UNIVERSITÀ DEGLI STUDI DI MILANO

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# INDICATORS TO ASSESS EQUIDS WELFARE ON FARM AND DURING TRANSPORT

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PhD Candidate: **Francesca Dai**
R11873

Tutor: **Prof Michela Minero**
Co-tutor: **Prof Barbara Padalino**
PhD Coordinator: **Prof Valeria Grieco**

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*The universe we observe*
*has precisely the properties we should expect*
*if there is, at bottom, no design, no purpose,*
*no evil and no good,*
*nothing but blind, pitiless indifference.*

*(Richard Dawkins, 1995)*

# Contents

# Abstract

Research on equids demonstrated how routine on-farm procedures, resources availability, facilities design, sport and work activities, and transport present several risk factors potentially hampering horses and donkeys welfare. To guarantee horses and donkeys welfare, the first step is to evaluate it in a reliable way, using scientifically sound indicators and assessment protocols to be applied on-farm and during transport. To sustain an effective change towards more welfare friendly practices, it is necessary to propose modifications which are both scientifically proved to be effective and acceptable by the stakeholders. This thesis aimed to experiment solutions to enhance equids welfare both on farm and during transport. Guidelines to improve dairy donkey management and welfare have been developed, taking into consideration the existing scientific literature and stakeholders involvement. The guidelines, translated in different languages and freely available online, have been officially presented at the European Parliament, and represent the first step for a targeted dissemination of information about appropriate management procedures for dairy donkeys, to assist donkey farmers in preventing welfare problems. Then the focus has been posed on effective training of new welfare assessors. The HGS (a tool for pain assessment in horses) has been chosen as a pilot indicator to evaluate the efficacy of the training method, since on-farm pain assessment represents a continuous challenge for owners and veterinarians. Then, an infrequent horse management system, on pasture group housing, has been evaluated from an animal welfare point of view, since this management system is anecdotally considered better than single-box housing for horse welfare. Finally, the research work focussed on transport procedures, investigating the effects of training in reducing transport-related stress in horses and donkeys kept for meat production, using behavioural and physiological indicators. As for horses, a positive reinforcement-based training has been applied to teach foals to self-load and its efficacy has been evaluated. Such a training can be effective in reducing stress related behaviours during loading, but may not alleviate the overall impact of preslaughter transportation. As for donkeys, the impact of habituation to transport procedures in reducing transport-related stress has been analysed, revealing that habituation could mitigate stress during loading, but may be not effective in reducing stress related to travel and unloading. Moreover, a new physiological indicator of stress (Chromogranin A) was used for the first time to evaluate transport-related stress in donkeys. In conclusion, the thread of this thesis is the performance of evidence based research to be directly applied to the development of possible solutions to improve equids welfare on farm and during transport. As such, it tackles the design of animal management guidelines, the development of effective welfare assessment training programs, the evaluation of the impact of specific management systems and the development of training strategies to limit transport stress.

