 1-3
	Part 2	Tests 4-6
"Order" (n)	Test order: 1–7 for Part 1. Test order 1-4= personality tests analysed in another study (Briefer Freymond et al., in preparation, Chapter III of the PhD)	Test 1-3
	Test order: 8–12 for Part 2. Test order 11-12= personality tests analysed in another study (Briefer Freymond et al., in preparation, Chapter III of the PhD)	Test 4-6
"Hour" (n)	The hour at which saliva was collected (for cortisol analyse)	
"Base" (n)	The first saliva sample collected before the tests, i.e. baseline (for cortisol analyse)	

p: proportion; n: number; s : second, b: binary; c: category; a: average

Table 4. Fixed and control factors for the directly scored behavioural measures. The abbreviations are described in Table 3. The crosses indicate which factor were used in the models to investigate the variation observed in the directly scored behavioural measures. The response variable was Time for Test 1-6, Wood and Touch for Test 5 and Bucket for Test 2 and 6. In this table, Group is used for Group CB-C or A-B-C.

Factors		Test 1	Test 2	Test 3	Test 4	Test 5	Test 6
Fixed	**Group**	x	x	x	x	x	x
	Trial	x	x	x	x		x
	Trial * Group	x	x	x	x		x
	Bucket * Group	x	x				x
	Trial * Bucket	x	x				x
Control	**CBnb**	x	x	x	x	x	x
	Age	x	x	x	x	x	x
	Sex	x	x	x	x	x	x
	Side	x	x				
	Arena	x	x	x	x	x	x
	Sideb	x	x				
	Bucket	x	x				x

Over all six learning tests, 14 of 19 crib-biters displayed stereotypic behaviour at least once (tables 1 and 2). We therefore reran the LMMs and GLMMs as described above, to compare crib-biters that did ("A") and did not ("B") show stereotypic behaviour in each test (tables 1 and 2) and control subjects.

Learning progress

To assess if horses improved their performance throughout the different trials in the learning tests (*Test 1-4* and *6*), we compared the time that the horses took to go to the bucket filled with grain (*Test 1-4*) or waiting time in front of the corridor (*Test 6*) during the first and the last trial of each test ("Time"), using linear mixed-effects models (LMM; lmer function, lme4 library). These LMMs included "Time" as a response variable and the same fixed, control and random factors as for the *behavioural measures (directly scored)* except that this time, Group was considered as control factor.

Behaviours (scored from the videos)

Two response variables were analysed (*Test 1, 2, 3 and 6*); frustration ("Frustr") and standing attentive ("Att"), using either LMM or GLMM depending on the transformation needed in R 3.0.2 (tables 3 and 4). Group CB-C and Group A-B-C were considered as fixed parameters. The other control parameters are defined in tables (3- 4). Farm type was added as random factor.

Physiological measures

To compare the physiology of crib-biters and controls during the learning tests, we used linear mixed-effects models (LMM; lmer function, lme4 library), including as response variables (3 separate models) the saliva cortisol value ("Cort") the average HR and RMSSD values for each learning tests (hereafter "HR" and "RMSSD", respectively; tables 3 and 5). Group CB-C and Group A-B-C were considered as fixed parameters. For the cortisol analyses, horses were included in Group A (crib-biters that did crib-bite) when they did crib-bite in the corresponding part where cortisol was measured. For HR and RMSSD, because we calculated an average HR and RMSSD value for each test, crib-biters were included in Group A as soon as they started to crib-bite in the corresponding part. The fixed, control factors and interactions terms are described in tables 3 and 5. To control for repeated measurements of the same subjects, the identity of the horses nested within their respective farms was included as a random factor.

Table 5. Fixed and control factors for the physiological measures. The abbreviations are described in Table 3. The crosses indicate which control or fixed factors were used in the models investigating the variation observed in the physiological parameters (response variable: Cort, HR, RMSSD). In this table, Group is used for Group CB-C or A-B-C.

Factors		**Cort**	**HR**	**RMSSD**
Fixed	**Group**	x	x	x
	Order * Group	x	x	x
Control	**Part**	x		
	Base	x		
	Sex	x	x	x
	Age	x	x	x
	Order		x	x
	Hour	x		
	CBnb		x	x

The residuals were checked graphically for normal distribution and homoscedasticity. To satisfy model assumptions, we used a log transformation for "Time" in *Test 6,* "RMSSD", and for "Att" in *Test 1*, a square-root transformation for "Att" in *Test* 2 and *Test* 6 and for "Frustr" in *Test 6* (abbreviations defined in table 3). "Time" in *Test 2* was cube-root transformed. Finally, "Time" in *Test 1* and *3,* which did not meet statistical assumption despite log, square-root or cube-root transformation, was split in six categories of equal duration (i.e., 1: 1–30 s, 2: 31–60 s, 3: 61–90 s, 4: 91–120 s, 5: 121–150 s, 6: 151–180 s). All the resulting parameters satisfying model assumptions were then input into linear mixed-effects models (lmer function). Some parameters did not meet the statistical assumptions despite transformation. They were thus transformed to binomial data as follows; value equal or higher than median = 1 or value lower than median = 0. This was the case for "Time" in *Test 4,* for "Frustr" in *Test* 2 and 3 and for "Att" in *Test* 3. The parameters scored as binomial (Bucket, Wood, Touch), as well as parameters transformed to binomial data were input into GLMM fit with binomial family distribution and logit link function (glmer function).

For the LMMs and GLMMs, we used a standard model simplification procedure by removing each non-significant term, until the deletion caused a reduction in goodness of fit (at which point, the term was left in the model). *P*-values were calculated based on Satterthwaite's approximations (anova function, lmerTest package in R). The significance level of the factors was set at $\alpha = 0.05$ for all models.

For all the models, when significant interaction effects between fixed and/or control factors were found and when Group A-B-C had a significant effect, further post-hoc analysis were carried out using LMMs and GLMMs. Bonferroni correction was then applied.

RESULTS

One control horse was not interested in rewards and did not perform any of the learning tests. It was therefore removed from the analyses.

Behaviours (directly scored)

In the test *place learning* (*Test 1*), there was no difference between crib-biters ("CB") and controls ("C) in the time the horses took to go to the bucket containing grain ("Time") (LMM: effect of Group CB-C on Time, X_1^2 = 0.74, p = 0.39). However, there was an interaction effect on Time between the Group A-B-C ("A", crib-biters that did crib-bite in Part 1 (table 2); "B", crib-biters that did not crib-bite in Part 1 (table 2); and "C", controls) and the corresponding trial ("Trial"), as well as between Group A-B-C and if the horses went to look at the empty bucket or not ("Bucket") (LMM: interaction effect between Groups A-B-C and Trial on Time, X_2^2 = 13.45, p = 0.001; interaction effect between Groups A-B-C and Bucket on Time, X_2^2 = 12.68, p = 0.002). Post hoc analysis per Trial and Bucket did not show further significant differences between Groups (LMMs: p = 1.0 for all).

In the test *reversal learning* (*Test 2*), there was no effect of Group CB-C, nor of Group A-B-C, on Time (LMM: effect of Group CB-C on Time, X_1^2 = 1.36, p = 0.24; LMM: effect of Group A-B-C on Time, X_2^2 = 1.52, p = 0.47). Because the sides where the bucket containing food and the empty bucket were situated during Test 1 were swapped (figure 2), we also analysed if there was some difference between Groups in Bucket. There was no effect of

Group CB-C, nor of Group A-B-C on Bucket (GLMM: effect of Group CB-C on Bucket, X_1^2 = 0.0012, p = 0.97; effect of Group A-B-C on Bucket, X_2^2 = 1.06, p = 0.59). For the all the horses, Bucket decreased with Trial (GLMM: effect of Bucket on Trial, p <0.0001; slope estimate ± SD: -0.53 ± 0.09) indicating that horses improved their performance over trials as shown by a decrease in going to look at the empty bucket.

In the *detour learning* (*Test 3*), there was an interaction effect between Group CB-C and Trial on Time and the same for Group A-B-C (LMM: interaction effect between Group CB-C and Trial on Time, X_1^2 = 4.05, p = 0.04; interaction effect between Group A-B-C and Trial on Time, X_2^2 = 13.35, p = 0.001). Further post-hoc analyses per trial did not show any significant effect of the Group factors on Time, and post-hoc analyses per Group did not show any significant effect of Trial on Time (LMMs: p ≥ 0.31 for all).

In the test *food motivation* (*Test 4*), there was an interaction effect between Group CB-C and Trial on Time and the same occurred for Group A-B-C (GLMM: interaction effect between Group CB-C and Trial on Time, X_1^2 = 6.96, p = 0.008; interaction effect between Groups A-B-C and Trial on Time, X_2^2 = 7.15, p = 0.03). Further post-hoc analyses per trial did not show any significant effect of the Groups on Time, and post-hoc analyses per Group did not reveal any significant effect of Trial on Time (GLMMs: p ≥ 0.07 for all).

In the test *distractibility* (*Test 5*), there was no effect of Group CB-C, nor of Group A-B-C, on either looking at the piece of wood or not ("Wood") or in touching the piece of wood or not ("Touch") (GLMM: effect of Group CB-C on Wood, X_1^2 = 46.461, p = 0.08; effect of Group CB-C on Touch, X_1^2 = 0.07, p = 0.80; effect of Group A-B-C on Wood, X_2^2 = 46.03, p = 0.13; effect of Group A-B-C on Touch, X_2^2 = 0.43, p = 0.80).

In the test *extinction* (*Test 6),* there was no effect of Group CB-C, nor of Group A-B-C, on Time (LMM: effect of Group CB-C on Time, X_1^2 = 0.22, p = 0.64; effect of Group A-B-C on Time, X_2^2 = 3.37 p = 0.19). Because in this test, the bucket previously containing food had been replaced by an empty bucket, we also analysed if there were some differences between Groups on Bucket. There was no difference between Group CB-C, nor between Group A-B-C on Bucket (GLMM: effect of Group CB-C on Bucket, X_1^2 = 0.36, p = 0.55; effect of Group A-B-C on Bucket, X_2^2 = 2.27, p = 0.32). For all the horses, Bucket decreased with Trial (GLMM: effect of Bucket on Trial, p <0.0001; slope estimate ± SD: -0.49 ± 0.07), indicating that horses improved their performance over trials as shown by a decrease in going to look at the empty bucket.

For *Tests 1, 2* and *3*, in which horses did crib-bite, Time increased with the number of instances of crib-biting ("CBnb") (slope range between 0.13 to 1.26; p ≤ 0.04 for all). CBnb also affected Bucket in *Tests 2* and *6* (*Test 2;* GLMM: p = 0.031; slope estimate ± SD: 2.30 ± 1.23; *Test 6;* GLMM: p = 0.002; slope estimate ± SD: -0.61 ± 0.22). In contrast, there was no effect of CBnb on Time for Tests 4 and 6 (*Test 4*; GLMM: p = 0.46; *Test 6*; GLMM: p = 0.37).

Learning progress

For each test, there was a significant difference between the first and the last trial of the test in the time required to go to the bucket (*Test 1-4*) or in the time waiting in front of the corridor (*Test 6*) ("Time"; LMMs: effect of Trial on Time; p ≤ 0.04 for all; for *Test 1-4,* slope estimate, range between –8.32 to -0.16; for *Test 6:* slope estimate = 0.40 ± 0.03). Therefore, all horses improved their performances throughout the trials as shown by a decrease in time between the first and the last Trial for *Tests 1-4,* and an increase in the time waiting in front of the fence for *Test 6.*

Behaviours (scored from the videos)

In the test *place learning (Test 1),* there was no effect of either Group CB-C or Group A-B-C on frustration behaviours ("Frustr") (GLMM: effect of the Group CB-C on Frustr, X_1^2 = 1.68, p = 0.19; effect of the Group A-B-C on Frustr, X_2^2= 2.12, p = 0.35). There was also no effect of either Group CB-C or Group A-B-C on standing attentive "Att" (LMM: effect of Group CB-C on Att, X_1^2 = 0.001, p = 0.98; effect of Group A-B-C on Att, X_2^2 = 2.29, p = 0.32).

In the test *reversal learning (Test 2),* there was no effect of Group CB-C on Frustr (GLMM: effect of the Group CB-C on Frustr, X_1^2 = 0.09, p = 0.77). However, there was an effect of Group A-B-C on this behaviour (GLMM: effect of the Group A-B-C on Frustr, X_2^2 = 7.40, p= 0.02). Further post-hoc comparisons showed that more Group B horses did show frustration behaviours in this test compared to Group A horses (proportion of horses showing frustration behaviour: Group A, 0.40 ± 0.52; Group B, 0.78 ± 0.44; GLMM: effect of the Group A versus B on Frustr, X_1^2 = 7.76, p= 0.015), while there was no difference between Group A and C and between Group B and C (proportion of horses showing frustration behaviour: Group C, 0.50 ± 0.51; GLMM: effect of Group A versus C on Frustr, X_1^2 = 0.18, p= 1.0; effect of Group B versus C on Frustr, X_1^2 = 4.33, p= 0.12). Finally, there was no effect of either Group CB-C or Group A-B-C on Att (LMM: effect of Group CB-C on Att, X_1^2 = 1.58, p= 0.21; effect of Group A-B-C on standing attentive, X_2^2 = 3.33, p= 0.19).

In the *detour test (Test 3)*, there was no effect of either Group CB-C or Group A-B-C on Frustr (GLMM: effect of Group CB-C on Frustr, X_1^2 = 0.091, p = 0.76; effect of Group A-B-C on Frustr, X_2^2 = 0.39, p = 0.82). There was also no group effect on Att (GLMM: effect of the Group CB-C on Att, X_1^2 = 0.07, p = 0.79; effect of the Group A-B-C on Att, X_2^2 = 0.50, p = 0.78).

In the *extinction* test (*Test 6*), there was no effect of either Group CB-C or Group A-B-C on Frustr (LMM: effect of the Group CB-C on Frustr, X_1^2 = 2.60, p = 0.11; effect of the Group A-B-C on Frustr, X_2^2 = 4.83, p = 0.09). There was also no group difference in Att for Group CB-C, nor for Group A-B-C on Frustr (LMM: effect of the Group CB-C on Att, X_1^2 = 0.23, p = 0.63; effect of the Group A-B-C on Att, X_2^2 = 2.75, p = 0.25).

Physiological measures

Cortisol

There was no difference in cortisol levels ("Cort") between Groups CB and C (LMM: effect of Groups CB-C on cortisol, X_1^2 = 0.03, p = 0.87). However, in the model including CB-C as a Group, Cort tended to be higher after Part 1 than after Part 2 (part 1 (*Tests 1 to 3*) = mean ± SD, 0.62 ± 0.20 ng/ml; Part 2 (*Tests 4* to *6)* = 0.54 ± 0.18 ng/ml; "Part"; LMM: effect of Part on Cort, X_1^2 = 6.49, p = 0.01). There was an interaction effect on Cort between Part and Group A-B-C (LMM: interaction effect between Part and Group A-B-C on Cort, X_2^2 = 8.56, p = 0.014). Post-hoc comparisons showed that Cort differed between the Groups A-B-C after Part 1 (LMM: effect of Group A-B-C on Cort in Part 1, X_2^2 = 8.71, p = 0.03), but not after Part 2 (LMM: effect of Group A-B-C on Cort in Part 2, X_2^2 = 0.90, p = 1.28 (figure 3)). Further two-by-two comparisons showed that for Part 1, Group A had a lower cortisol values than Group B (effect of Group A versus B on Cort; LMM: X_2^2 = 11.012, p = 0.024) and also than Group C (effect of Group A versus C on Cort; LMM: X_2^2 = 12.55, p = 0.012). By contrast, Group B and Group C did not differ (effect of Group B versus C on Cort; X_2^2 = 5.75, p = 0.17; figure 3).

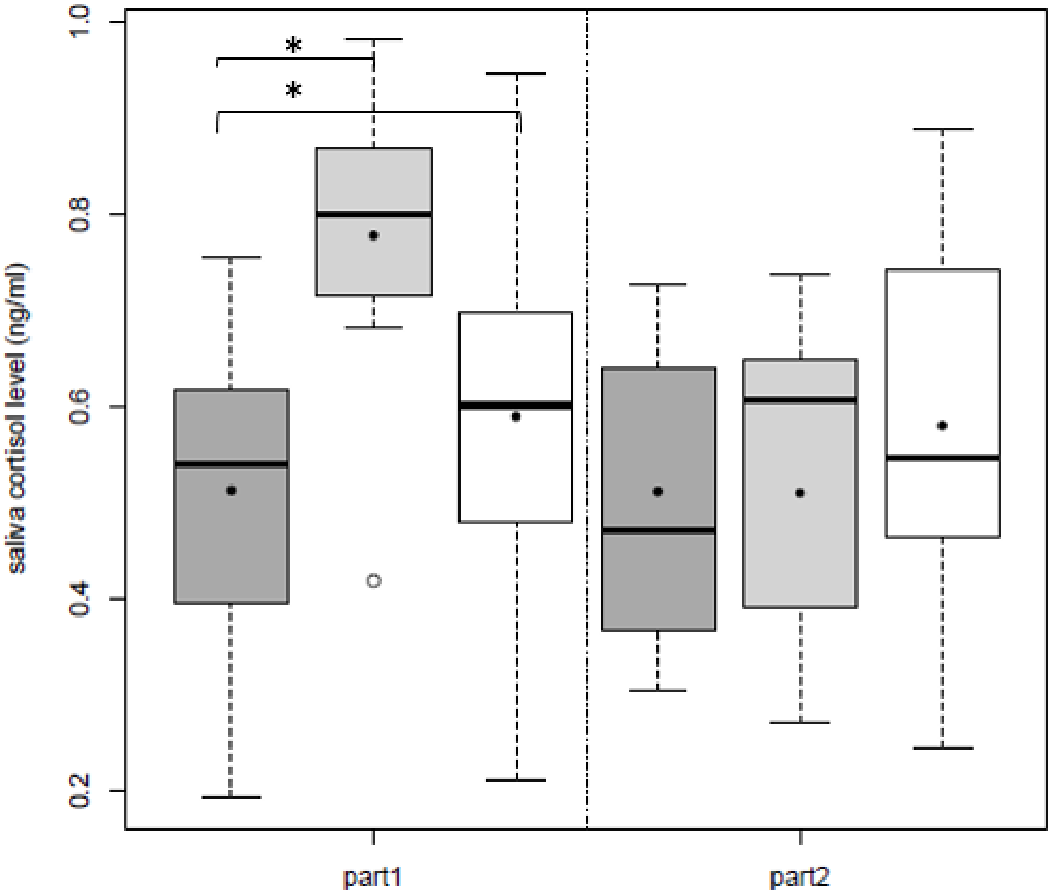


Figure 3. Saliva cortisol level as a function of the crib-biting group. Saliva cortisol level in crib-biters that did crib-bite (dark grey; *N* = 10 in Part 1 and *N* = 12 in Part 2), crib-biters that did not crib-bite (hell grey; *N* = 9 in Part 1 and *N* = 7 in Part 2) and control horses (white; *N* = 18); box-and-whiskers plot (the horizontal line shows the median, the box extends from the lower to the upper quartile, and the whiskers to 1.5 * the interquartile range above the upper quartile or below the lower quartile). The black dots indicate the means. Significant differences between Phase are indicated as * 0.05 ≤ p.

Heart rate (HR)

There was no effect of Group CB-C, nor of Group A-B-C, on the average heart rate (“HR”) value per test (LMM: effect of Groups CB-C on HR, X_1^2 = 0.56, p = 0.21, effect of Groups A-B-C on HR, X_2^2 = 1.67, p = 0.44). Only the order of the tests (“Order”) had an effect on HR (LMM: p < 0.001).

Root mean square of successive inter-beat interval differences (RMSSD)
There was no effect of Group CB-C, nor of Group A-B-C, on the average value of root mean square of successive inter-beat interval difference ("RMSSD") per test (LMM: effect of Group CB-C on RMSSD, X_1^2 = 0.16, p = 0.69; effect of Groups A-B-C on HR, X_2^2 = 1.72, p = 0.42).

For all the model, the effect of the fixed factors and interactions not mentioned above were not significant.

The means and standard deviations of behaviours (scored from the video), Cortisol, HR and RMSSD for crib-biters and control horses, for which there were no differences between the groups, are summarised in Annex III of the PhD.

DISCUSSION

In the current study, we investigated the learning abilities of a large sample of horses, half of which showed a stereotypy (crib-biting behaviour). The horses performed six spatial learning tasks, including some that have been considered as challenging for horses, that is, reversal learning, detour and extinction. Across experiments, all subjects (crib-biters and controls) improved their performance, with no between-group differences. However, within the stereotypic group, we found differences between subjects that did or did not perform stereotypic behaviour. In particular, crib-biters that did crib-bite showed lower levels of frustration than those that did not crib-bite in the reversal learning test and lower cortisol levels after the first experimental part compared to those that did not crib-bite and controls. These results corroborate previous findings (Briefer Freymond et al., 2015) that suggest that stereotypic behaviour is an adaptive strategy to reduce stress.

Our results did not show any difference between stereotypic and non-stereotypic horses, indicating a stronger reward dependency in crib-biters compared to controls in contrast with our expectations (Ijichi et al, 2013). One explanation is that the learning tests were not challenging enough to reveal any learning differences between crib-biters and controls. Indeed in previous studies animals were over-trained, before showing an effect of habit formation (e.g. 40 trials in Parker et al. (2008) and horses have been shown to excel in finding food sources (Martin et al., 2006; Hothersall et al., 2010; Brubaker and Udell, 2016). For this reason, the limited training the horses received and the tests that have been performed were perhaps too simple to reveal any learning difference.

There is a general consensus that, across animal species, cognitive performance decreases with increasing levels of stress (Valenchon et al., 2013). Similarly, learning under stress can negatively impact on subsequent performance (humans: (Schwabe and Wolf, 2010)), possibly because stress acts as a distractor during encoding and, instead, favours inflexible habit formation (McBride et al., 2017). Another explanation for the absence of group differences in our study is therefore that we allowed stereotypic subjects from executing stereotypic behaviour (i.e. on a solid fence), which might have decreased their stress levels and helped them to have a less strong dependency on rewards. These results corroborate our previous findings that suggest that allowing crib-biters to crib-bite may improve their learning performance through a decrease in stress, even in highly challenging tasks (reversal learning based on visual cues; (Briefer Freymond et al., 2018)).

Comparisons can be made between our findings and human psychological disorder, namely attention-deficit/hyperactivity disorder (ADHD), which involves non-goal oriented motor movements. Interestingly, performing such movement has been shown to improve cognitive performance (Sarver et al., 2015). Our results lead us to recommend that, as previously suggested (Hausberger et al., 2007; Briefer Freymond et al., 2018), stereotypic horses and perhaps also human showing stereotypic behaviours may require specific training adapted to their needs. Letting stereotypic individuals the possibility to perform their stereotypy might be one specific feature to incorporate in learning protocols, which might then allow them to perform successfully.

The lower stress level experienced by crib-biters in our study was confirmed by our physiological analyses. Indeed, although our results did not reveal any difference in cortisol between crib-biters and control horses, crib-biters that did crib-bite might have a lower cortisol levels after the first experimental part, indicating lower stress levels, than crib-biters that did not crib-bite and even control horses. This could suggest that crib-biting functions as a coping strategy to deal with stressful (e.g. frustrating) situations (McBride and Cudderford, 2001; Briefer Freymond et al., 2015) and to avoid pathogenic outcomes (Cabib and Puglisi-Allegra, 2012). This suggestion is strengthened by the fact that the crib-biters that did crib-bite also seem to show less behaviours indicating frustration than crib-biters that did not crib-bite in the reversal learning test. Stereotypic behaviour may therefore act as a way to reduce stress and increase well-being (Mason and Latham, 2004; Briefer Freymond et al., 2015). We suggest that the absence of cortisol difference between crib-biters that did crib-bite, crib-biters that did not crib-bite and controls after the second experimental part could be due to the fact that the learning tests (*Tests 4 –6*) were followed by other tests aimed at testing difference in personality (Briefer Freymond et al., in preparation, Chapter III of the PhD), which likely induced less frustration.

In accordance with a previous study, crib-biters and control horses had similar HR and RMSSD levels (Briefer Freymond et al., 2015), despite differences in cortisol levels (figure 2). This could be due to the fact that these two kinds of physiological parameters were not measured at the same time. HR and RMSSD were measured continuously throughout the study, while cortisol levels were only measured twice (at the end of first and second part). Alternatively, HR may be additionally influenced by behavioural activity, suggesting that it is an imprecise measure of stress in tests involving movement (von Borell et al., 2007; Koolhaas et al., 2010), particularly since horses were required to move in some tests to find the food.

Why some crib-biters in our study refrained from showing stereotypic behaviour during frustrating situations is not clear. One possibility is that they were at an earlier stage of stereotypy development and, correspondingly, might not have had suffered from a clear dopaminergic dysfunction yet. Further experimental studies are required to test this hypothesis, by exploring the impact of preventing stereotypic behaviour to different degrees on stress parameters. Such parameters could include for instance spontaneous blink rate (SBR) (Roberts et al., 2015), an indicator of dopamine transmission, striatal functioning and an index of dopaminergic component of sustained attention and fatigue in humans (Maffei and Angrilli, 2018).

However, because the horses were allocated in three groups according to their crib-biting performances during the tests, i.e. crib-biters that did versus crib-biters that did not crib-bite, a-posteriori instead of randomly, our results should be interpreted with precaution. To validate them, it will be necessary to replicate our tests in a more controlled manner in order to investigate the coping hypothesis. Further experimental studies should include a subset also in the control group. For instance, crib-biters could be, in the same way as in our study, tested against a control non-stereotypic group in learning tasks causing frustration, but this time half of the crib-biters could be provided with a solid support to give them the possibility to crib-bite, while the other half would not have this option. The control horses could thus also to be tested with the two treatments consisting in the presence or not of a solid support. Such a study could also include the collection of stress parameters and the number of times horses perform the stereotypic behaviour could be coded by an experimenter who is blind to which horse is stereotypic or not.

CONCLUSION

In this study, we did not find clear differences in learning capacities between crib-biting and control horses during a battery of learning test. Our tests all involved spatial problems, with limited training, which might be ecologically more relevant for horses, and thus easier to solve, compared to paradigms used in other studies (Hemmings et al., 2007; Parker et al., 2008; Parker et al., 2009). In our study all crib-biters were allowed, and some did, execute stereotypic behaviour during testing. Overall, we could suggest that executing stereotypic behaviour both lowered stress (as assessed by cortisol levels) and frustration, suggesting that engaging in stereotypic behaviour is a strategy to prevent stress, which might result in helping them in learning tests. In accordance with our previous findings (Briefer Freymond et al., 2015; Briefer Freymond et al., 2018), we thus conclude that crib-biting might be a viable strategy that enables certain individuals to lower stress, in order to deal with sub-optimal captive conditions. However, to validate these findings, it will be necessary to replicate our tests in a more controlled manner in order to investigate the coping hypothesis.

ETHICS

The experimental procedure for the horses was approved by the Federal Veterinary Office (approval number VD 26777 bis; Switzerland)

ACKNOWLEDGMENTS

We are grateful to the owners of the horses, who offered to participate in this study. We are also grateful to Matthew Parker, who helped us with the design of the experiment.

COMPETING INTERESTS

We declare we have no competing interests.

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CHAPTER V

Based on:

Stereotypic horses (*Equus caballus*) are not cognitively impaired

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Published in Animal Cognition

ABSTRACT

Stereotypies in animals are thought to arise from an interaction between genetic predisposition and sub-optimal housing conditions. In domestic horses, a well-studied stereotypy is crib-biting, an abnormal behaviour that appears to help individuals to cope with stressful situations. One prominent hypothesis states that animals affected by stereotypies are cognitively less flexible compared to healthy controls, due to sensitization of a specific brain area, the basal ganglia. The aim of this study was to test this hypothesis in crib-biting and healthy controls, using a cognitive task, reversal learning, which has been used as a diagnostic for basal ganglia dysfunction. The procedure consisted of exposing subjects to four learning tasks; first and second acquisition, and their reversals. For each task, we measured the number of trials to reach criterion and heart rate and heart-rate variability. Importantly, we did not try to prevent crib-biters from executing their stereotypic behaviour. We found that the first reversal learning task required the largest number of trials, confirming its challenging nature. Interestingly, the second reversal learning task required significantly fewer trials to reach criterion, suggesting generalisation learning. However, we did not find any performance differences across groups; both stereotypic and control animals required a similar numbers of trials and did not differ in their physiological responses. Our results thus challenge the widely held belief that crib-biting horses, and stereotypic animals more generally, are cognitively impaired. We conclude that cognitive underperformance may occur in stereotypic horses if they are prevented from crib-biting to cope with experienced stress.

Keywords Crib-biting · Basal ganglia · Learning capacity

INTRODUCTION

Stereotypies exist in humans and non-human animals and have been defined as repetitive, relatively invariant patterns of behaviour with no apparent goal or function (Mason and Latham 2004). In animals, they seem to be artefacts of a captive environment involving restricted and sub-optimal housing conditions (McGreevy et al. 1995). They exist in various forms, the most common forms being oral and locomotor stereotypies (Mason and Rushen 2006). One classic ethological model of motivation suggests that restrictive environments can prevent the animal from reaching the "consummatory phase" of a behaviour (e.g., feeding) (Hughes and Duncan 1988). As a consequence, a number of appetitive behaviours (e.g., locomotor action to access food) are being attempted in an effort to reach unobtainable goal states (e.g., elevated blood glucose). When the goal is available, its consummation results in a negative feedback that decreases motivation. In contrast, in cases where the goal is unachievable, the lack of consummation and resulting absence of negative feedback increases the level of motivation to perform appetitive behaviours. If such frustration-inducing situations occur repeatedly, stereotypies can develop (Mason and Rushen 2006; McBride and Parker 2015). Along similar lines of reasoning, it has been suggested that privation (as experienced in captivity) increases the desire for rewards, which in turn enhances appetitive behaviours such as locomotor or feeding behaviours (Spruijt et al. 2001). Excessive use of appetitive behaviours, can, in turn, lead to a loss of regulatory control, which means that these behaviour will become environmentally irreversible (inflexible) and can develop into stereotypies (Toates 2004).

Other studies have focussed on the impact of chronic stress on the brain, and in particular, on parts of the basal ganglia (the mesoaccumbens dopaminergic system) (Spruijt et al. 2001). The reasoning behind this research is that stressful negative experiences lead to an increased tendency to seek rewards via a neurobiological process of sensitization (van der Harst et al. 2003), a possible common principle underlying stereotypies in evolutionarily distant species such as mice and human (Cabib et al. 1998). For example, some studies have shown an association between stressors, stereotypy development and dopamine receptor function in the basal ganglia in rodents (Cabib et al. 1998) and crib-biting horses, *Equus caballus* (McBride and Hemmings 2005), suggesting that the basal ganglia plays a general role in the development of stereotypic behaviour (McBride et al. 2017).
However, the exact causal relationship between basal ganglia alterations and the emergence of stereotypic behaviour is currently unknown (McBride and Hemmings 2005).

Each part of the basal ganglia plays a specific role during instrumental learning (Dickinson 1985). The first stage ("acquisition") is mediated by the ventral and dorsomedial striatum of the basal ganglia (McBride et al. 2017; Parker et al. 2008; Yin and Knowlton 2006), the second stage ("action-outcome") by the dorsomedial striatum (McBride et al. 2017; Parker et al. 2008; Yin and Knowlton 2006), and the final stage ("habit formation") by a shift in activation from the dorsomedial towards the dorsolateral striatum of the basal ganglia. To summarize, the ventral striatum and dorsomedial striatum seem to be important in the acquisition and execution of goal-directed actions, that is, in establishing the link between stimulus response and outcome (McBride et al. 2017; Yin et al. 2008). By contrast, the dorsolateral striatum seems to control subsequent habit formation, independently of the response outcome (Yin and Knowlton 2006). Therefore, due to its primary functional role, dysfunctions in the basal ganglia might lead to behavioural abnormalities linked to impaired action selection and also to impairments in controlling instrumentally learned behaviour (McBride and Hemmings 2005; Vickery and Mason 2005).

Although there are strong reasons to assume that an impaired basal ganglia function is related to the development of stereotypic behaviour, research is challenging for financial, logistical, and ethical reasons, which has led to the development and use of indirect and non-invasive methods (McBride et al. 2017). For instance, stereotypy levels (frequency of stereotypy performance) have been shown to constitute strong predictors of the latency to extinguish conditioned responses or of the tendency to inappropriately repeat responses, both of which constitute indirect measures of basal ganglia dysfunction in several species such as bears, *Ursus thibetanus* and *Helarctos malayanus* (Vickery and Mason 2005), Orange-winged amazon parrots, *Amazona amazonica* (Garner et al. 2003) and bank voles, *Clethrionomys glareolus* (Garner and Mason 2002). In humans, poor abilities to suppress learnt behaviour (perseveration) have been shown in autistic patients who are prone to stereotypic behaviour (Boyd et al. 2009; Lopez et al. 2005).

Domesticated horses are subject to management practices that make them prone to develop stereotypic behaviours. Understanding the nature of stereotypies and their impact on learning abilities is therefore of considerable importance for horse owners. Crib-biting, an oral stereotypy, is one of the most common forms of stereotypy in horses (Luescher et al. 1991; Wickens and Heleski 2010). The performance of this behaviour varies between horses in terms of the percentage of time occupied by the stereotypic behaviour (Houpt and McDnnell 1993). Crib-biting has been linked to learning impairments in extinction paradigms (Hemmings et al. 2007; Roberts et al. 2015). In particular, stereotypic horses need more trials compared to healthy individuals before extinction of a previously learnt action occurs, and this may be linked to a basal ganglia dysfunction. In one study, crib-biting horses appeared to exhibit altered dopamine receptor sensitivity in the basal ganglia (McBride and Hemmings 2005), due to the higher number of dopamine receptors in the ventral striatum and the lower number of receptors in the dorsomedial striatum. Since the dorsomedial striatum mediates action–outcome learning, it is possible that crib-biting horses are simply unable to maintain this type of learning and show an accelerated shift from action–outcome learning to habit formation (Parker et al. 2008, 2009; Roberts et al. 2015), and a reduced ability to learn about outcomes (Schwabe and Wolf 2011). Additionally, another study including many different kinds of stereotypies in horses (locomotor and oral) showed that stereotypic horses need more time to learn an instrumental task (opening a chest by raising the lid using the nose) compared to non-stereotypic horses (Hausberger et al. 2007).

As described above, previous studies have found differences in the learning capacities of crib-biting and control horses (Hemmings et al. 2007; Parker et al. 2008, 2009; Roberts et al. 2015), and other work has shown that crib-biting horses appear to have alterations in the dopaminergic system (McBride and Hemmings 2005). However, the link between these alterations and cognitive performances has remained unclear (Roberts et al. 2017). Reversal learning paradigms are of particular relevance, as they have been used as a diagnostic tool for dopaminergic dysfunction and as general measure of cognitive flexibility in rodents, nonhuman primates and humans (Izquierdo et al. 2017; McBride et al. 2017). To our knowledge, reversal learning has not been investigated in crib-biting horses and has been shown to pose a challenge to this species when based on visual cues (Brubaker and Udell 2016; Hothersall et al. 2010; Martin et al. 2006; McBride et al. 2017; Sappington et al. 1997; Voith 1975), unlike reversal learning tasks based on spatial cues, which seem to be fairly easy for horses due to their ecological relevance (e.g., finding natural food sources) (Brubaker and Udell 2016; Fiske and Potter 1979; Martin et al. 2006; Voith 1975; Warren and Warren 1962). We therefore tested crib-biting and control, non-stereotypic horses in two subsequent reversal learning tasks based on visual cues. First, we predicted that crib-biting horses would need less trials than controls to perform the first and second acquisition task, because they might be more prone to habit learning than to response–outcome learning (Hemmings et al. 2007; Parker et al. 2008, 2009; Roberts et al. 2015). Second, we predicted that crib-biting horses would need more trials than controls to perform the reversal learning tasks, suggesting learning disabilities, if they suffered from dopaminergic dysfunction. By contrast, similar performances between crib-biting and control horses would suggest that the stereotypic horses are not suffering from such a dysfunction. Importantly, we did not try to prevent crib-biters from executing their stereotypic behaviour, based on our previous finding that crib-biting reduces stress (Briefer Freymond et al. 2015), to avoid any confounding influence of stress on learning (Schwabe and Wolf 2010).