# Sintesi

La ricerca sugli equidi ha dimostrato come le procedure aziendali di routine, la disponibilità di risorse, la progettazione delle strutture, le attività sportive e lavorative, e il trasporto presentino diversi fattori di rischio per il benessere di cavalli e asini. Per garantire il loro benessere, il primo passo è una valutazione affidabile, utilizzando indicatori e protocolli scientificamente validi da applicare in azienda e durante il trasporto. Per sostenere il cambiamento verso pratiche più rispettose del benessere animale, è necessario proporre modifiche che siano sia efficaci, come dimostrato scientificamente, sia accettabili per gli *stakeholders*. Questa tesi ha lo scopo di valutare soluzioni per migliorare il benessere degli equidi sia in allevamento che durante il trasporto. Sono state sviluppate delle linee guida per migliorare la gestione e il benessere degli asini da latte, prendendo in considerazione la letteratura scientifica esistente e il coinvolgendo gli *stakeholders*. Le linee guida, tradotte in diverse lingue e liberamente consultabili online, sono state ufficialmente presentate al Parlamento Europeo, e rappresentano il primo passo per una diffusione mirata di informazioni in merito alle procedure gestionali più appropriate per gli asini da latte, con lo scopo di aiutare gli allevatori nella prevenzione dei problemi di benessere. Quindi l'attenzione è stata posta sulla formazione di nuovi valutatori. L'HGS (uno strumento per la valutazione del dolore nei cavalli) è stato scelto come indicatore pilota per valutare l'efficacia del metodo di formazione, poiché la valutazione del dolore in azienda rappresenta una sfida continua per proprietari e veterinari. In seguito, è stato valutato da un punto di vista di benessere animale un sistema di gestione del cavallo poco diffuso, la stabulazione in gruppo al pascolo, poiché questo sistema è considerato aneddoticamente migliore per il benessere del cavallo rispetto alla stabulazione in box singoli. Infine, il lavoro di ricerca si è concentrato sulle procedure di trasporto, indagando gli effetti dell'addestramento nella riduzione dello stress correlato al trasporto nei cavalli e negli asini allevati per la produzione di carne, utilizzando indicatori comportamentali e fisiologici. Per quanto riguarda i cavalli, è stata valutata l’efficacia di un addestramento basato sul rinforzo positivo per insegnare ai puledri a salire autonomamente sul trailer. Tale addestramento può essere efficace nel ridurre i comportamenti legati allo stress durante il carico, ma potrebbe non alleviare l'impatto complessivo del trasporto pre-macellazione. Per quanto riguarda gli asini, è stato analizzato l'impatto dell’abituazione alle procedure di trasporto nel ridurre lo stress, rivelando che l'abituazione potrebbe mitigare lo stress durante il carico, ma potrebbe non essere efficace nel ridurre lo stress legato al viaggio e allo scarico. Inoltre, per la prima volta è stato utilizzato un nuovo indicatore fisiologico (Cromogranina A) per valutare lo stress correlato al trasporto negli asini. In conclusione, il filo conduttore di questa tesi è lo svolgimento di ricerche basate sulle evidenze per sviluppare possibili soluzioni per migliorare il benessere degli equidi in allevamento e durante il trasporto. In quanto tale, affronta la progettazione di linee guida per la gestione degli animali, lo sviluppo di efficaci programmi di formazione sulla

valutazione del benessere, la valutazione dell'impatto di specifici sistemi di gestione e lo sviluppo di strategie di addestramento per limitare lo stress da trasporto.

# 1. Introduction

Animal welfare can be defined as “a state of complete mental and physical health in which the animal is in harmony with its environment” (1). Since the publication of the Brambell Report in 1965, public awareness and concerns about animal welfare has been constantly growing, mainly for ethical reasons (2). Consumers are increasingly interested in the way animals are treated in the production chain, so that certification schemes, aiming at guaranteeing animal welfare, have been developed in many Countries (3). Nowadays, poor animal welfare is considered to be the third most important cause for unsustainable livestock production (4).

A reliable assessment, able to determine the actual welfare of animals, constitutes the first step to improve animal welfare on farm, providing information regarding the existing weak points of the analysed farming system. Since animal welfare includes both physical and mental states (5), animal welfare assessment requires a multi-dimensional approach (6). In the last decades, scientists put efforts to the development of reliable and feasible animal welfare assessment protocols, composed by scientifically sound indicators. In 2008 the European founded project Welfare Quality® defined four welfare Principles, divided into twelve different Criteria (7,8) (figure 1).

Risk factors for animal welfare have been defined as “any aspect of the environment of the animals in relation to housing and management, animal genetic selection, transport and slaughter, which may have the potential to impair their welfare” (5). Animals can be exposed to several risk factors for their welfare and each factor may contribute to a variety of consequences (5).

When evaluating animal welfare, it is possible to assess the environment where the animal live (resource-based indicators; i.e. food, water, lying space), the on-farm management practices (management-based indicators; i.e. vaccination protocol, milking scheme, farrier visits calendar) or the animal itself (animal-based indicators; e.g. physiological and behavioural parameters) (5). While the use of resource-based and management-based indicators is attractive, since their measurement is generally quick, easy and reliable (9), the use of animal-based indicators should be preferred. These measures, indeed, “are linked to welfare-related outcomes and they can be considered as a form of toolbox from which to select the range of measures necessary to address the specific objectives of the assessment for that particular species and category of animal at that time” (5). Animal-based indicators refer to the animal itself rather than the environment, therefore their use allows to assess animals in different housing conditions; resource-based indicators and management-based indicators may be used to highlight risk factors related to the observed welfare conditions (5).