METHODS

Subjects and management conditions

The study was carried out on six crib-biters ("CB") and seven control horses ("C") (*N* = 13) of various breeds, sexes (mares, geldings and stallions), and ages (10–25 years old), housed in five different farms in Switzerland, between January and May 2016 (Table 1). Eight horses were privately owned, and five horses were owned by the Swiss National Stud Farm. All the horses had been at their respective farms for at least 1 year. To be eligible for inclusion in the study, crib-biters were required to have demonstrated crib-biting behaviour for a minimum of 1 year, as reported by their owners. All the crib-biters eventually included in the study had been crib-biting for at least 4 years. Controls were horses that had never been observed crib-biting or performing other kinds of stereotypies (e.g., weaving or box-walking). All but two animals participated in a previous study, which involved a spatial learning task (Briefer Freymond et al., in preparation; one crib-biter, one control). Each crib-biter was matched with a control horse of similar breed (except for one pair), sex, age, and housing conditions (individual or group, single box or box with paddock, and if possible in the same farm) (Table 1). One supplementary control horse was tested to lower the average age of the controls, which was originally higher than the age of the crib-biters (final mean age (years old): controls = 17.6; crib-biters = 13.5). Routine care of the study animals was provided by the farm/horse owner.

Experimental design

Experimental protocol

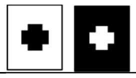
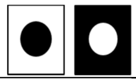
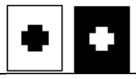
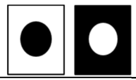
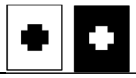
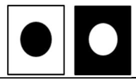
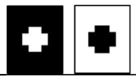
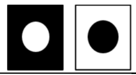
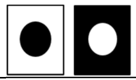
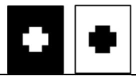
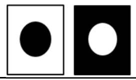
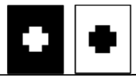
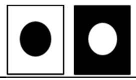
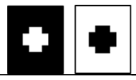
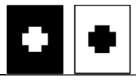
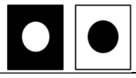
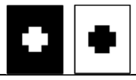
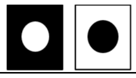
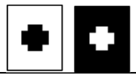
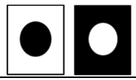
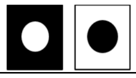
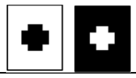
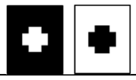
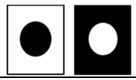
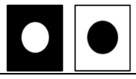
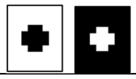
Before the start of the learning experiment, all the CB horses were filmed in their home pen, while undisturbed, during a 48-h period (excluding times when horses were ridden or in pasture), to assess their crib-biting frequency per hour (see in "Stereotypy level" below and Table 1). For the learning experiment, each horse was led individually, to a delimited (8/10 m) familiar arena, after equipping it with a heart-rate monitor in its home pen (see in "Physiological measures" below). The arena was divided into a waiting area and a test area, separated by a start rope. The learning apparatus was placed at one end of the test area (Fig. 1). The horses were filmed from the back with a video camera fixed on a pole to score their behaviour (see in "Behavioural measures" below). Two experimenters were present during the study sessions; Experimenter 1 was located in the arena and handled the subjects (see in “Discrimination procedure” below), while Experimenter 2 was located outside the arena and was entering comments on the collected physiological data (see in “Physiological measures” below) as well as preparing the learning apparatus for the next trial (see in “Discrimination procedure” below).

Apparatus

Because horses are very skilled at reading subtle unintentional human behaviour (Ringhofer and Yamamoto 2017), we built an apparatus which allowed the experimenters to remain in the back of the area (Fig. 1). Following Gabor and Gerken (2010), the apparatus consisted of a wooden box measuring 1 m (height) × 1.6 m (length) × 0.4 m (width) with two flaps (45 × 61 cm) on the front side (Fig. 1). The horse could reach the food through these flaps. To prevent the horses from using olfactory cues, Experimenter 2 always filled both bowls situated at the back side of the apparatus with food (20 g of commercial concentrate), even though only one side was rewarded each time. This was achieved by closing the unrewarded flap automatically using an electromagnet that could be activated with an infrared remote control. In addition, in case of an incorrect choice, the positively reinforced flap was immediately closed from the other side by activating the electromagnet to prevent the horse from being rewarded for an incorrect choice. A vertical piece of wood was added in the middle of the apparatus between the two flaps to better separate the two sides of the apparatus and facilitate the scoring of the horse’s choices.

The visual stimuli (see “Two-choice visual discrimination tasks” for more details) were inserted on the front side of the apparatus inside a plastic window fixed on the wooden boards. The stimuli consisted of sheets of laminated paper (21 × 29.5 cm) on which either a black cross on top of a white background, a white cross on top of a black background (first set of stimuli), a black circle on top of a white background, or a white circle on top of a black background (second set of stimuli) were drawn. The same amount of sheet area as covered by the cross (13.5 × 13.5 cm) and by the circle (13 cm in diameter) (Table 1).

Table 1. Characteristics of the horses used in the experiment. Group (controls = “C”; crib-biters = “CB”; strong crib-biters = “S”, medium crib-biters = “M”, low frequency crib-biters = “L”, controls = “C”), Sex (female = f; gelding = g, stallion = s), Breed (Franches-Montagnes = “FM”; English thoroughbred = “ET”; warmblood = “WB”; Camargue horse= “CA”; Hispano-Arabian = “HA”), year of birth, stimuli used in the first set of learning tasks (Acquisition 1 = ”Acq1”, Reversal 1 = “Rev1) and in the second set of learning tasks (Acquisition 2 = “Acq2”, Reversal 2 = “Rev2”), and presence of a change in colour between Rev1 and Acq2 or not (change of colour = “Y”, no change of colour “N”).

Horses	Group	Breed	Sex	Birth	Acq1/Rev1	Acq2/Rev2	Colour
1	C	FM	f	1993			Y
2	C	FM	s	1996			Y
3	C	ET	g	1991			Y
4	C	FM	g	2001			Y
5	C	WB	g	2002			N
6	C	CA	f	2000			N
7	C	FM	s	2006			N
8	CB-S	FM	f	1997			Y
9	CB-M	FM	s	2002			Y
10	CB-L	FM	s	2005			Y
11	CB-M	FM	g	2004			N
12	CB-S	ET	g	2003			N
13	CB-S	HA	f	2004			N

Acclimation and pre-training (2–6 days)

During the acclimation and pre-training phases, the horses were habituated to the experimental arena and trained to move from the starting point to the apparatus and to open the flaps in the absence of any stimuli. The horses were trained during two 10-min sessions each day, for 2–6 days. The horses were first trained to touch a target (a tennis ball fixed on a stick) with their noses and then the flaps on the apparatus, using a shaping procedure. This shaping procedure, also called "successive approximations", consisted of reinforcing behaviours directed towards the desired response (McGreevy and McLean 2010). The first step of the pre-training phase lasted until each horse was acclimated to the apparatus and touched both flaps easily with its nose. The second step consisted in shaping the horse to open the flaps, by rewarding it each time it pushed the flaps. When the horse had learnt to open the flaps with its nose, Experimenter 1 led the horse to the waiting area, and it was trained to go alone from the waiting area to open the flaps (third step). The pre-training lasted until the horses opened the flaps at least five times on both sides of the apparatus.

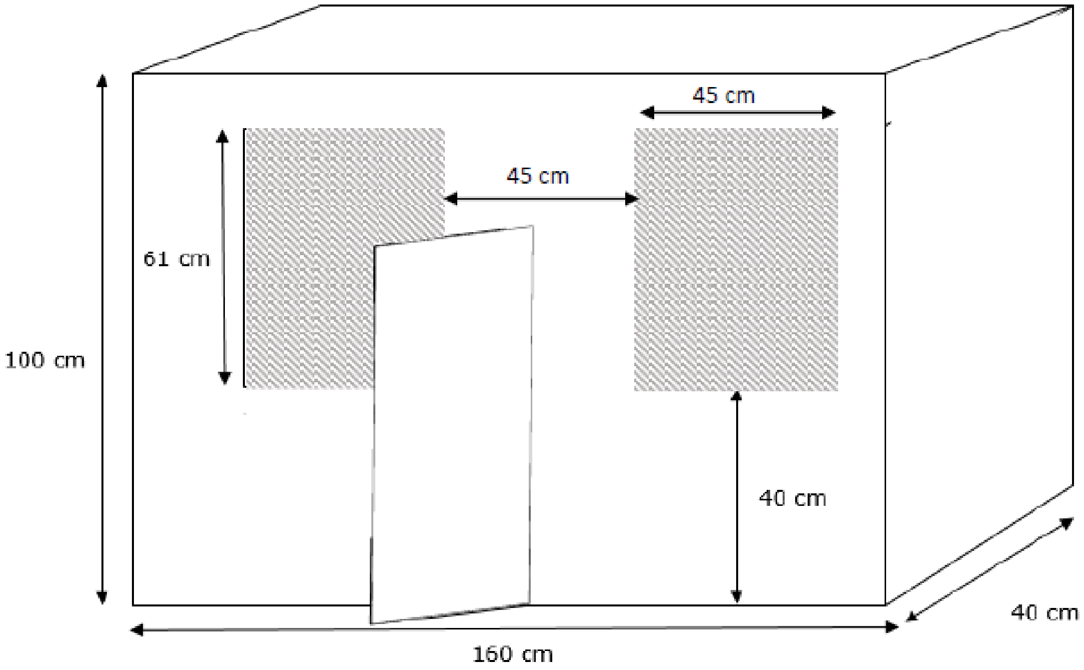


Fig. 1 Test apparatus for the visual discrimination task. The visual stimuli were inserted on the front side of the apparatus inside a plastic window (indicated in dash line). In case of a correct choice, the horses could reach the food through the corresponding flap. A vertical piece of wood was added in the middle of the apparatus.

Discrimination procedure

The discrimination procedure took place during the following 3–7 weeks. The horses were tested each Monday, Tuesday, Thursday and Friday. They were tested one by one with two sessions per day (15–20 min), each containing between 20 and 23 trials, with a break between the respective sessions of about 20 min, during which time another horse from the same farm was tested. Before each trial, Experimenter 2 inserted the two stimulus sheets into the plastic windows in a "pseudo-randomized" order (established a priori). This order ensured that each given stimulus was not presented for more than three consecutive trials at the same position (left or right). After inserting the stimuli sheets, the feeding bowls at the back side of the apparatus were filled and the unrewarded flap was remotely blocked. During this time, Experimenter 1 led the horse to the waiting area and released it after closing the waiting area with a rope. As soon as the setting for the next trial was ready, Experimenter 1, who was blind to the correct stimulus at the beginning of each session, opened the start rope while facing and looking away from the horse and the test area. In case of a correct choice, the horse was led back to the waiting area after reaching and eating the reward. In case of an incorrect choice, the horse was led back to the waiting area and allowed to choose again with the same arrangement of stimuli ("correction trial")(Flannery 1997). After three wrong decisions, the horse was led to the correct stimulus where it could open the flap to reach the reward (Flannery 1997). In this case, if necessary, Experimenter 1 pointed at the correct flap with the hand. Each trial was limited to a 90-s duration, during which all subjects made stopped before a choice was made.

Two-choice visual discrimination task

The learning procedure consisted of four different phases ("Phase"). At the start of the learning phase 1 ("Acq1"), two initial stimuli (a black versus a white cross or a black versus a white circle) were presented to the horses. The learning criterion was set at six consecutive correct responses in one learning session (i.e., probability of doing this by chance = 0.02) (McBride et al. 2016). Once the horse had reached the learning criterion for Acq1, the colour of the stimuli were reversed ("Rev1") and the next session started. Once the horses had reached the learning criterion for Rev1, they were then tested with a second set with novel stimuli (i.e., a black versus a white circle or a black versus a white cross; second acquisition phase, "Acq2"). Acq 2 was then followed by a second, final reversal ("Rev2"), after reaching the learning criterion for Acq2. Therefore, the two reversals, Rev1 and Rev2, consisted in rewarding the previously unrewarded stimulus (during Acq1 and Acq2, respectively), while Acq2 consisted in presenting circles to horses who received crosses during Acq1 and Rev1, and vice versa, with or without changing the colour compared to Rev1 (Table 1). The rewarding stimuli for the different phases were assigned randomly to each horse before the study. For three of the six crib-biters and four of seven control horses, there was a colour change between Rev1 and Acq2 (Table 1).

In previous studies, it was shown that extended sessions of concentrated training could lead to a lack of motivation or to inappropriate and inefficient learning behaviour (McCall 1990; Rubin et al. 1980). To ensure that horses stay motivated, we decided, based on some preliminary tests (*N* = 7 non-stereotypic horses, not used in this study) to perform two sessions of 20 trials per day, and also to reward horses in cases when they chose the incorrect flap three times in a row (and thus did not obtain any reward).

Response measures

Stereotypy level

We scored the number of crib-biting events over time from the video recordings collected over a 48 h period before the start of the experiment (see in "Experimental protocol" above), to assess the stereotypy level of the crib-biters at the time of the study. This score was converted into a frequency of crib-biting events per hour per horse. Based on these frequencies, we made three groups of crib-biters for the analyses: "S", strong crib-biters (58.37–65.76 crib-biting events per hour, N = 3 horses); "M", medium crib-biter (25.03 crib-biting events per hour, N = 1 horse); and "L" low crib-biters (1.09–9.06 crib-biting events per hour; N = 2 horses).

Behavioural measures

All the learning tests were video recorded using a GoPro HERO3 to control for eventual errors of scoring for all the following behaviours, which were directly scored during the tests by Experimenter 2; the choice of stimulus (correct or incorrect), the side chosen (right or left), and crib-biting events. Crib-biting was defined as instances when the horse grasped the top of the apparatus with its incisors, pulled back, contracted the neck muscles and drew air into its oesophagus, emitting an audible grunt (McGreevy et al. 1995). Three stereotypic horses did crib-bite during at least one trial, while the others never did so (Table 2).

Table 2. Number of crib-biting events ("CBnb") and number of trials ("Trial") needed to attain the learning criterion for the corresponding phase ("Phase"; Acq1, Rev1, Acq2, Rev2). "Horses" refer to the number attributed to each horses in Table 1.

Horses	**CBnb**	**Trials**	**Phase**
8	1	144	Acq1
8	0	94	Rev1
8	0	124	Acq2
8	0	105	Rev2
9	1	109	Acq1
9	4	247	Rev1
9	0	229	Acq2
9	0	9	Rev2
10	0	14	Acq1
10	0	149	Rev1
10	0	65	Acq2
10	0	1	Rev2
11	0	74	Acq1
11	0	420	Rev1
11	0	49	Acq2
11	0	220	Rev2
12	0	111	Acq1
12	0	478	Rev1
12	0	27	Acq2
12	0	157	Rev2
13	121	53	Acq1
13	745	298	Rev1
13	380	73	Acq2
13	2634	473	Rev2

Physiological measures

Before bringing a horse into the testing arena, it was equipped with a wireless heart-rate monitor (MLE120X Bioharness Telemetry System, Zephyr) fixed on a specific girth. During the acclimation and pre-training phases, the horses did also wear the girth for habituation, but without recording any data. During the tests, we collected the ECG trace continuously, allowing us to obtain the heart rate (HR) and the root mean square of successive inter-beat interval differences (RMSSD) as indicator of the physiological stress level of the subjects (von Borell et al. 2007). ECG gel was applied on the electrodes before each use. The data were transmitted in real time to a laptop using AcqKnowledge software v.7.2

(Biopac), and stored for later analyses. This allowed Experimenter 2 to add live comments during the visual discrimination task indicating when each session and each trial started. This enabled us to measure the physiological parameters precisely for each phase and each trial. We extracted HR and RMSSD from good-quality sections with clearly visible heartbeats on the ECG trace. We divided each session into five parts of equal duration, and analysed, when possible, three segments of 10 s each per part (at the beginning, middle, and end of each part). We checked visually that the software was tracking the heartbeats properly, and extracted HR and the inter-heartbeat (RR) intervals (ms). RR intervals were then used to calculate RMSSD (ms). We then calculated an average value per phase ("Acq1", "Rev1", "Acq2", "Rev2") for HR and for RMSSD. The total duration over which we were able to extract HR and RMSSD for the analyses was comparable between crib-biters and controls ("CB" group; means ± SD = 41.20 ± 18.59 s and "C" group = 48.28 ± 10.56 s).

Statistical analysis

All our data were analysed using generalised linear mixed models (GLMMs) or linear mixed-effect models (LMMs), in R 3.0.2, as described below. The list of the fixed and control factors as well as interaction terms included in each model are given in Tables 3 and 4. Because many studies have shown that stereotypy levels (frequency of stereotypy performance over time) can be strong predictors of learning abilities (Garner and Mason 2002; Garner et al. 2003; Vickery and Mason 2005), we first ran the models described below to investigate differences between crib-biters ("CB") and control horses ("C") ("GroupCB-C"), and then reran the same models again to investigate, this time, differences between the four groups defined by the frequency of crib-biting events per hour over 48 h (see "Stereotypy level" above) as follows; "S" strong crib-biters, "M" medium crib-biter, "L" low-frequency crib-biters and "C" control ("GroupSMLC") (Tables 3, 4).

Table 3. Abbreviations of the parameters used in the different analysis.

parameter abbreviation	definition
"Phase" (c)	Different phases of learning until the learning criterion (LC) is reached;
"Acq1"	First acquisition: phase during which the horses learned to choose the rewarded signal among a first set of two stimuli.
"Rev1"	First reversal: phase during which the horses learned to choose the signal that was unrewarded in Acq1.
"Acq2"	Second acquisition: phase during which the horses learned to choose the rewarded signal among a novel set of stimuli.
"Rev2"	Second reversal: phase during which the horses learned to choose the signal that was unrewarded in Acq2.
"Correct" (f)	Frequency of correct choices during the last session of Acq1 and Acq2, and during the first session of Rev1 and Rev2
"Sessions" (n)	Number of sessions of 10 min during the acclimation and pre-training (two sessions per day)
"Trial" (n)	Number of trials needed until the learning criterion is reached (6 correct trials in a row)
"Signal" (c)	Cross or circle
"Col" (c)	Colour of the signal (black or white)
"Person" (c)	Person leading the horse
GroupCB-C ("CB" or "C") (c)	Crib-biting or control group
GroupSMLC ("S", "M", "L" and "C") (c)	Groups of crib-biters based on the frequency of crib-biting events per hour over 48h (4 groups; strong crib-biters, medium crib-biters, low-frequency crib-biters and controls)
"Colour": "Y" or "N" (c)	Whether the colour changed between Rev1 and Acq2 (yes or no)
"HR" (m)	Heart rate (average value per horse per phase, in BPM)
"RMSSD" (m)	Root mean square of successive inter-beat interval differences (average value per horse per Phase, in ms)

c: category, f: frequency; m: mean; n: number

Table 4. Response variables, fixed and control parameters used in the different models. The abbreviations are described in Table 2. The crosses indicate which parameters and which response variable were used in the different models. The fixed parameters are the GroupCB-C or GroupSMLC, the Phase and the Colour depending on the model. The others parameters are control parameters. For the model with Correct as response variable, we selected only Rev1 and Acq2 among the other Phases. "1" indicates that we used either Phase or Colour as fixed parameters in the model.

Response variable	***Acclimation***	***Learning performance***	***Learning capacities***	***physiology***
Session	x			
Correct		x		
Trial			x	
HR				x
RMSSD				x
Fixed factors	***Acclimation***	***Learning performance***	***Learning capacities***	***physiology***
GroupCB-C	x	x	x	x
GroupSMLC	x	x	x	x
Phase		x	x[1]	x[1]
Colour			x[1]	x[1]
Phase x GroupCB-C		x	x[1]	x[1]
Phase x GroupSMLC		x	x[1]	x[1]
Colour x GroupCB-C			x[1]	x[1]
Colour x GroupSMLC			x[1]	x[1]
Control factors	***Acclimation***	***Learning performance***	***Learning capacities***	***physiology***
sex	x	x	x	x
age	x	x	x	x
Person	x	x	x	x
Col		x	x	x
Signal		x		

We first investigated group differences (GroupCB-C, or GroupSMLC, Tables 3, 4) in the number of training sessions the horses needed for the acclimation and pre-training phases ("Session"). The fixed and control factors as well as interactions terms included in this GLMM are described in Tables 3 and 4. The place where the horses were housed was added as a random factor. Two-by-two comparisons between the different groups of the factor GroupSMLC (“S”, “M”, “L”, and “C”) were then carried out using Tukey post-hoc tests (function glht, package multcomp in R, multiple comparisons of means).

To investigate learning performances, we then tested for group differences (GroupCB-C, or GroupSMLC, Tables 3, 4) in the frequency of correct choices (i.e. number of correct choices divided by the total number of trials; “Correct”) during the last session of acquisition for the first (“Acq1”) and second (“Acq2”) sets of learning tasks, and during the first session of reversal for the first (“Rev1”) and second (“Rev2”) sets of learning tasks. The fixed and control factors as well as interaction terms included in these LMMs are described in Tables 3 and 4. The horse identity nested within the place where the horses were housed was included as a random factor. Two-by-two comparisons between the different learning phases (between “Acq1” and “Rev1”, and between “Acq2” and “Rev2”) were then carried out using Tukey post-hoc tests (function glht, package multcomp in R, multiple comparisons of means).

Finally, to compare the learning abilities (i.e. number of trials needed to reach the learning criterion for each learning phase, “Trial”) and the physiological stress level of GroupCB-C and of GroupSMLC, we ran two separate sets of LMMs with Trial, HR or RMSSD as response variables. The first set of LMMs was aimed at testing the learning abilities and stress levels of crib-biters and controls during each learning phase (Acq1, Rev1, Acq2 and Rev2). In this set, we investigated group differences (CB-C or SMLC) in Trial, HR or RMSSD during the four learning phases. The fixed, control factors and interaction terms included in these LMMs are described in Tables 3 and 4. Because of the small sample size, we additionally carried out a power analysis for the effect of Group CB-C and GroupSLMC on Trial, HR and RMSSD to calculate if the power of our analysis was large enough (pwr.f2 function, pwr library in R). Two-by two comparisons between the different learning phases were then carried out using Tukey post hoc tests (function glht, package multcomp in R, multiple comparisons of means). The second set of LMMs was aimed at testing the effect of the change in the colour of the signal that some horses experienced between Rev1 and Acq2 on Trial, HR or RMSSD (Table 1). Indeed, this change in colour (hereafter “Colour”, change in colour “Y”, no change of colour “N”) could have also been perceived as a reversal by the horses. Since the factors Phase and Colour are correlated, we tested their effects on the response variables in different sets of models. In the set used to test the effect of the change in colour, the same fixed and control factors were included as in the set used to test the effect of the phase (Tables 3, 4), except for the fixed factor Phase, which was replaced by Colour. In this second set, only the data for Rev1 and Acq2 were included, because we were interested specifically in the colour change or not between Rev1 and Acq2. Because of the small sample size, we again carried out a power analysis for the effect of GroupCB-C and GroupSLMC on Trial, HR and RMSSD to calculate if the power of our analysis was large enough (pwr.f2 function, pwr library in R). For all these models (first and second sets), the horse identity nested within the place where the horses were housed was included as a random factor.

For all models described above, the residuals were checked graphically for normal distribution and homoscedasticity. To satisfy model assumptions, we used a square-root transformation for Trial, and a cube-root transformation for RMSSD. All the resulting parameters satisfying model assumptions were then entered into linear mixed-effects models fit with Gaussian family distribution and identity link function (lme function, nlme library in R). The session did not meet the statistical assumptions despite transformation. It was thus transformed to binomial data as follows; value equal or higher than median = 1 or value lower than median = 0. This parameter transformed to binomial data was input into a generalized linear mixed model fit with binomial family distribution and logit link function (glmer function, lmerTest library in R). For all models, we used a standard model simplification procedure by removing each non-significant term, until the deletion caused a reduction in goodness of fit (at which point, the term was left in the model). We assessed the statistical significance of each factor by comparing the model with and without the factor included using likelihood-ratio tests (LRT). The significance level of the factors was set at $\alpha = 0.05$.

RESULTS

Acclimation and pre-training

Crib-biters required significantly more sessions ("Session") to fulfil the learning criterion before starting the discrimination learning task than controls (CB: 6.83 ± 2.99 sessions; C: 4.57 ± 0.79 sessions; GLMM: effect of GroupCB-C on Session; $\chi 2 = 7.29$, *df* = 1, $p = 0.007$). There was also a significant effect of the four groups defined by the frequency of crib-biting events per hour over 48 h ("GroupSLMC": "S" strong crib-biters, "M" medium crib-biter, "L" low-frequency crib-biters and "C" control) on sessions (GLMM: Effect of GroupSMLC on Session, $\chi 2 = 11.94$, *df* = 4, $p = 0.008$). However, further two-by-two comparisons did not show any significant difference between the four groups "S", "M", "L" and "C" in the number of sessions needed to fulfil the learning criterion ($p \geq 0.98$ for all).

Learning performance

All 13 animals completed the four learning phases ("Phase": "Acq1", "Rev1", "Acq2", "Rev2"). There was a significant effect of Phase on the frequency of correct choices ("Correct") in the last session of acquisition for the first and second sets of learning tasks, and the first session of reversal for the first and second sets of learning tasks (LMM: effect of Phase on Correct, $F3,36 = 15.51$, $p < 0.0001$; Fig. 2). Further post hoc analyses showed a significant drop in the frequency of correct choices per session between the last session of Acq1 (mean ± SD = 0.72 ± 0.18) and the first session of Rev1 (Rev1 = 0.40 ± 0.17; multiple comparisons of means; effect of Phase Acq1 versus Rev1 on Correct; $Z = -4.80$, $N = 13$, $p < 0.0001$; Fig. 2). There was also a significant drop in the frequency of correct choices per session between the last session of Acq2 (0.76 ± 0.08) and the first session of Rev2 (0.44 ± 0.22; multiple comparisons of means: effect of Phase Acq2 versus Rev2 on Correct; $Z = -4.80$, $N = 13$, $p < 0.0001$, Fig. 2). On the other hand, there was no effect of group CB versus C on the frequency of correct choice (LMM: effect of GroupCB-C on Correct; $F1,7 = 1.77$, $p = 0.31$) nor of GroupSMLC (LMM: effect of GroupSMLC on Correct; $F3,5 = 0.59$, $p = 0.65$).

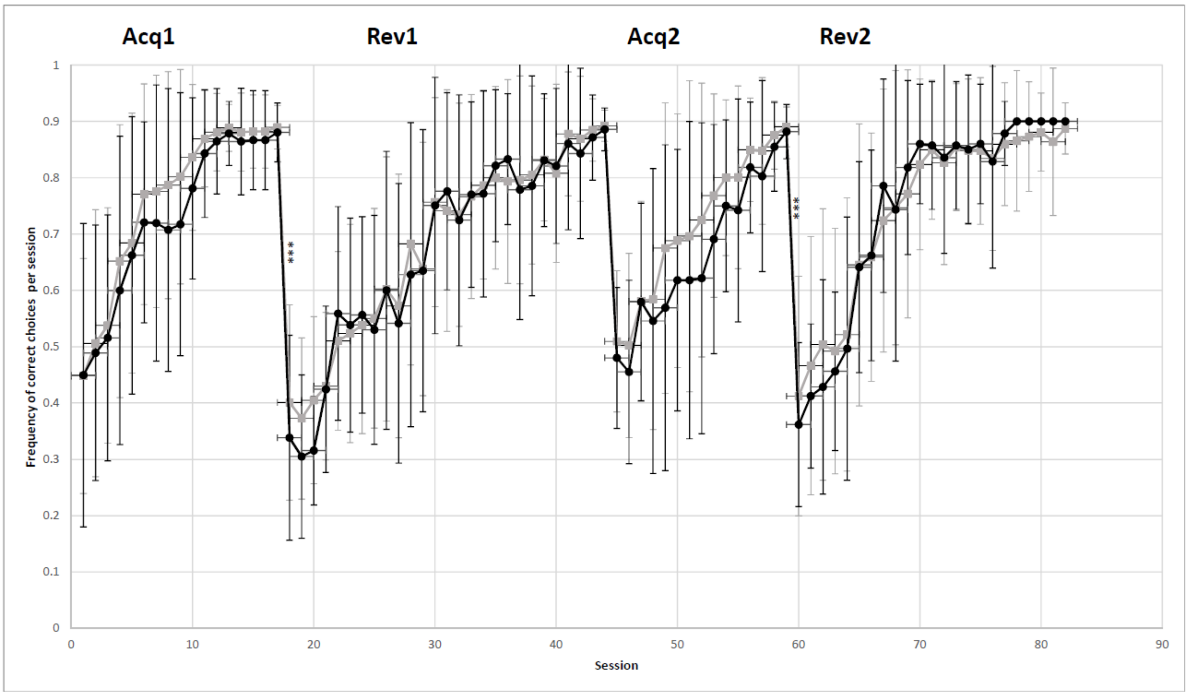


Fig. 2 Session-by-session summary of the performance of all horses. Data are the frequency (±SD) of correct choices per session for all horses of a group (control horses in black and crib-biters in grey) combined. The dots indicate the different session. For each horse, once the learning criterion (6 correct trials in a row) was reached, it was assigned a score of 90% until all remaining animals reached the criterion within that acquisition or

reversal phase. The different phases are the first acquisition (Acq1), the first reversal (Rev1), the second acquisition (Acq2) and the second reversal (Rev2). Significant differences between the last session of Acq1 and the first session of Rev1 and between the last session of Acq2 and the first session of Rev2 are indicated (*** $p < 0.001$).

Learning capacities

There was neither an effect of GroupCB-C (LMM: effect of GroupCB-C on Trial; $F_{1,7} = 1.77$, $p = 0.23$; Fig. 3), nor of GroupSMLC (LMM: effect of GroupSMLC on Trial; $F_{3,5} = 0.962$, $p = 0.48$), on the number of trials per phase needed to reach the learning criterion (“Trial”). However, there was a significant difference between phases (Acq1; Rev1; Acq2; Rev2) in Trial for all horses (LMM: effect of Phase on Trial; $F_{3,36}= 5.05$, $p = 0.005$; Fig. 3). The number of trials needed until the learning criterion was reached is shown in Fig. 3. Post-hoc comparisons showed that all horses needed significantly more trials for Rev1 than for Acq1 (multiple comparisons of means: $Z = 3.64$, $N = 13$, $p = 0.002$; Fig. 3), and more trials for Rev1 than for Acq2 (multiple comparisons of means: $Z = 2.92$, $N = 13$, $p = 0.018$; Fig. 3) and for Rev2 (multiple comparisons of means: $Z = – 2.65$, $N = 13$, $p = 0.041$ Fig. 3). The other two-by-two comparisons were not significant ($P \geq 0.76$ for all). In addition, considering only the first reversal and second acquisition, horses needed more trials when there was a colour change (“Colour”) (mean ± SD = 169.86 ± 95.12 trials) than when there was no change in colour (100.67 ± 87.13 trials) between Rev1 and Acq2 (LMM: effect of Colour on Trials; $F_{1,12} = 6.603$, $p = 0.025$).

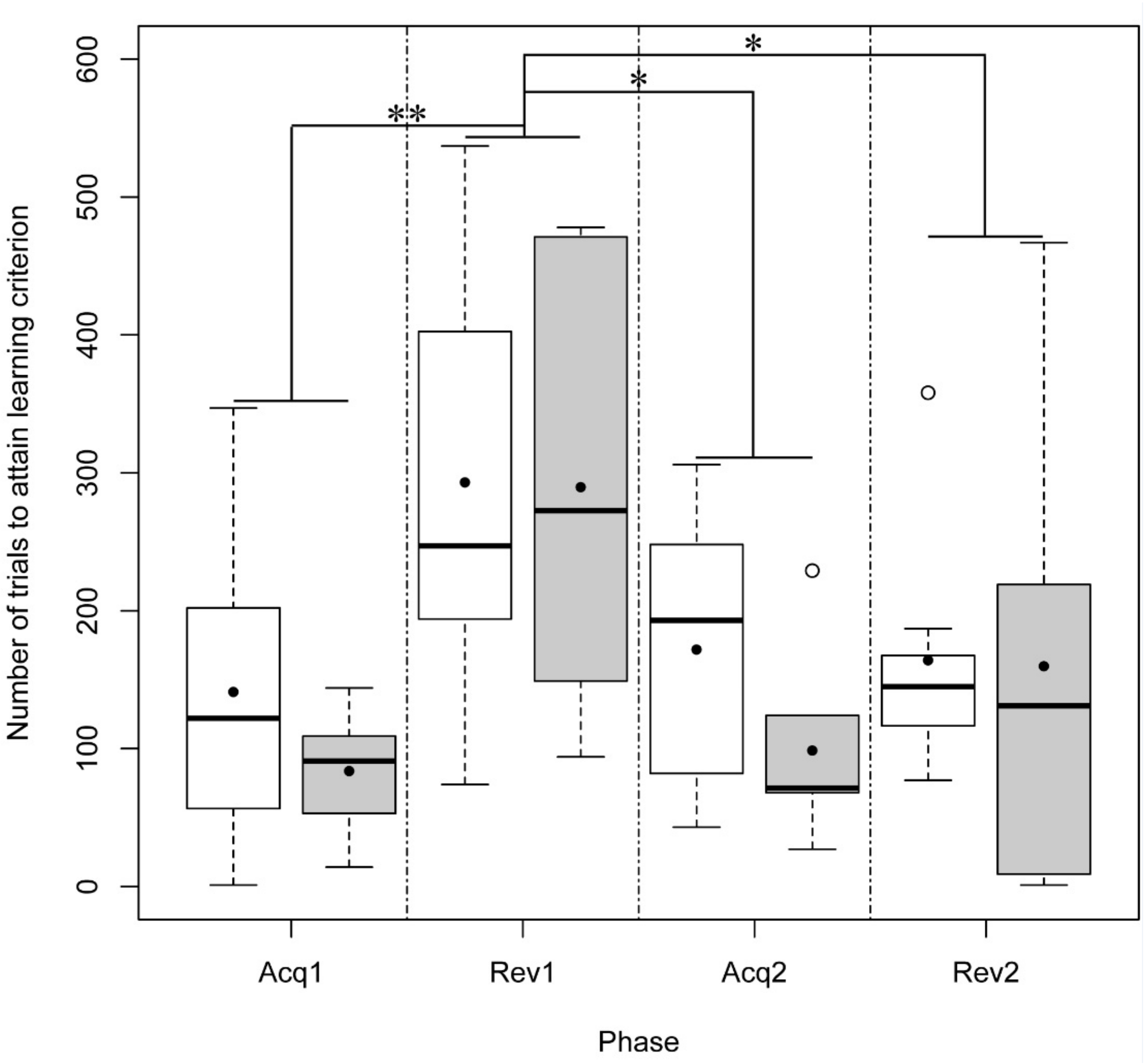


Fig. 3 Learning capacities of crib-biters and control horses. Number of trials until the learning criterion was attained for all crib-biters (group CB: *N* = 6 horses, in grey) and control horses (group C: *N* = 7 horses, in white), for each Phase (Acq1, Rev1, Acq2, Rev2). The different phases are the first acquisition (Acq1), the first reversal (Rev1), the second acquisition (Acq2) and the second reversal (Rev2). The learning criterion was fixed at six correct trials in a row. The black dots indicate the means. Significant differences between Phase are indicated as * $0.05 \leq p < 0.01$; ** $0.01 \leq p < 0.001$.