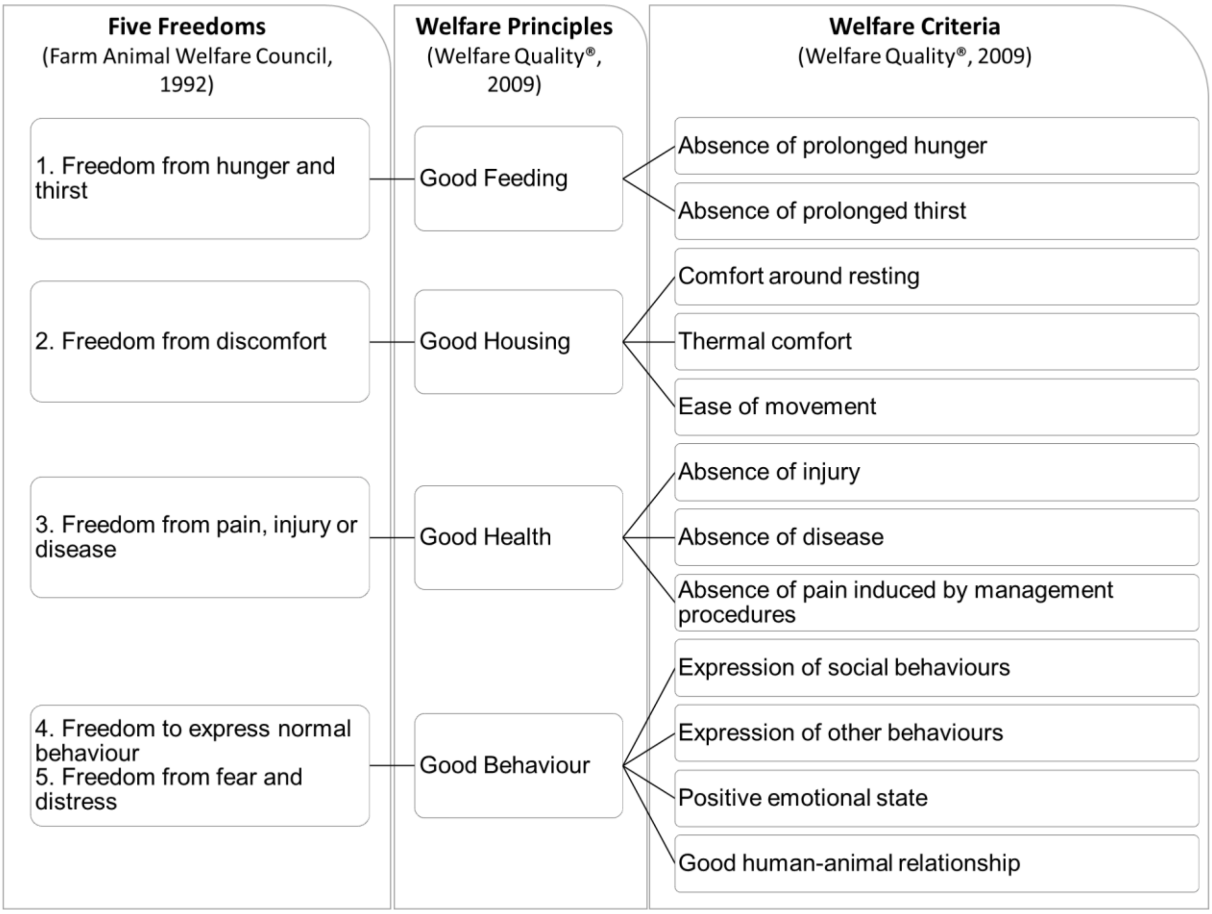


**Figure 1.** Animal welfare Principles and Criteria formulated by Welfare Quality®, and their link with the Five Freedoms proposed by the Farm Animal Welfare Council (modified from (10))

Taking into consideration the result of the assessment and the identified risk factors, a second, fundamental, step to improve animal welfare, is to apply changes in management or resources, in order to better meet the animal needs. The final goal is to observe constant improvement in animal welfare (11). To do so, the focus must be at the people involved in animal farming, and the process should include motivating people to implement changes to their systems, management and daily routines (11). To perform substantial and positive changes, people cannot only be forced by legislation or financial penalties, they need to be truly confident in the necessity of changes they are adopting (11,12).