For all the LMMs carried out on Trial, neither the interaction between Phase and GroupCB-C or GroupSMLC, nor the sex, the age, the person leading the horse (two different persons), the type of signal (cross or circle), or the colour of the signal (black or white) had a significant effect. These terms were thus removed during model selection. Power analyses conducted on non-significant models revealed that the various LMMs testing the effect of GroupCB-C and GroupSMLC on Trial had a power ≥ 0.94, suggesting that a larger sample size would not have led to a significant result.

Physiological parameters

Heart rate (HR)

There was no difference in HR between the different learning phases (LMM: effect of Phase on HR; $F3,35 = 1.03$, $p = 0.39$), no effect of GroupCB-C (LMM: effect of GroupCB-C on HR; $F1,7 = 2.09$, $p = 0.20$), and no effect of GroupSMLC (LMM: effect of GroupSMLC on HR; $F3,5 = 1.88$, $p = 0.25$). In addition, considering only the first reversal and second acquisition, Colour had no effect on HR (LMM: effect of Colour on HR; $F1,12 = 0.30$, $p = 0.59$).

Root-mean square of successive inter-beat interval differences (RMSSD)

There was no difference in RMSSD between the different learning phases (LMM: effect of Phase on RMSSD; $F3,32 = 0.97$, $p = 0.41$), no effect of GroupCB-C (LMM: effect of GroupCB-C on RMSSD; $F1,7 = 0.05$, $p = 0.84$), and no effect of GroupSMLC on RMSSD (LMM: effect of GroupSMLC on RMSSD; $F3,5 = 0.91$, $p = 0.50$). In addition, Colour had no effect on RMSSD (LMM: effect of Colour on RMSSD; $F1,12 = 1.35$, $p = 0.27$).

For all the LMMs carried out on HR or RMSSD, neither the interaction between Phase and Group, nor the interaction between Colour and Group, nor the sex, the age, the person leading the horse (two different persons), the signal (cross or circle), or the colour of the signal had a significant effect. These terms were thus removed during model selection. Power analyses conducted on non-significant models revealed that the various LMMs testing the effect of GroupCB-C and GroupSLMC on HR or RMSSD had a power ≥ 0.87, suggesting that a larger sample size would not have led to a significant result.

DISCUSSION

In this study, we used a reversal learning task, which has been used as a diagnostic for one aspect of basal ganglia dysfunction, to compare the learning performances of crib-biting and control horses. According to our results, there is no indication that crib-biters suffer from such a dysfunction. Except for the acclimation phases, which took longer for crib-biters compared to the controls to achieve, we did not find any differences between crib-biters and control horses in the number of trials necessary to reach the learning criterion in any phase of the experiment. In fact, all horses reached the learning criterion and performed the two reversals. Interestingly, they also performed the second reversal in fewer trials compared to the first one, suggesting that they learned to learn. Unlike in other studies that found that crib-biting horses have altered learning abilities compared to other horses (Hemmings et al. 2007; Parker et al. 2009; Roberts et al. 2015), our subjects had the opportunity to crib-bite during the experiment, and hence to potentially reduce their stress levels (Briefer Freymond et al. 2015), as shown by the absence of differences between the two groups in the physiological parameters that we measured. We could therefore suggest that discrepancies between our study and previous studies on learning performance could result from differences in stress levels experienced by crib-biters and control horses in these former studies, although they did not collect physiological measures of stress.

Acclimation and pre-training

In this study, crib-biters needed a longer time than the controls to be acclimated to the learning apparatus, and to attain the conditions required to start the discrimination procedure (i.e., pushing the flaps alone five times on both sides of the apparatus without any intervention of the experimenter). This could be explained by the fact that crib-biters seem to be more stress sensitive (Briefer Freymond et al. 2015), and might thus need more time to be acclimated to a new situation. However, we did not collect any physiological indicators of stress during the pre-training phase, because in this phase, the movement of the horses was not standardised, unlike during the learning phase. Indeed, it is recommended that only measures made during times of similar behavioural pattern should be compared (von Borell et al. 2007). Future studies that additionally measure stress parameters during habituation could inform us on the stress levels of crib-biters in such situations.

Learning capacities

All the horses (crib-biters and controls) in our study performed the two reversal tasks, and needed significantly more trials to reach the learning criterion for the first reversal (“Rev1”) than for the other phases (first acquisition, “Acq1”, second acquisition, “Acq2”, and second reversal, “Rev2”). In addition, the significant drop that we observed in the number of correct responses between the acquisition phases and their following respective reversals suggests that learning had taken place (McBride and Parker 2015). Although few studies suggest that horses possess the ability to perform reversal learning based only on visual cues (Sappington et al. 1997; Voith 1975), this task seems to be more challenging, and in some cases not achievable, compared to reversal learning based on spatial cues (Hothersall et al. 2010; Martin et al. 2006). The fact that control and even stereotypic horses learned the reversal task let us suggest that reversal learning based only on visual cue is possible under certain conditions. Moreover, the fact that horses performed the second reversal in less trials than the first one, confirmed that horses learned to learn as it was also demonstrated in other studies testing horses in visual or spatial discrimination tasks (Fiske and Potter 1979; Martin et al. 2006; Voith 1975; Warren and Warren 1962). As underlined by Brubaker and Udell (2016), the study protocol and nature of the visual stimuli appear to affect a horse's ability to perform any given cognitive task. In our study, we adapted the experimental protocol, based on preliminary tests and previous studies (Flannery 1997; Gabor and Gerken 2010; Hall et al. 2003) to keep the horses motivated, as follows. First, we chose to oppose two signals that differed only in whether they were black or white, because such colours seem to be easy to differentiate by horses. Indeed, horses' ability to discriminate between different colours is limited due to their dichromatic vision (Blackmore et al. 2008). Second, we ensured that the stimuli were presented close to the ground level (40 cm in our study). Indeed, former studies demonstrated that horse performance was improved when stimuli were presented close to the ground level (22 cm), compared at the height of 90 cm from the ground (Hall et al. 2003). Third, we adapted the number of trials per sessions and rewarded the horses during the study if they had been choosing the wrong stimulus three times in a row, to maintain their attention span and motivation (Flannery 1997; Rubin et al. 1980; Sappington et al. 1997). Finally, we waited until all the horses reached the learning criterion before stopping the study, even when a relatively high number of trials was required (*N* max = 537). We suggest that similar precautions might help improve the motivation of horses in future cognitive studies.

Learning performance of crib-biters compared to control horses

We did not find any difference in the number of trials needed to reach the learning criterion between crib-biter and control horses, contrary to previous studies on the same topic (Hemmings et al. 2007; Parker et al. 2008, 2009; Roberts et al. 2015). Indeed, previous studies found that crib-biters might be more prone to habit learning than to response–outcome learning (Hemmings et al. 2007; Parker et al. 2008, 2009; Roberts et al. 2015). Therefore, we expected that they would reach the learning criterion during Acq1 or Acq2 faster compared to the controls. In addition, since previous studies also demonstrated that crib-biting horses need more operant responses compared to the other horses before the extinction of a previously learnt action (Hemmings et al. 2007; Roberts et al. 2015), and were unable to maintain response–outcome learning in a continuously applied learning paradigm (Parker et al. 2008), we expected that the crib-biters would need more trials in Rev 1 and Rev 2 to reach the criterion compared to controls. In contrast to these predictions, in our study, all the crib-biters were able to achieve the different phases (Acq1, Rev1, Acq2, Rev2) in a similar number of trials compared to the controls. An explanation for these discrepancies between our studies and previous ones could be that in our experiment, the crib-biters had the opportunity to crib-bite on the learning apparatus. It is not always clear whether stereotypic horses had the opportunity to crib-bite, and did so, during previous studies (Hemmings et al. 2007; Parker et al. 2008, 2009). However, in Roberts et al. (2015), crib-biting straps were removed prior to the tests, although no information about crib-biting events is specified. If crib-biting is indeed a coping strategy (Briefer Freymond et al. 2015), reducing stress levels could, as a result, improve their learning capacities, allowing them to achieve the same performances as non-stereotypic horses (Schwabe and Wolf 2010; Valenchon et al. 2013). Even if other studies did not measures stress parameters during learning tasks (Hemmings et al. 2007; Parker et al. 2009, 2008; Roberts et al. 2015), this suggests that allowing crib-biting horses to perform their stereotypic behaviour during learning could improve their cognitive abilities.

Since stereotypies in animals are often likened to human developmental, neurological or severe psychiatric disorders (e.g., autism, obsessive compulsive disorder (OCD) or schizophrenia) (McBride and Parker 2015), comparisons between our results and human disorders can be made. Even if OCD patients usually report that they get a sort of relief by performing their rituals, and that preventing performance increases their anxiety (Boyer and Lienard 2006), the literature on autism in humans suggests that, on the contrary, children exhibiting high levels of stereotypy fail to learn while engaged in stereotypy (Cunningham and Schreibman 2008). On the other hand, our findings could be related to results found in another human psychological disorder, named attention deficit hyperactivity disorder (ADHD). In the same way as animals suffering from stereotypies, ADHD patients show non-goal-oriented motor movements. These movements are however, unlike stereotypies, not executed as repetitive invariant patterns. A recent paper showed that performing such movements is associated with an improvement in cognitive performance (Sarver et al. 2015). Such findings are in accordance with our results in crib-biting horses. As suggested in Hausberger et al. (2007), stereotypic horses differ from other horses in their behaviour and may require specific training. Letting these horses the possibility to perform their stereotypy might be, as our study suggests, one specific feature to incorporate in learning protocols, which might then allow them to perform successfully. Future studies could compare the learning capacities of crib-biter horses prevented or not to crib-bite and of a corresponding number of control horses subjected to the same treatment. To summarize, the results of our study do not support the hypothesis that crib-biters display alterations in learning abilities, which could result from impaired dopaminergic system. In addition, our findings suggest that, in the same way as the performance of non-goal-oriented motor movements improves cognitive performances of

ADHD human patients (Sarver et al. 2015), the performance of stereotypic behaviour might improve crib-biting horse learning abilities.

During the acclimation and pre-training phase, however, crib-biters needed more sessions than controls to attain the learning criterion, even if they also had the possibility to crib-bite. Since we did not collect measures of stress indicators (e.g., HR, RMSSD) during this period, we cannot make inferences about the stress level of stereotypic horses compared to controls during this phase. Nevertheless, we could suggest that crib-biting horses were less focussed on the task during the acclimation and pre-training compared to the test phase, because horses had to manage too many other external stimuli. A general difficulty of maintaining a task focus (i.e., attention) in stereotypic compared to control horses has been suggested by Hausberger et al. (2007). These authors proposed that the time invested in performing stereotypic behaviour throughout the day and at night is likely to affect sleep quality and quantity in stereotypic horses. This could lead to a general lower attention span in these horses than in non-stereotypic horses. Attention state and motivation being primordial for learning (Cowan 1998; Rochais et al. 2014), we could hypothesise that crib-biters might needed a longer time than control horses to habituate to novel situations and be able to ignore and exclude external stimuli (i.e., new area, apparatus). However, after a longer habituation than for the controls, they might be able to focus on the cognitive task (i.e., test phase). During the test phase itself, as a result of our protocol that was designed to maximise attention span and motivation (e.g., short training sessions of around 20 min, "correction trials" (Flannery 1997)), all horses, including crib-biters, seemed very attentive and motivated, which might have boosted their performances. Attention deficits have also been demonstrated in ADHD disorder, which has the particularity to induce difficulties in maintaining task focus (Sarver et al. 2015). To test the hypothesis that crib-biters are generally less attentive than other horses, future studies could evaluate the distractibility (i.e., how much can an individual be distracted by external stimuli) of these horses compared to non-stereotypic individuals (e.g., using a "distractibility test"; (Rochais et al. 2017)). Another indicator of attention that could be used to test such hypothesis is spontaneous blink rate (SBR; (Roberts et al. 2015)). SBR is a basic measure of dopamine transmission utilised to determine striatal functioning in stereotypy-performing humans and could also be applied to animals (Roberts et al. 2015). Using this indicator, Roberts et al. (2015) demonstrated that crib-biter horses display lower SBR than other horses (Roberts et al. 2015). Interestingly, SBR has been recently shown to constitute an index of dopaminergic component of sustained attention and fatigue in humans (Maffei and Angrilli 2018). By comparing the distractibility and attention of stereotypic and control horses when performing cognitive tests, future studies might be able to further highlight the need to adapt the design of training sessions to horses suffering from stereotypies, in order to maximum their learning abilities and improve their welfare (e.g., by avoiding frustration).

A last explanation for the discrepancies between our studies and other studies investigating learning alteration in crib-biting horse could be that reversal learning tests are perhaps not appropriate for assessing dopaminergic alterations that might be present in crib-biters. Such alterations, including a higher number of dopamine receptor subtypes in the ventral striatum or nucleus accumbens *(Nac)* and a lower number of such receptors in the dorsomedial striatum (DMS) or caudate have been shown using post-mortem analyses in crib-biting horses (McBride and Hemmings 2005). However, behavioural flexibility, or the ability to adjust responses according to a change in the environment, is mediated by a large neural network, including prefrontal-basal ganglia circuits in addition to the dorsal and ventral striatum. As mentioned previously, the DMS has been identified as an important structure for flexible responding (Castane et al. 2010). Indeed, DMS lesions, due to the role of this brain structure in learning, might result in a switch from goal-directed to habit formation and thus in the impairment of the development of habits (Yin et al. 2008). However, the role of *Nac* in instrumental performance remains nowadays controversial (Yin et al. 2008). For example, some studies found that lesions in the *Nac* do not impair spatial, visual or motor reversal in monkeys, *Macaca fascicularis* (Stern and Passingham 1995), while other studies found that such lesions impaired both an initial discrimination and its reversal in Lister hooded rats (Annett et al. 1989). A reason for these discrepancies between studies could be that most studies on DMS or dorsolateral (DLS) lesions have used rats, despite the fact that it is difficult to compare the physical location of dorsal or ventral striatum in rat and other species such as primates (Yin et al. 2008). To conclude, reversal learning paradigms are among the most widely used tests for cognitive flexibility and there is accumulating evidence that DMS is involved in this type of learning (Castane et al. 2010; Izquierdo et al. 2017; Ragozzino et al. 2003). However the role of *Nac*, which has been suggested to be impaired in crib-biters (McBride and Hemmings 2005), in reversal learning tasks is controversial (Yin et al. 2008). Therefore, testing crib-biters with other cognitive tests than reversal learning might be perhaps more valuable to investigate the suggested impairment in *Nac* (McBride and Hemmings 2005).

Effect of the change in colour

In our study, the colour of the signal always changed from white to black or vice versa, between the acquisition and the corresponding reversal. However, for half of the horses (half of the crib-biters and four of the seven controls), a change in colour occurred also in the middle of the learning procedure, between Rev1 and Acq2 (Table 1; Fig. 1). This change in colour in the middle might have been experienced as an additional reversal (based on colour only). In accordance with this hypothesis, the results showed a significant effect of colour change between Rev1 and Acq2, with horses submitted to the colour change needing more trials than the other horses. We suggest that further studies including several acquisition phases with different visual stimuli should be aware that changes in colours between phases might be perceived by the animals as reversals.

Stereotypy level and performance

The crib-biters in this study differed with regard to the strength of their stereotypy (Table 1). Many studies have reported more cognitive difficulties in animals displaying a higher frequency of stereotypic behaviour compared to less stereotypic ones (Garner and Mason 2002; Garner et al. 2003; Vickery and Mason 2005). Indeed, stereotypic levels have been shown to correlate with an increase in the persistence of inappropriate responses in an extinction learning test in bears, *Ursus thibetanus* (Vickery and Mason 2005). However, our results did not show such a trend. Our three groups based on the frequency of crib-biting of the horses assessed over 48 h before the experiment started (GroupSMLC, "S", strong crib-biters, "M", medium crib-biters and "L", low crib-biters), did not differ in their learning performance. Therefore, we did not find any evidence showing that the frequency of crib-biting is a factor that influences the cognitive abilities of horses. This absence of group difference could also suggest that the stereotypic level is not a good indicator of dopaminergic system alterations. Similar results have been found in rhesus macaques, *Macaca mulatta* (Pomerantz et al. 2012). Interestingly, in this study, the authors found that some type of stereotypies did correlate with perseveration while some did not. Future studies could investigate the potential links between learning performance and the time since a horse started crib-biting instead of its stereotypic level.

Physiological parameters

We did not find any evidence for group differences in the sympathomedullary (SAM) axis parameters measured in this study (HR and RMSSD) during the phases of acquisitions and their respective reversals. Because one potential cause of stereotypies is a previous exposure to a chronic stress situation that could induce higher sensitivity to stress (Bhatnagar and Vining 2003), we would have expected crib-biters to be more stressed than controls. However, neither the results of the present study, nor those of our previous study revealed any difference in SAM parameters between crib-biters and controls (Briefer Freymond et al. 2015). It also suggests that the crib-biters were experiencing similar stress levels as controls during the experiment, possibly as a result of crib-biting on the apparatus, which might have reduced their stress levels (Briefer Freymond et al. 2015). We could also have expected horses to be more physiologically stressed during the first reversal compared to the other learning phase as this learning phase might be more challenging for them, as displayed by the increased number of trials required to achieve this task. However, we did not find any effect of the learning phase (acquisition or reversal) on HR or RMSSD. Finally, it is possible that other parameters than HR and RMSSD might be more adequate to measure stress during a learning task involving locomotor behaviour. Indeed, HR and RMSSD are also influenced by physical activity (von Borell et al. 2007). For this reason, only measures made during times of similar behavioural pattern should be compared (von Borell et al. 2007). Our assumption is that this is the case in our study, because all horses (crib-biters and controls) had to perform the same trajectory, and the same number of trials per session. However, further studies could aim at designing tasks involving less movement and take also additional measures of stress, such as behavioural measures (e.g. ‚Equine Facial Action Coding Systems (FACS) (Wathan et al. 2015), behavior scores (Young et al. 2012)) during learning tasks as well as during habituation.

Animal welfare

Animal welfare being of increasing public and scientific concern, it is important to understand the link between stereotypic behaviour and animal welfare. It has been proposed hat stereotypic behaviour might indicate poor welfare only if a dopaminergic dysfunction is present (Mason and Latham 2004). Indeed, even if stereotypies develop under contexts of chronic stress state, their performance, once fully developed, might not necessarily indicate poor welfare. For instance, in this case, the performance of stereotypic behaviours might serve as coping mechanisms, helping individuals to reduce their stress level (e.g., *"mantra effect"*, (Mason and Latham 2004)). Mason and Latham (2004) proposed that stereotypies correlate with poor welfare only when they have become a habit and, only when behaviours have changed in control and have become environmentally insensitive. At this developmental stage, stereotypies are performed in a more diverse set of situations and are harder to interrupt. However, such a stage is not easy to assess because the performance of stereotypies may vary between individuals, in terms of stereotypy level over time. Mason and Latham (2004) additionally argued that perseverative responding, resulting from basal ganglia dysfunction, also indicates poor welfare. In fact, with perseveration, individuals may produce unnecessary and inappropriate responses to environmental cues. In humans, as mentioned earlier, perseveration is also correlated with human disorders, like schizophrenia, autism and other brain injuries. In conclusion, if neurobiological changes are linked to stereotypies, resulting in alterations in the learning profile of animals, stereotypic behaviour should indicate poor welfare. However, in this study, we could not conclude to the existence neurobiological alteration in crib-biters, since these horses did not need more trials to perform the reversal learning tasks compared to control horses. Further studies on cognitive abilities of crib-biter horses are thus required to determine the impact of this stereotypy on horse welfare.

CONCLUSION

Our study did not reveal any difference in cognitive abilities between crib-biters and controls and therefore we cannot conclude that stereotypic horses suffer from a dopaminergic dysfunction. Indeed, our results show that all horses, including stereotypic horses and controls, were able to perform reversal discrimination tasks based on visual cues, and that they even learned to learn (i.e., improve their performance from one reversal to the next). An explanation for the discrepancies between our study and the previous ones could be that, in our study, the crib-biters had the opportunity to crib-bite on the learning apparatus, which might have enabled them to reduce their stress level, as suggested by the lack of group difference in physiological stress parameters. Further studies could test the learning capacities of crib-biters that are prevented or not to perform the stereotypic behavior against a group of non-stereotypic horses subjected to the same treatment. Finally, our results point towards several parallels between horse crib-biting behaviour and human developmental, neurological or psychiatric disorders, such as ADHD disorders, suggesting that the study of horse crib-biting behaviour could serve as a good animal model to better understand such disorders in humans (Brace et al. 2015).

ACKNOWLEDGMENTS

We are grateful to all the owners of the horses who offered to participate in this study. We are also grateful to Matthew Parker, who gave us the idea to perform this study and helped us with the design of the experiment.

ETHICS

Ethical approval the experimental procedure for the horses was approved by the Federal Veterinary Office (approval number VD 26777 bis; Switzerland).

CONFLICTS OF INTEREST

The authors declare that they have no competing interest.

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CHAPTER VI

OVERALL DISCUSSION AND FUTURE PROSPECTS

Stereotypies are abnormal behaviours characterised by repetitive, relatively invariant patterns of behaviour usually described as having no apparent ultimate and proximal function (Mason and Rushen, 2006). They constitute major impairments in humans, often associated with human developmental disorders, and in a wide range of animal species in captivity (McBride et al., 2017). The proximate function of these behaviours is not well understood. It is debated whether they serve to cope with a stressful environment, and the available experimental evidence is yet far from clear (Ladewig, 1987; McGreevy and Nicol, 1998; McBride and Cudderford, 2001; Wickens and Heleski, 2010; Sarrafchi and Blokhuis, 2013). Additionally, the causes of the development of stereotypies and the consequences of this behaviour on individuals are only partially known. Such stereotypic behaviour may appear in context of chronic-stress, which could modify the stress physiology (e.g. modification of the stress pathways) and sensitize a specific brain area, the *basal ganglia* (Spruijt et al., 2001). Such modifications of the *basal ganglia* would indicate a malfunction or maladaptation and its consequence might be an alteration of the learning profile of animals.

Domesticated horses are often confined for up to 23h per day. This confinement time exceeds their ability to adapt, for example, by strongly limiting foraging time, and makes them prone to develop stereotypies. The most prevalent stereotypy in horses is crib-biting behaviour, an oral stereotypy, which is common in domesticated ungulates and for this reason a major issues. The aim of the present dissertation was to answer some questions that remain elusive regarding this specific stereotypy. This was done in four steps during which I tested almost the same horses (Annex II); I first investigated if crib-biting horses show differences in their physiological response in an ACTH challenge test, compared to control horses (Chapter II). Secondly, I investigated if some individual variation in behaviour may predispose animals to develop crib-biting behaviour (Chapter III). Thirdly, I tested the potential consequences of such behaviour on the learning profile of the animals (Chapter IV and V). Animal welfare being of increasing public and scientific concern, it is important to assess whether or not stereotypies are adaptive, maladaptive or both. The results I found might indicate that crib-biting is a coping strategy helping horses to reduce physiological stress. Moreover, based on previous studies (McBride and Hemmings, 2005; Hemmings et al., 2007; Parker et al., 2008; Roberts et al., 2015) and because I did not find evidence of learning alterations between stereotypic and control horses in the reversal learning task (Chapter V), it seems that crib-biting horses might be more likely impaired in one specific part of the *basal ganglia*, the *ventral striatum*.

In this general discussion, I will first integrate and discuss the results of the first four studies conducted during my PhD, focussing on the different questions remaining elusive about stereotypic behaviour: the physiology and function, the personality and coping style of stereotypic horses and the potential consequences of stereotypies on learning abilities. One of my aims was also to study the ontogeny of the crib-biting behaviour. For this reason, a fifth study was conducted, in which I followed foals at weaning time. However, due to a limited sample size and collinearity between breed and management conditions, no detailed analyses could be carried out on this part. These results appear thus in an annex (Annexe I), and I will only briefly present and discuss them here. Subsequently, I will also discuss the limitations of the studies performed during the PhD and propose some ideas and perspectives for future research. Finally, based on my results, I will propose guidelines on how to help reducing the frequency of stereotypies in horses. These guidelines could help to improve the welfare of horses displaying crib-biting behaviour.

DEFINITION – FUNCTION OF CRIB-BITING BEHAVIOUR

The Chapter II of my PhD thesis consisted in investigating the physiological reactivity of crib-biters and the same number of non-stereotypic horses. I thus performed an ACTH challenge test on 43 horses. I found that the crib-biters that did not perform any stereotypic behaviour during the three-hour ACTH challenge test had higher HPA axis reactivity than the controls, which was not the case for crib-biters showing stereotypic behaviour. As explanation, I suggested that crib-biting might be, for some horses at least, an adaptive strategy and concluded that crib-biting in horses might be a coping strategy helping individuals to reduce cortisol level caused by stressful situations (Briefer Freymond et al., 2015). I discussed the fact, that some of the crib-biters tested in this study, and not others, were at the stage where full-blown stereotypies serve their coping function of reducing stress, while in earlier stages of stereotypy development might show a different pattern (Briefer Freymond et al., 2015). It would be interesting for further studies to investigate individual differences in physiology among crib-biters. Such studies might reveal differences in stress responses between an early stage of development and full-blown stereotypies. Alternatively, another explanation for these results could be that some crib-biters did not crib-bite because they were too stressed during the experiment.

The results described in Chapter IV, may also support this coping hypothesis. In this Chapter, I investigated differences in cognitive performances between crib-biters and the same number of non-stereotypic individuals in some challenging spatial learning tasks (Briefer Freymond et al., in preparation). Apart from one horse, a crib-biter, all horses had previously been tested in Chapter II (Annex II). In Chapter IV, (similarly to Chapter II), some stereotypic horses did crib bite during some of the tasks on a solid fence used as part of the experimental set-up, while other ones did not. I therefore investigated once more if differences in physiological responses to the tests existed between individuals identified as crib-biters that responded by crib-biting (Group A) or not (Group B) and control horses (Group C). These comparisons revealed that Group A had lower saliva cortisol levels than the other horses at the end of the learning tests. Furthermore, there was more horses of Group B showing frustration behaviours during one of the challenging spatial task than the crib-biters of Group A, suggesting that crib-biting on the fence helped the crib-biters to handle their frustration. It has been argued that stereotypies that have reached the full-blown developmental stage can be performed in a more diverse set of situations than stereotypies at earlier stages of development (Mason and Latham, 2004); at that advanced stage, horses will for example not only perform the stereotypy in their box at a specific place, but also outside on a solid fence. Potentially existing differences in developmental stages among the stereotypic horses tested in my studies might explain why some of them only used the solution they developed to cope in with stress in another situation than in their usual environment (i.e. in their box). Based on results described in Chapter II and IV, I therefore conclude that the performance of stereotypies, once developed, might decrease stress and hence improve horse cognitive abilities and welfare in some situations (e.g. during a learning session). However, again an alternative explanation could be that crib-biters did not crib-bite because they were more stressed than other horses during the experiment.

LIMITATIONS OF THE RESULTS

Because in these two studies (Chapter II and IV), the horses were allocated in three groups according to their crib-biting performances during the tests, i.e. crib-biters that did versus crib-biters that did not crib-bite, a-posteriori instead of randomly, my results should be interpreted with precaution. To validate them, it will be necessary to replicate the tests in a more controlled manner in order to investigate the coping hypothesis. Further experimental studies should include a subset also in the control group. For instance, crib-biters could be tested against a control non-stereotypic group, in the same way as in these two studies, in an ACTH challenge test, versus in learning tasks causing frustration, but this time half of the crib-biters could be provided with a solid support to give them the possibility to crib-bite, while the other half would not have this option. The control horses could thus also be tested with the two treatments consisting in the presence or not of a

solid support. Moreover, such studies have to record carefully stress parameters and the number of times horses perform the stereotypic behaviour could be coded by an experimenter who is blind to which horse is stereotypic or not.
Another potential limitation of my results that needs to be highlighted is the fact that cortisol concentrations were measured from saliva samples. In humans, saliva cortisol concentration has been shown to be independent from saliva flow rate (Chiappin et al., 2007). Yet, also in humans, it has been suggested that food consumption 90 mins before saliva cortisol sampling might influence the cortisol determination (Toda et al., 2004). In contrast to these findings, in animals only few studies have examined potential effects of feeding, drinking and possible dilutions effects on saliva concentration. One recent study showed that feeding and drinking seems to have no influence on saliva cortisol measurements (Schwinn et al., 2016). Crib-biting behaviour being hypothesised to increase saliva flow (Nicol, 1999), this phenomenon needs to be discussed in relation to cortisol saliva measurements in crib-biters because, it could potentially have a dilution effect on saliva concentration. Indeed, it has been shown that crib-biting and wood chewing might increase saliva production (Moeller et al., 2008), while another study showed no evidence (Houpt, 2012). In my PhD project, I decided to use saliva cortisol instead of blood serum cortisol, because this measure has many advantages compared to the other one. The first advantage is that collection of saliva cortisol is less invasive than blood serum cortisol, which can be stressful for horses and thus alter the results (Peeters et al., 2011). The reliability of saliva cortisol in comparison to blood serum cortisol measurements have been validated in horses during an ACTH challenge test (Peeters et al., 2011), as well as in other situations such as during competition and semen collection in stallions (Lebelt et al., 1996; Peeters, 2011). The second advantage is that measurements of free cortisol concentration in saliva have been shown to reflect changes in the HPA axis activity more accurately than blood serum cortisol measurements (Alexander and Irvine, 1998). A disadvantage however, is that salivary cortisol measure has been suggested to be inappropriate during equine acute physiological stress (Pell and McGreevy, 1999). To this aim, it would be useful to perform additional studies comparing blood serum with saliva cortisol measurements in crib-biters versus non-stereotypic horses in acute and in higher stress situations.

In conclusion, my results suggest that crib-biting might help horses to reduce physiological stress. However, more studies are needed to confirm these results.

CAUSES OF CRIB-BITING BEHAVIOUR: COPING STYLE – PERSONALITY- GENETIC PREDISPOSITION

Although the genes involved in stereotypic behaviour remain unknown, the causes of stereotypic behaviour have been suggested to consist in genetic predispositions associated with chronic stress experience due to environmental restriction of species-specific behaviour (McGreevy et al., 1995; Cabib et al., 1998; Bachmann et al., 2003). Since genetic differences could induce differences in personality, individual variation in behaviour may predispose animals to develop stereotypies (Joshi and Pillay, 2016). Therefore, I hypothesised that some differences in personality, coping style and/or physiology would exist between crib-biters and non-stereotypic horses.

The results described in Chapter II, give some information about the potential genetic predisposition of the crib-biting behaviour (Briefer Freymond et al., 2015). In fact, I found that crib-biters reacted with higher cortisol responses than non-stereotypic horses in an ACTH challenge test, indicating a higher hypothalamo-pituitary-adrenocortical (HPA) axis reactivity, and suggesting a higher sensitivity to stress (Briefer Freymond et al., 2015). This higher HPA axis reactivity could be the result of chronic-stress-induced alterations, but also of a genetic predisposition. I thus conclude that future studies investigating genes involved in this higher sensitivity to stress would be very valuable.

In Chapter III, I compared the personality of horses displaying crib-biting behaviour to the personality of the same number of non-stereotypic, control horses (Briefer Freymond et al., in preparation). The personality of my subjects was assessed following a previously validated model, which proposes five personality traits in horses (reactivity to humans, tactile sensitivity, fearfulness/curiosity, social reactivity and locomotor activity; (Lansade and Bouissou, 2008; Lansade et al., 2008b; a; Lansade et al., 2008c). According to the results described in Chapter II, showing that crib-biters display physiological characteristics of reactive individuals, i.e. high HPA-axis reactivity in crib-biters (Briefer Freymond et al., 2015), I expected these horses to also display behavioural characteristics of reactive coping individuals, i.e. being less fearful than the control group (Koolhaas et al., 2010; Nagy et al., 2010). However, based on the results of Chapter III, I could not conclude that crib-biters showed more characteristics of reactive individuals compared to non-stereotypic horses (i.e. to be less anxious (e.g. fearful), to interact less with unfamiliar humans, to be less active, to show less exploratory behaviours, and to be more social compared to control horses) (Koolhaas et al., 2010; Nagy et al., 2010; Górecka-Bruzda et al., 2011; Briefer Freymond et al., 2015; Finkemeier et al., 2018). I suggest that further behavioural or physiological indicators than the ones I used to assess fearfulness could help to detect differences between reactive and proactive individuals. Indeed, behavioural reactions to fear-inducing situations could be less strongly expressed in reactive individual than in proactive ones (Koolhaas et al., 2010). In fact, and as recently suggested by Squibb et al. (2018), reactive horses might appear less fearful than proactive ones, despite a similar or stronger physiological reaction. It would therefore be useful in further studies to measure, in addition to behavioural responses, other types of fear indicators (e.g. physiological responses, Equine Facial Action Coding Systems (FACS) (Wathan et al., 2015)), in order to increase the accuracy of fear assessment. On the other hand, the results of Chapter III provided new knowledge regarding the personality profile of crib-biting horses (Briefer Freymond et al., in preparation). Indeed, it seems that crib-biters might be more sensitive to tactile stimulation than non-stereotypic horses. A first explanation could be that they share some characteristics of autistic people, and especially those with Asperger's syndrome, which are often described as being highly disturbed by their environment due to a more intense perception of external stimuli compared to other people (Blakemore et al., 2006). A second explanation is that such elevated tactile sensitivity could be due to altered dopamine physiology, resulting from chronic stress exposition. Because all these personality tests were performed only one time, they should be replicated in future studies for validation.

In previous studies, it has been proposed that stereotypic behaviour reflects a proactive coping response to stress, following characterisation of different line of mice as being proactive or reactive and linked to stereotypy development (Cabib and Puglisi-Allegra, 2012; Ijichi et al., 2013). However, according to the results reported in Chapter II, (Briefer Freymond et al., 2015), I expected crib-biters to display characteristics typical for reactive individuals, i.e. being more flexible, showing less cue dependency and being less routine forming than other non-stereotypic horses (Koolhaas et al., 2010) in the studies conducted in Chapter IV and V. Again, the results described in those chapters did not allow me to draw any conclusion regarding the coping strategy (proactive or reactive) of crib-biters (Briefer Freymond et al., in preparation and Briefer Freymond et al., 2018). Indeed, stereotypic horses did not show any differences in flexibility, cue dependency or routine formation compared to the controls during the learning tasks (Koolhaas et al., 2010). Therefore, more studies are needed in order to assess if crib-biters are characterised by a proactive or reactive coping strategy.

I conclude that further investigations are required to fully characterise the personality of stereotypic horses (e.g. to characterise them as reactive or proactive individuals). This could allow an early detection of individuals prone to develop stereotypies, and hence might help to prevent them to develop this abnormal behaviour.

DEVELOPMENT OF CRIB-BITING BEHAVIOUR – ONTOGENY

During my PhD, I performed a preliminary study investigating the development of stereotypic behaviour during weaning. Studies on crib-biting behaviour mostly focus on mature horses with an established history of performing the behaviour, and never on younger horses during earlier stages of the stereotypy, before the emancipation (Wickens and Heleski, 2010). Such method does not provide knowledge about the development of stereotypic behaviour in relation to cortisol release in stressful situation. Therefore, longitudinal studies are required to establish whether the development and later performance of crib-biting reduce cortisol levels from even higher original level and to determine if a transient peak in stress level occurs prior to the emergence of stereotypic behaviour (Pell and McGreevy, 1999; Clegg et al., 2008; Wickens and Heleski, 2010).

Weaning marks a very stressful event in a horse's life. The weaning of foals usually results in deprivation of maternal care, social isolation, and in a sudden transition in the source of nutrients. The stressful impact (short or long term) of separation from their mothers on foals, has been clearly demonstrated (Moons et al., 2005; Weary et al., 2008; Henry et al., 2012). It has also been shown that the mother-infant relationship is an important factor in the modification of the HPA axis (Kaufman et al., 2000; Moons et al., 2005). As consequence of this highly stressful period, weaning is closely associated with an increased incidence of stereotypic behaviour (Bachmann and Stauffacher, 2002). For example, it was shown that foals develop oral stereotypies in relation to frustrated suckling attempts (Nicol and Badnell-Waters, 2005).