## 1.1. Equids industry

To better understand the impact that welfare assessment and subsequent welfare improvement in equids may have internationally, a brief portrait of the equids industry may be useful.

World equids population is estimated in more than 71 million of heads, including horses, donkeys and mules (13). Europe alone hosts more than 5 million of horses, almost 400 thousand of donkeys and about 164 thousand of mules (13). In Italy, registered equids are 479,250, including 376,676 horses, 94,908 donkeys, 7,495 mules and even 171 zebras and zebra-hybrids (14).

The estimated annual economic impact of the equids industry in Europe is around 100 billion euros; for example, horse sector provides employment to at least 896.000 people in Europe (15) and occupies 6 million hectares of land for horse grazing around the world (16).

While in several Countries equids are still used as draught animals, pack animals, working animals (17–20), in Europe horses and donkeys are raised with different purposes. Horses are mainly kept for sport and leisure activities: horses are involved in several different sport disciplines, ridden, driven and non-ridden, from amateur to high-level professional competitions, such as, just to name a few, show jumping, dressage, eventing, endurance, reining, tent pegging, western pleasure, vaulting, flat racing, harness racing, and so on. The European Equestrian Federation estimates that equestrian sports alone occupy 900.000 people with an economic impact of 34 billion euros (15). Moreover, horses are also involved in therapy programs (15,21) or kept as pets.

Donkeys may be involved in different assisted activities with children with autism or motor disabilities (22,23), or people with mental disorders (24). Donkeys are also kept as pets, or involved in leisure and tourism activities (15,25). Donkey milk and meat is also traditionally produced in several countries (26–29).

Nevertheless, equids in some European Countries are still used as working animals, even if there are no official data regarding the amount of animals employed in this sector (15).

The equids industry not only involves people and animals directly employed on-farm, but also comprises a large number of animals and professionals involved in transport (figure 2). Equids are the most transported animals in Europe, since they do not only travel to reach the slaughterhouse, as other farm animals, but they are also transported for competitions, breeding and training (30–33). They are also transported by their own owners for leisure activities, reaching veterinary clinics, or change facility (15).