In the preliminary study I conducted during my PhD, it was unfortunately not possible to run an advance statistical analysis, due to the limited sample size and the collinearity between breed and management conditions (Annex 1). Moreover, none of the foals started to crib-bite. A preliminary analysis of the data suggests that faecal cortisol metabolites decrease after weaning in the Franches-Montagnes breed (Figure 1, Annexe 1). It was however not the case for Swiss warmblood horses, for which faecal cortisol metabolite seem to decrease at an early stage followed by an increase (Figure 2, Annex 1). Such breed difference might be the consequence of the differences in management conditions between the two farms where the foals were weaned (Annex 1, Table 1).

CONSEQUENCES OF CRIB-BITING BEHAVIOUR – BRAIN DYSFUNCTION – LEARNING ALTERATION

One prominent hypothesis states that animals affected by stereotypies might be cognitively less flexible compared to healthy controls, due to sensitization of a specific brain area, the *basal ganglia mesoaccumbens* dopaminergic system, part of the *basal ganglia* (Spruijt et al., 2001). In fact, many studies found a link between stereotypies in animals or human and dopaminergic dysfunction (Frith and Done, 1983; Turner, 1997; Garner and Mason, 2002). As consequence, due to its primary role in allowing animals to learn about outcomes associated with objects, and to select the most appropriate motor response for survival (action selection)*,* dysfunctions of the *basal ganglia* should lead to alteration in learning performance (McBride and Parker, 2015). For example, perseveration, defined as inappropriate repeat responding, was shown to correlate with the intensity of stereotypy performance in schizophrenia (Frith and Done, 1983) and autism (Turner, 1997), in many different species (Garner and Mason, 2002; Garner et al., 2003; Vickery and Mason, 2005) and also in crib-biters (Hemmings et al., 2007; Parker et al., 2008; Parker et al., 2009; Roberts et al., 2015). To assess such brain dysfunction, I conducted two different studies presented in Chapter IV and V, using cognitive tests, as indirect measures of some aspect of *basal ganglia* dysfunction (McBride et al., 2017).

In Chapter IV (Briefer Freymond et al., in preparation), crib-biters and non-stereotypic horses were submitted to some challenging spatial learning tasks. The results did not show any differences in learning performance between crib-biters and control horses. I suggested that the different learning tasks even though frustrating were perhaps not challenging enough to allow me to find differences between the two groups. Indeed, all the tests involved spatial problems, with limited training, which may be ecologically more relevant for horses and easier to solve, compared to paradigms used in other studies, with long lasting training (Hemmings et al., 2007; Parker et al., 2008). Based on the results of this study, therefore, there is no evidence for a *basal ganglia* dysfunction. However, the validity of this study is reduced by the fact that all the tests were performed on one day potentially leading to proactive inferences between tasks. Such limitations need to be taken under consideration and these tests could be repeated independently in future studies. Additionally one test could be interesting to be repeated in future studies comparing crib-biters and non–stereotypic horses including some adaptations. This is the case of the *distractibility test* (Chapter IV of the PhD) which has not been previously looked at in the context of place/response learning. The horses could be trained, many times to go to a bucket with food and after overtraining, a distractor with food could be placed in the maze.

Different predictions could be made depending on the “*dopamine*” or “*basal ganglia*” models (Garner and Mason, 2002; McBride and Hemmings, 2005). The “*dopamine*” model will predict that crib-biters hypermotivated by food, will stop and eat the food and the “*basal ganglia”* model will predict habit formation and thus that crib-biters will go to the place to which they were overtrained to go.

Because spatial learning tasks seem not to be challenging enough, to see potential differences in learning performance between crib-biters and control horses, I then performed a similar study in Chapter V (Briefer Freymond et al., 2018), with a more challenging task and a better survey of the daily percentage of the crib-biting level of the subjects at the time of the experiment. In this study, the horses had to perform two reversal tasks (considered as indirect measures of one aspect of the *basal ganglia* dysfunction, and more accurately as a measure of flexibility of learning, (McBride et al., 2017)) using visual cues only. Unlike reversal learning tasks using spatial cues, which seem to be easy for horses, reversal learning tasks using visual cues have been shown to pose a challenge to this species (Martin et al., 2006; Brubaker and Udell, 2016). It was also the first time that crib-biters were compared to non-stereotypic horses in such a task. In this study the crib-biters were again not prevented to execute their stereotypic behaviour and some of them did crib-bite on a solid apparatus (Briefer Freymond et al., 2018). Interestingly, the results again did not reveal any performance differences across groups; both stereotypic and control animals required similar numbers of trials (Briefer Freymond et al., 2018). It can be suggested that the dopamine dysfunction shown in previous studies testing learning abilities of crib-biting horses (McBride and Hemmings, 2005; Hemmings et al., 2007; Parker et al., 2008) is not affecting the performance of crib-biters in reversal learning tests, indicating that, likely, this dysfunction does not impact on the learning flexibility of these horses. This finding gives rise to a new hypothesis suggesting that the *accumbens* reward pathway might be affected in crib-biters and not the *dorsal loop* as previously suggested, responsible for habit formation (Garner and Mason, 2002; Parker et al., 2009; Roberts et al., 2017). Such hypothesis is supported by the results of a recent study, Hemmings et al. (2018), suggesting an increased dopaminergic activity within the *ventral striatum* in crib-biters. Such a dysfunction could result in a hyper-motivated endophenotype (Hemmings et al., 2018). This could also be the cause of the higher stress sensitivity reported in Chapter II of the PhD (Briefer Freymond et al., 2015) and of the poor ability of crib-biters to differentiate reinforcer value (Parker et al., 2008) and would have no impact on reversal learning tasks. Such higher stress sensitivity could also explain why crib-biters needed more time than controls to be acclimated and pre-trained in Chapter V of the PhD (Briefer Freymond et al., 2018).

To conclude, the results of the Chapter V, challenge the hypothesis that crib-biting horses might have a dysfunction in the *dorsal loop* of the *basal ganglia* and therefore might be more prone for habit formation and let suggest that crib-biters might have a dysfunction in one specific part, the *ventral striatum.* Such dysfunction might explain the fact that crib-biters showed a hypersensitivity to stress (Briefer Freymond et al., 2015) and needed more time than controls to be acclimated and pre-trained (Briefer Freymond et al., 2018).

LINK TO WELFARE

According to Mason and Latham (2004), a stereotypy could be classified as adaptive if it serves a coping function and maladaptive or pathological if it is caused by a dysfunction of the nervous system. It could also be suggested that stereotypies could be both. Indeed, such abnormal behaviour could develop as a result of chronic stress frustration, be maintained by proximal reinforcement and become a habit, thus indicating alteration in the nervous system. Mason and Latham (2004) propose that a stereotypy correlates with bad welfare when behaviours have changed in control and have become environmentally insensitive. At this developmental stage, stereotypies are performed in a more diverse set of situations and are harder to interrupt. However, as discussed earlier (see: Section *Function of stereotypic behaviour*) such stage is not easy to assess because the expression of stereotypies may vary between individuals, in terms of percentage of time occupied by the activity, of vigour and of persistence of the behaviour. Mason and Latham (2004) argue that perseverative responding or more generally the switch in behavioural control from actions to habits (see: Section Function of crib-biting behaviour *Consequences of crib-biting behaviour - Brain dysfunction - learning alteration*) could be an indirect indicator of this developmental stage. In fact, with such dysfunction, individuals may produce unnecessary and inappropriate response to environmental cues, i.e. behaviours become environmentally incentive. In human, this switch in behavioural control from actions to habits is a symptom of disorders, like schizophrenia, autism, addiction and various clinical disorders resulting from *basal ganglia* abnormalities (Yin and Knowlton, 2006).

Therefore, if neurobiological changes are present in stereotypic behaviours and alter the learning profile of animals, we could presume that stereotypic behaviour correlates with bad welfare. Moreover, the review by Mason and Latham (2004) suggests assessing the degree of perseveration (i.e. alteration of learning capacity) of animals to identify whether stereotypies could be considered as good or bad welfare. To this point, the results described in Chapter V challenge the hypothesis that crib-biters are not able to perform reversal learning tasks, an indirect behavioural measures for flexibility in learning. Together with the results of previous studies (McBride and Hemmings, 2005; Hemmings et al., 2007; Parker et al., 2008), it can be suggested that crib-biters are impaired in one specific part of the *basal ganglia*, the *ventral* part or *nucleus accumbens.* By the way, the results of Chapter II and IV may suggest, despite limitations in the statistical analysis (see: Section *Function of crib-biting behaviour*) that crib-biting is an adaptive strategy, since in both studies, the horses might be less stressed when they did crib-bite than when they did not crib-bite.

To conclude, crib-biting might be adaptive helping horses to reduce stress and might not reduce flexibility of learning as suggested in previous studies (Garner and Mason, 2002; Parker et al., 2009; Roberts et al., 2017). However, based on previous studies (McBride and Hemmings, 2005; Hemmings et al., 2007; Parker et al., 2008) it can be suggested that crib-biters are impaired in the *ventral part* of the *basal ganglia* and could thus be suggested as being more sensitive to stress than non-stereotypic horses. To this aim, this specific stereotypy could be classified as adaptive with a dysfunction. Yet, as stated in Mason and Latham (2004) and as recently suggested by Hemmings et al. (2018), it is important to avoid generalisation. Indeed, stereotypies may have different underlying causes, resp. neurological basis and mechanisms and therefore might only be rarely comparable between species and sometimes even within the same species.

GUIDELINE ON HOW TO CURE OR TO REDUCE CRIB-BITING FREQUENCY IN HORSES

To close the general discussion, I will propose a guideline on how to cure crib-biting or at least reduce the frequency of performance of the behaviour over time, based on the results of my thesis. Many horse owners try to prevent or suppress their horses' crib-biting behaviour with coercive measures. However, if crib-biting is a coping strategy, as discussed earlier (i.e. Section *Function of stereotypic behaviour*), it might be better to let the horses cope and to improve their environment. In fact, preventing stereotypic horses from crib-biting, without reducing the underlying motivation, could be counterproductive.

In Mason and Rushen (2006), for example, some treatment recommendations for compulsive behaviour in dogs and cats are outlined, which could be adapted for other animals. The main principles are to identify and remove the cause of conflict that triggers the stereotypic behaviour. This could be done even before the emancipation of the stereotypic behaviour. Another principle is to reduce the cause of stress within the environment. This could be done by improving the domesticated animal's environment. As a reminder, chronic stress occurs when a situation exceeds the natural regulatory capacity of the organism for an extended period, particularly in situations that include unpredictability and uncontrollability (Koolhaas et al., 2010). Therefore, finding ways to increase the animal’s feeling of having some control over their environment or to make their environment more predictable could help to improve their welfare (Wemelsfelder, 1993). Many studies have worked on different kinds of 'enrichment' for captive animals such as ungulates, carnivores, primates and other species. These 'enrichments' include mimicking nature, increasing complexity of the environment, increasing sensory stimulation, removing sources of stress or providing coping options such as appropriate hiding places (Mandel et al., 2016). Studies investigating 'enrichment' of captive animals’ housing systems compared the percentage of time occupied by stereotyping before and after enrichment. The conclusion is that zoo 'enrichment' works quite well (Hutchins et al., 1984). Among carnivores, primates and other species, a 50-60% reduction in stereotypic behaviour was observed following the 'enrichment' programme, although stereotypies were not completely abolished (Mason and Rushen, 2006).

Many studies in welfare science aim to improve the housing conditions of captive horses, by providing them with an 'enrichment' in which they are able to display more natural behaviours. Such more natural housing conditions generally reduce incidences of crib-biting. For example, increasing feeding time is a form of environmental 'enrichment' that mimics a more natural setting. Such enrichment has been shown to effectively reduce frustration, stereotypic as well as other abnormal behaviours (Rochais et al., 2018). To improve horses’ welfare, another possibility could be to house them in groups (Hartmann et al., 2012), which has been shown to reduce their physiological stress levels (Yarnell et al., 2015). Alternatively, housing systems that allow horses to have close contact with their neighbours (through grids for example), can increase their possibilities of interacting with each other and hence also increase welfare (Benhajali et al., 2014). The opportunity to move freely is another important horse ethological need that is not fulfilled in captivity. Housing horses in paddocks results in animals that are less aggressive towards humans (Rivera et al., 2002) and that develop less stereotypies or abnormal behaviours compared to box housing (Chaya et al., 2006). Another possibility to give horses more control over their environment and to satisfy their locomotion needs is to house them in boxes with a small outdoor area. Such housing systems enable for instance horses to choose if they want to stay inside their stable or outside. However, to date, no studies on such housing system in relation to the evolution of stereotypies have been conduct. Furthermore, more longitudinal studies are needed to test the effect of these different 'enrichment' possibilities on the development and evolution of stereotypies in horses.

To conclude this part, improving horses’ welfare using 'enrichment' could help to reduce sources of chronic thwarting that initially give rise to stereotypies. Consequently, this could reduce sources of frustration and help to reduce the frequency of stereotypic behaviour, as shown in studies on other species in captivity (Mason and Rushen, 2006). Such 'enrichment' could improve the welfare of stereotypic animals, but also of the other horses. Indeed, as stated Mason and Latham (2004) a lack of stereotypy does not necessary imply good welfare or even low stress. This is crucial because, even if crib-biting is a coping strategy, as long as the sources of chronic thwarting that initially give rise to stereotypy have not been removed, stereotypic animals may continue to suffer (Mason and Latham, 2004). Additionally, 'enrichment' leads to further positive effects, like for example reducing negative emotional reactions and increasing exploratory behaviour with novel objects and places (Renner and Rosenzweig, 1987).

FINAL CONCLUSION

Stereotypies exist in humans as well as in captive animals. There is a lack of understanding of the function of these behaviours. In fact, stereotypies are defined as having no apparent goal or function. They are known to develop in captive animals in order to compensate for the restrictive environment imposed. Animal welfare being of increasing public and scientific concern, it is of relevance to assess whether or not stereotypic behaviour has an adaptive function such as a coping strategy and/or if it is a malfunction or maladaptation, and to assess the consequences of such behaviour, such as learning alterations.

Based on the results obtained in the course of my PhD project, it could be suggested that stereotypies in horses, or at least crib-biting behaviour, may have an adaptive coping function that consists in helping the horses to reduce physiological stress. This hypothesis was supported by the results described in Chapter II (Briefer Freymond et al., 2015) and also in Chapter IV (Briefer Freymond et al., in preparation). However, due to the statistical limitations of these results it will be necessary to replicate these tests in a more controlled manner in order to investigate the coping hypothesis. Based on the results of Chapter IV and V (Briefer Freymond et al., in preparation and Briefer Freymond et al., 2018), in which cognitive tests were used as diagnosis for some aspects of *basal ganglia* dysfunction, I could not concluded to learning alterations in crib-biters even in reversal learning tests. However, together with previous studies testing learning abilities of crib-biting horses (McBride and Hemmings, 2005; Hemmings et al., 2007; Parker et al., 2008), this finding gives rise to a new hypothesis suggesting that crib-biters might suffer from a dysfunction located in one specific part of the *basal ganglia,* the *ventral part* and not in the *dorsal loop* as previously suggested (Garner and Mason, 2002; Parker et al., 2009; Roberts et al., 2017). Such a dysfunction could be the cause of the higher stress sensitivity reported in Chapter II of the PhD (Briefer Freymond et al., 2015) and of the poor ability of crib-biters to differentiate reinforcer value (Parker et al., 2008) and would have no impact on reversal learning tasks. However, large differences exist among stereotypic horses and future research should differentiate forms and levels of performance over time of repetitive behaviours in order to examine the underlying neurophysiological mechanisms. Additionally, my results give some indications on how to improve the housing conditions of stereotypic horses. The conclusion drawn is that it may be more useful to remove the sources of chronic thwarting that initially give rise to the stereotypy rather than to prevent horses from crib-biting. This can be done by improving the captive environment, mimicking nature, increasing feeding time or giving horses some kind of control over their environment. This should be done for all horses, in order to prevent the development of stereotypies or to cure stereotypies, as well as to improve the welfare of horses that do not crib-bite, but are housed in the same environment as crib-biters. This is crucial, since the absence of stereotypies does not necessarily indicate good welfare. Similarly, the presence of stereotypies does not necessarily indicate poor welfare, since they could have developed in a another environment, in which the horse was housed previously, and be performed, once fully blown, even in the absence of the original source of stress (Mason and Latham, 2004).

This thesis gives also some promising results regarding what predisposes individuals to develop stereotypy. Some individuals seem to have a higher susceptibility to develop stereotypies than others, despite being exposed to similar environments (Ijichi et al., 2013), although the genes involved remain unknown. My results highlighted two phenotypes that could be explored. The first one is a higher sensitivity to stress (Briefer Freymond et al., 2015). The second one, seems to be a higher tactile sensitivity that could due to altered dopamine physiology, resulting from chronic stress exposition (Briefer Freymond et al., in preparation, Chapter III of the PhD).

However, the question of the ontogeny of stereotypies remains elusive and further studies are needed to assess the development of stereotypic behaviour in relation to cortisol release in stressful situations. To answer this question, longitudinal studies are required, in order to establish whether the development and later performance of crib-biting behaviour results in a reduction of cortisol levels from even higher original levels.

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ACKNOWLEDGMENTS

It is now time to thank all the people who helped me through this PhD.

En premier lieu je dois remercier Iris Bachmann et Stefan Rieder, qui ont tout fait pour que je puisse réaliser ma thèse en cours d'emploi au Haras national suisse, Agroscope, ainsi que Klaus Zuberbühler et Redouan Bshari, qui ont accepté de superviser un travail sur les chevaux et Matthew Parker, qui m'a aidé lors du design des études sur l'apprentissage et qui a accepté d'être mon expert externe!