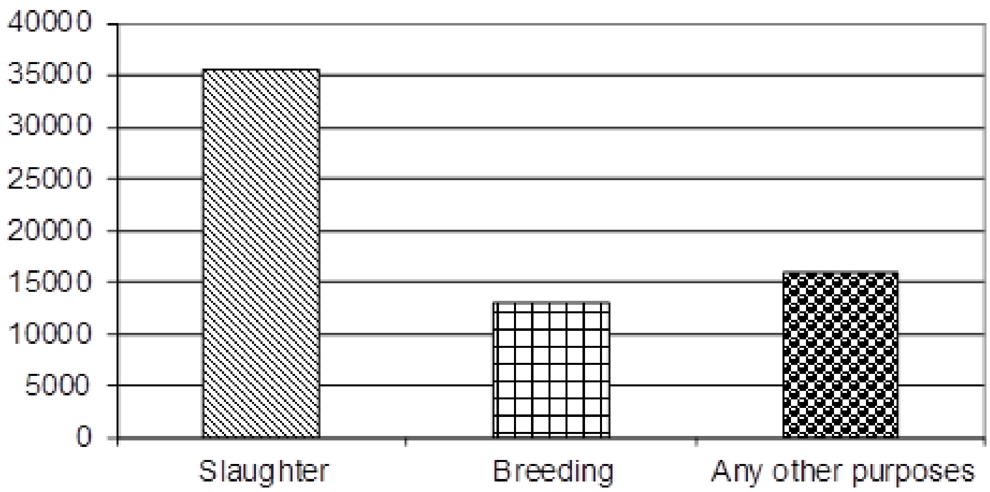


**Figure 2.** Number of equids transported in Europe in 2010 (modified from (15))

## 1.2. On-farm welfare evaluation of Equids

Being equids used for many different purposes and consequently kept in various different management systems, these animals face several challenges which may affect their welfare. Inadequate housing, feeding, management, harness, and equipment can cause stress and painful conditions (34). It is also reported that some European countries face a lack of support services, such as veterinary practitioners, farriers and equine dentists (25), and this may represent a serious risk factor for equids welfare. Moreover, despite the growing body of law in the field of animal welfare, the equids sector is still fundamentally unregulated (25,35).

Horses are generally housed in single stables for all day long, frequently with no social contact or possibility to graze (36–40). This housing system represents a serious risk for horse welfare (41), since it is not possible for horses to satisfy highly motivated behaviours such as movement (42) and social relationship (43). Consequently, it is reported a high prevalence of undesired behaviour, such as stereotypies (44,45), ranging from 14.4% (41) to 32.5% (44). Sport activities may also represent a risk factor for horse welfare: in Countries in which horses are mainly used for competition and for pleasure, welfare problems related to lameness, back pain, overweight, respiratory disease and bad human-animal relationship have been reported (40,46–50).

While horse conditions are largely investigated in scientific literature, donkeys seem to be forgotten by research. Limited information regarding welfare condition of donkeys in Europe is available in the literature (26,51–53). Also, limited information is available regarding the correct management of donkeys, causing huge variability in the professionalism of different donkey farmers, which can treat donkey welfare (15,27). Reported welfare issues in donkeys kept for different purposes are obesity, incorrect hoof management, integument alterations, and scarce human-animal relationship (26,27).

Several species- or context-specific protocols have been developed to assess welfare of equids kept for different purposes and assessed in different stages of their lives.

In 2005, thanks to  a collaboration between Brooke and the University of Bristol, the Working Equid Welfare assessment (WEWA) protocol was published (54). The protocol is intended to be used to evaluate the welfare of working horses, donkeys and mules, and includes 41 observations of health and behaviour, recorded either as present/absent or as scores of severity (54). The WEWA protocol represents the first animal-based welfare assessment tool for equids (55) and, according to the developers, it presents some limitations such as the presence of parameters with limited use, missing parameters, low sensitivity of measures of abnormality (only presence/absence was recorded) (55).

To overcome these limitations, in the following six years, a review of WEWA was conducted, removing several indicators with limited use, adding new indicators to evaluate previously unconsidered conditions, refining scoring systems. As a result, the Standardised Equine-Based Welfare Assessment Tool (SEBWAT) was published (55). The protocol is dedicated to working equids and includes 40 animal-based measures.

SEBWAT has been used extensively in the field by Brooke and their partners to evaluate welfare of working equids in low income countries (55). The assessment takes 5-10 minutes per animal, making it easy to apply also to a high number of animals. While the protocol has proven useful in these conditions, it is not applicable in different management systems (e.g. production, pet, tourism, sanctuary/retired, feral equids). Moreover, as highlighted by the authors, a correct assessment requires a comprehensive training and it may be difficult to train local assessors with limited background knowledge (55).

Researchers from Wageningen University published in 2011 the Welfare Monitoring System For Horses. The protocol is based on the Welfare Quality® framework (4 Principles, 12 Criteria) and comprises both animal-based and resource-based indicators, for a total of 44 indicators (56). An aggregation of data collected, similar to the one proposed by the Welfare Quality® researchers (57–59), is proposed, in order to obtain a single score per farm. The Welfare Monitoring System For Horses includes a number of scientifically validated indicators, however, some Criteria are not covered by animal based indicators (e.g. Absence of prolonged thirst, Comfort around resting, Thermal comfort, Ease of movement, Expression of social behaviours, Positive emotional state), while one is not covered at all (e.g. Good human-animal relationship). Moreover, it requires 5.9–7.6 hours for the evaluation of 20 horses (56); therefore, as highlighted by the authors, it is not possible to evaluate more than 20 horses a day. Time constraint is a clear limitation to welfare assessment, especially when a large number of horses is kept in the same facility and the same farm needs to be visited for several days. Finally, the protocol is specifically designed for horses kept in box, no suggestions are available for the adaptation to horses kept on pasture, and donkeys nor mules are included.

In 2015, at the end of the European founded Animal Welfare Indicators project (AWIN), the AWIN welfare assessment protocol for horses (60) and the AWIN welfare assessment protocol for donkeys were published (61). For the first time, the AWIN project considered several species not specifically protected by welfare-related legislations and not included in previous European projects: not only horses and donkeys but also goats, sheep and turkeys (62–64). Aim of the project was to develop welfare assessment protocols based on valid, reliable and feasible animal-based indicators, following the framework of the Welfare Quality® protocols (57–59). The AWIN welfare assessment protocol for horses is dedicated to horses older than three years, kept in box and used in different activities and includes 25 welfare indicators. An adaptation for group-housed horses is also suggested. The AWIN welfare assessment protocol for donkeys is intended to be used in different management conditions and on donkeys over one year old kept for different purposes and includes 22 animal-based indicators. Unlike Welfare Quality ®, the AWIN approach proposes a two-step evaluation: a first-level assessment is recommended as a screening tool in each farm, it is quick and easy to apply (about 5 minutes *per* animal). When some risks for animal welfare are identified, it is suggested to apply the second level assessment, which is more comprehensive and informative, but also more time consuming and requires a specific training (65). Moreover, AWIN researchers did not propose any results aggregation methods to obtain a single score; the proposed analysis of the results gives an overview of animal welfare in the evaluated farm (% of animals fulfilling the welfare Criteria) and makes a comparison with a “reference population” (data collected during the AWIN project), with the aim of facilitating the discussion with the stock people (65). Embracing the technological innovation, the AWIN researchers also developed specific mobile applications, freely available on Google Play Store, to give anyone the possibility to collect, store and analyse data related to welfare assessment (66). Perceived limitations of the AWIN protocols are the semi-exclusive use of animal-based indicators, since data regarding housing and husbandry are not collected, and the target age of horses and donkeys, since foals are not included (67). Moreover, the AWIN protocols focus on the most common farming systems (e.g. single box for horses and group housing for donkeys) and may, therefore, need refinements to be applied in different conditions (e.g. working and feral equids, semi-extensive housing systems). Finally, some indicators developed during the project, such as Horse Grimace Scale (HGS), would benefit of additional research and refinement to further validate them.

More recently, the Equid Assessment, Research and Scoping (EARS) tool has been developed by The Donkey Sanctuary (67). The EARS protocol is intended as a “one size fits all” tool, being applicable to horses, donkeys and their hybrids, kept for work, production, leisure, retirement, and even to feral equids. EARS incorporates pre-existing validated welfare assessment indicators and include the collection of several management and resource information; it comprises 290 questions, divided into 19 welfare indicators. These questions are organised into protocols (defined as “a set of questions that relates to a particular context”) to accommodate the user needs (67). Developers proposed an aggregation method to obtain a score per each domain of animal welfare (Nutrition, Environment, Health and Behaviour), with the aim of guiding actions towards animals with the poorest levels of welfare (68). A possible limitation of the EARS tool is the requirement of a complete training to be used (67), and, at the present moment, the training is provided only by The Donkey Sanctuary to selected new assessors. Moreover, the protocol includes 290 questions, requiring about 20 minutes *per* animal to be completed; factors such as extensive time requirement and large numbers of indicators are deemed to make the assessment economically expensive (69).