Ensuite je remercie ma sœur chérie, qui m'a tout d'abord donné goût à la recherche en me proposant il y a quelques années d'écrire un article sur les étalons en groupe, puis qui a toujours été là pour répondre avec professionnalisme à mes nombreuses questions et qui a été d'accord de relire et corriger ma thèse.

Un grand merci également à toutes mes collègues qui ont pris sur elles, car elles ont dû prendre beaucoup de travail à leur charge, pour me libérer et pour que je puisse mener à bien mes études. Je cite avant tout Anja et Christa. Merci également à toute mes autres collègues pour être aussi adorables et avoir répondu également à mes questions: Anne-laure (pour avoir relu une partie de mon Phd), Christelle (pour ces magnifiques photos), Annick et Markus (pour avoir répondu à mes questions de génétique), Sabrina, Clara, à la clinique vétérinaire et au personnel du haras (dont Jean-Philippe, qui m'a aidé à fabriquer la boîte pour la dernière étude).

Je remercie ensuite toutes ces stagiaires tellement extraordinaires qui m'ont accompagné sur le terrain, Alice, Déborah, Maurine, Sandrine, Livia, Camille, Julia, Margaux… ainsi que les propriétaires de chevaux qui m'ont fait confiance et ont toujours répondu positivement à chaque nouvelle étude et à leur chevaux formidables.

Je remercie ensuite mes amies et amis qui m'ont permis de décompresser Sandra, Nicole, Sonia, Franziska, Laurence, Vanessa, Jean-Bernard, Hélène, Audré…

Quand à ma famille, je remercie ma fille Alexia qui est si adorable et emphatique, mon fils Kylian qui m'a donné toute cette inspiration dans mon ventre pour écrire mon doctorat, mon mari avec ses idées de génie, qui m'a fabriqué cette magnifique boîte pour ma dernière étude, Roi qui pose toujours tant de questions intéressantes, et sans oublier tous ces animaux qui m'accompagnent tous les jours et m'aident à décompresser, je cite : mes fidèles Mimi et Kira, mon nouveau fidèle Torent, mes chats petits et grands et mes chevaux pensionnaires qui sont si gentils.

ANNEX I

METHODS

Subject and management conditions

The study was carried out between October and December 2014 on 4 different places where foals are weaned in Switzerland (i.e. Sevaz, Nuvilly, Ponthaux, Chandon). In Ponthaux, 5 males and 2 females of different breeds and born between April and June 2014 were weaned in a loose housing system (Table 1). In Chandon, 2 Swiss warmblood females (born between April and Mai at this place) were weaned in a box next to their mother (Table 1). In Nuvilly, 7 females and 8 males, all Franches-Montagnes (born between January and April at this place), were weaned, in a loose housing system (Table 1). In Sevaz, 3 males and 2 females, all Swiss warmblood (born between April and July at this place) knowing each other were weaned in a box (Table 1). Routine care of the study animals was provided by the farm/horse owners. The study was approved by the Federal Veterinary Office (approval number VD 2899; Switzerland).

Table 1. Characteristics of the horses used in the experiment. Sex (M = mare; S = stallion), age (months: 1 for January), breed, housing system (loose housing, box) and place (each letter refers to a given farm).

horses	sex	month	breed	housing system	place
1	M	2	Franches-Montagnes	loose housing	w
2	S	3	Franches-Montagnes	loose housing	w
3	S	2	Franches-Montagnes	loose housing	w
4	S	3	Franches-Montagnes	loose housing	w
5	M	3	Franches-Montagnes	loose housing	w
6	S	1	Franches-Montagnes	loose housing	w
7	S	4	Franches-Montagnes	loose housing	w
8	S	4	Franches-Montagnes	loose housing	w
9	M	2	Franches-Montagnes	loose housing	w
10	M	4	Franches-Montagnes	loose housing	w
11	M	4	Franches-Montagnes	loose housing	w
12	M	2	Franches-Montagnes	loose housing	w
13	M	4	Franches-Montagnes	loose housing	w
14	S	2	Franches-Montagnes	loose housing	w
15	S	1	Franches-Montagnes	loose housing	w
16	M	4	Swiss warmblood	box	m
17	M	4	Swiss warmblood	box	m
18	M	4	Swiss warmblood	loose housing	p
19	S	5	Quarter horse	loose housing	p
20	S	5	Swiss warmblood	loose housing	p
21	S	6	Swiss warmblood	loose housing	p
22	S	4	Arabian	loose housing	p
23	S	4	Swiss warmblood	loose housing	p
24	S	5	Swiss warmblood	box	s
25	M	4	Swiss warmblood	box	s
26	S	4	Swiss warmblood	box	s
27	S	7	Swiss warmblood	box	s
28	M	5	Swiss warmblood	box	s

Experimental procedure

Faecal sample collection

Faecal cortisol metabolites (FCM) are non-invasive indicators of adrenocortical activity (Mostl et al., 1999). For this reason, I collected faecal samples when the foals were at home with their mother, two times in the morning, in order to establish the baseline value. The baseline value ("Base") was then calculated as the average measure for these two days. Then, I collected fresh faeces during seven days after weaning, every morning. I also collected samples on the tenth day after weaning, two weeks later, three weeks later and finally, four weeks after weaning (Figure 1). I used the method described in Malmkvist (2014), which found that non-invasive measurement of fecal cortisol metabolites can be used to estimate stress in 6-8 months old foals. This study also found that foals returned to their pre-weaning baseline concentration of cortisol after ten days post-weaning. After collection, the samples were frozen at -20 °C (Christensen et al., 2012). They were then sent to a lab in Vienna (Palme). The samples were weighted (0.5g) and analyzed there as described in Mostl et al. (Mostl et al., 1999) using an 11-oxoaetiocholanolone enzyme immunoassay validated for horses.

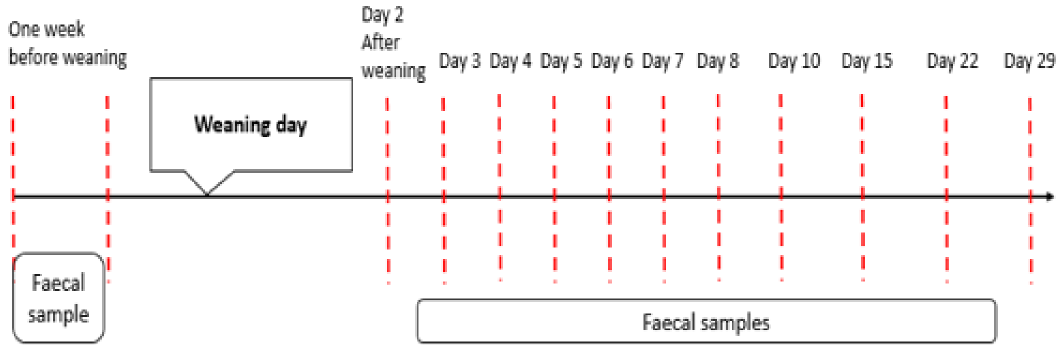

Figure 1. Experimental procedure for the part 4 of the PhD. The red dotted lines designate when the faecal samples were collected.

Perspective

The effect of the age at weaning time and of the management of the place, etc. on the physiology and behavior of the foals were analysed, in order to find the parameters that were more stressful for the foals during this weaning time.

Statistical analysis

I analysed the variation of the faecal cortisol metabolites over the days after weaning using linear mixed-effect models (LMMs) in R 3.0.2. The LMMs included the difference in faecal cortisol metabolites ("FCM") after weaning with the baseline value ("Base") as response variable. The fixed factors were the days after weaning ("Day"). The control factors were the breed, the sex, the age of the foal at weaning day and if the foals could go or not in an outside paddock after weaning (Table 1). To control for repeated measurements of the same subjects, the identity of the horses nested within the farms ("Farms") where they were housed was included as a random factors. When significant interaction effects between fixed and/or control factors were found, further post-hoc analysis were carried out using LMMs; these models included the same fixed and random factors as in the model. Bonferroni correction was then applied.

For the LMMs I used a standard model simplification procedure by removing each non-significant term, until the deletion caused a reduction in goodness of fit (at which point, the term was left in the model). The significance level of the factors was set at α = 0.05 for all models.

RESULTS

There was an interaction effect between breed and day after weaning ("Day") on the difference in faecal cortisol metabolite ("FCM") (LMM: interaction effect between breed and Day on FCM; LMM: $F_{3,264}$= 9.027, p < 0.0001). Further post hoc comparison per breed showed that Day had an effect on FCM in Franches-Montagnes (LMM: Effect of Day on FCM; LMM: $F_{1,141}$= 35.18, p < 0.0004;Figure 2). However, Day had no effect on FCM for the Swiss warmblood (LMM: Effect of Day on FCM; LMM: $F_{1,106}$= 7.53, p = 0.97; Figure 3).

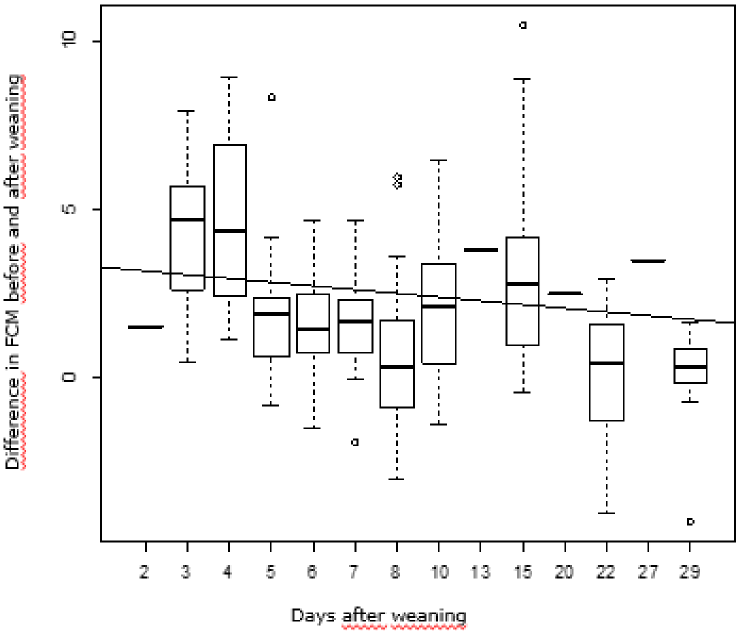

Figure 2. Faecal cortisol metabolites after weaning for Franches-Montagnes breed. Difference in faecal cortisol metabolites ("FCM") before and after weaning for each days after weaning.

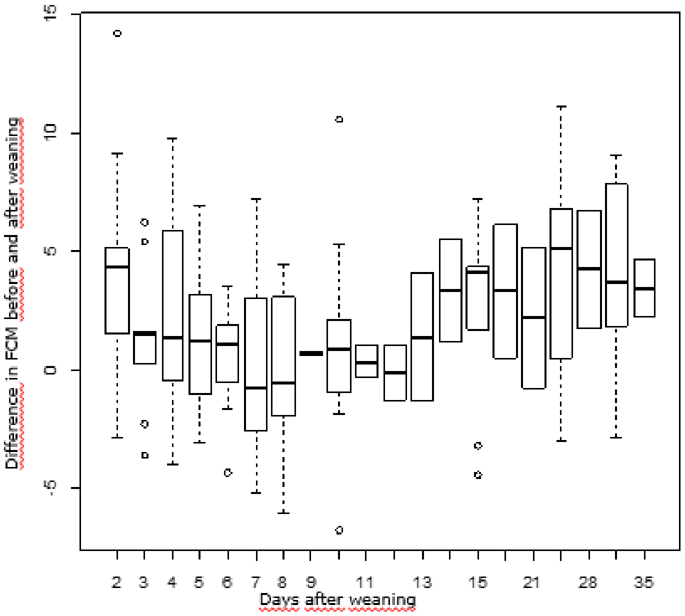

Figure 3. Faecal cortisol metabolites after weaning for Swiss warmblood. Difference in faecal cortisol metabolites ("FCM") before and after weaning for each days after weaning.

ANNEX II

Table 1. Characteristics of the horses used in the experiment Chapter II-V. Sex (M = mare; G = gelding, S = stallion), Group (CB = crib-biters; C= controls), age, breed, housing system (loose housing, paddock, box; alone or in group) and place (each letter refers to a given farm). The horses tested only in Chapter II of the PhD and not in Chapter III, IV and V of the PhD are represented in grey. The horses also tested in Chapter V are represented in red (the horse n°13 was tested in Chapter II, and V only, the horse n°44 was not tested in Chapter II and the horse n°45, was tested in Chapter V only).

horses	sex	Age (years)	Group	breed	housing system	alone / group	place
1	M	2000	CB	Shetland	loose housing	group	u
2	M	2007	CB	Warmblood	box paddock	alone	c
3	M	1991	CB	Criollo	box	alone	g
4	M	1997	CB	Franches-Montagnes	box	alone	y
5	M	2004	CB	Hispano-Arabian	box paddock	alone	b
6	M	2008	CB	Quarter horse	box	alone	s
7	M	2004	CB	Paint horse	box	alone	r
8	M	2008	CB	Paint horse	box paddock	alone	k
9	G	2004	CB	Franches-Montagnes	box	alone	d
10	G	2002	CB	Warmblood	box	alone	g
11	G	1990	CB	Franches-Montagnes	box paddock	group	n
12	G	2002	CB	Franches-Montagnes	box	alone	bo
13	S	2005	CB	Franches-Montagnes	box	alone	h
14	S	1996	CB	Franches-Montagnes	box	alone	h
15	S	1998	CB	Franches-Montagnes	box	alone	h
16	M	2008	CB	Franches-Montagnes	box paddock	group	m
17	M	1994	CB	Warmblood	box paddock	alone	w
18	G	1994	CB	Haflinger	box paddock	group	se
19	G	1995	CB	Warmblood	box	alone	a
20	G	2006	CB	Unknown origin	box paddock	alone	v
21	G	2003	CB	English thoroughbred	paddock	group	d
22	S	2002	CB	Franches-Montagnes	box	alone	h
23	M	2006	C	Quarter horse	box paddock	alone	s
24	M	1993	C	Franches-Montagnes	box	alone	y

25	M	1999	C	Warmblood	loose housing	group	h
26	M	1995	C	Apaloosa	box paddock	alone	b
27	M	1999	C	Warmblood	loose housing	group	h
28	M	1997	C	Trotter	box	alone	h
29	M	1995	C	Franches-Montagnes	loose housing	group	h
30	M	2003	C	Warmblood	box	alone	g
31	M	1994	C	Warmblood	box paddock	alone	w
32	G	2009	C	Friso-Arabian	box paddock	group	n
33	G	1989	C	Unknown origin	box paddock	alone	v
34	G	1991	C	English thoroughbred	paddock	group	d
35	G	2006	C	Quarter horse	loose housing	group	k
36	G	2007	C	Franches-Montagnes	box paddock	alone	di
37	G	2005	C	Franches-Montagnes	box	alone	d
38	G	1998	C	Warmblood	loose housing	group	h
39	G	2002	C	Warmblood	box	alone	h
40	G	2001	C	Frison	box paddock	alone	se
41	S	2010	C	Shetland	loose housing	group	u
42	S	1996	C	Franches-Montagnes	box	alone	h
43	S	2006	C	Franches-Montagnes	box	alone	h
44	M	2000	C	Camargue	loose housing	group	b
45	G	2001	C	Franches-Montagnes	Box paddock	alone	mo

ANNEX III

Table 3. Means and standard deviations for the parameters recorded in Chapter III and behaviours (scored from the video), Cortisol, HR and RMSSD recorded in Chapter IV for crib-biters and control horses, for which there were no differences between the groups. Group (CB=crib-biters, C=controls), Tests (personality, resp. learning tests of Chapter III and IV of the PhD), parameters abbreviations (See: Table 2, Chapter III and Table 3, Chapter IV of the PhD).

Chapters	**Tests**	**Parameters abbreviation**	**Group**	***N***	**Mean/ Median**	**SD**
Chapter III	**Test 1**	**Conth**	C	18	17.13	21.20
			CB	19	33.35	35.41
		Att	C	18	45.80	27.11
			CB	19	33.45	30.36
		Cat1	C	18	41.36	34.15
			CB	19	55.56	43.27
		Cat2	C	18	58.64	34.15
			CB	19	44.44	43.27
		Mov	C	18	2.74	2.35
			CB	19	5.11	13.95
	Test 3	**Conto**	C	18	19.81	19.79
			CB	19	17.55	18.61
		Att	C	18	33.76	16.85
			CB	19	39.27	24.36
		Cat1	C	18	26.85	25.01
			CB	19	25.44	24.03
		Cat2	C	18	73.15	25.01
			CB	19	74.27	24.09
	Test4	**Mov**	C	18	14.86	22.32
			CB	19	14.22	24.28
		Att	C	18	72.34	31.24

			CB	19	73.15	30.59
		Whin	C	18	1.85	6.63
			CB	19	5.14	11.94
	Test 5	**Time**	C	18	42.59	54.26
			CB	19	16.78	13.81
		Flight	C	16	2.75	1.13
			CB	19	2.22	1.00
	Locom	**Locom**	C	16	8.93	6.56
			CB	19	12.16	23.22
Chapter IV	**Test 1**	**Frustr**	C	17	0.27	0.42
			CB	19	0.37	0.90
		Att	C	17	19.87	19.64
			CB	19	21.75	21.79
	Test 2	**Att**	C	17	6.19	9.11
			CB	19	6.33	5.95
	Test 3	**Frustr**	C	17	1.60	3.53
			CB	19	0.99	1.90
		Att	C	17	12.65	14.65
			CB	19	12.39	17.45
	Test 6	**Frustr**	C	17	3.22	2.71
			CB	19	2.40	3.23
		Att	C	17	17.11	11.54
			CB	19	17.49	11.96
	Part 1	**Cortisol ng/ml**	C	17	0.60	0.20
			CB	19	0.64	0.19
	Part 2	**Cortisol ng/ml**	C	17	0.58	0.19
			CB	19	0.57	0.19
	Part 1 and Part 2	**HR BPM**	C	17	42.10	8.92
			CB	19	39.40	6.33
	Part 1 and Part 2	**RMSSD ms**	C	17	44.80	22.39
			CB	19	42.03	16.82

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