Finally, in 2020, Harvey and colleagues proposed a protocol to assess free-roaming horse welfare (70). The protocol aimed at giving scientists a method to evaluate the welfare of free-roaming wild animals during their normal day-to-day lives. Starting from the Five Domains Model (71), and on horse-specific recognised welfare needs, the Authors proposed a list of 28 measurable or observable indicators to evaluate various physical and affective states. Both animal-based and resource-based indicators were included, the former to provide welfare status information, and the latter to identify possible risk factors (70). Authors also suggests methods to identify individual animals in the wild (such as using individual coat colour or applying marks/tags). Finally, Authors proposed a grading system for grading welfare compromise (in a five-tier scale: A–E) and enhancement (in a four-tier scale: 0, +, ++, +++), within each domain, in order to standardise the assessment of animal welfare across different assessors, and to monitor animal welfare over time (70). While the Authors proposed a template for and describes the principle to making welfare assessments in free-roaming horses, the protocol has not been tested. Moreover, as Authors recognised, only specific indices and mental experiences can be assessed; moreover, in some contexts, very few welfare indices can be assessed and interpreted, significantly hampering welfare assessments (70).

## 1.3 Welfare assessment during transport procedures

Alongside farm conditions, transport represents an important moment in equids' lives, as previously described; it is worth remembering that equids are probably the most transported farm animals in Europe (15). Transport procedures can have substantial short-term and prolonged effects on animal welfare (72): several potential stressors are involved in transport, including loading, unloading and penning in a new and unfamiliar environment and confinement with and without motion, vibrations, changes in temperature and humidity, inadequate ventilation and, often, deprivation of food and water (73). Equids are subject to many potential stressors during transport causing disturbances to the autonomic nervous system, endocrine system, metabolic system and immune function (73). Horses subjected to transport stress can be more susceptible to a number of disorders, such as pneumonia, diarrhoeas, colics, laminitis, injuries and rhabdomyolysis (74), besides having their sports performance compromised (75). Therefore, transport-related stress not only severely affects animal welfare, but can also be costly for the owner.

Loading is considered to be one of the most stressful components of transport for most animals (76) and seems to be the major transport-related problem reported by horse owners (77,78). When the animal is required to enter into a trailer, it is subjected to an atavistic fear of the unknown, a fear potentially reinforced by recollections of previous unpleasant travelling experiences (73,74,79). Many horses fight during loading and owner's response often implies the use of physical force. This can produce a very dangerous situation: injuries to the handlers can include rope burns, lost fingers, broken bones, or bruises and bleeding; injuries to the animal can include lacerations to the head from banging into the trailer, scrapes and cuts on the legs, broken legs from falling, or even a broken back if the animal falls backwards (80–82). Often people use winches, whips, war bridles, chains, and other punitive methods to get horses to load; these methods are likely to cause aversive reactions to loadings (83,84) and are unlikely protecting humans from injuries.

Travelling phase can also induce stress, due to internal vehicle temperature, relative humidity, level of environmental contaminants, isolation or high stock density, confinement, movement and vibration of the vehicle, noise, unusual source of drinking water and food or fasting, orientation inside the vehicle, driver behaviour (75,85–89). Animals can fall or hit the trailer walls, getting injured during transport (81,90–92); transport represents one of the most common source of injuries to horses (93). Moreover, some horses move or kick inside the trailer, causing driving problems and fatal road accidents (75).

Finally, also unloading may represent a stressful event. Risk factors for unloading-related stress are ramp angulation and slipperiness, and facing a new environment (75,94,95). Moreover, it is worth noticing that while commercial transports are regulated (Council Regulation (EC) No 1/2005 on the protection of animals during transport and related operations), thousands of horses are moved short distances by their owners, meaning that the journey does not fall under 1/2005/EC (96).

Transport stress evaluation requires a multidisciplinary approach; assessment of transport stress and discomfort should involve both behavioural and physiological evaluation (73). In horses the most common evasive behaviour patterns reported in the literature during loading are plants, swings, pull-backs, attempts to escape, kicking, vocalise and nosing the ramp (75,80,94,97). Reported physiological modification related to transport stress in horses are: increase in cortisol levels (98–100), concentration of glucose (101), circulating T3, T4 and fT4 levels (101,102), heart rate (101,103), β-endorphin levels (104), ACTH levels (104), core temperatures (101), neutrophil:lymphocyte ratios (101), packed cell volume (103). A protocol specifically dedicated to the evaluation of transported horses during unloading was published in 2016 in the framework of the “Development of EU wide animal transport certification system and renovation of control posts in the European Union” project (30). Inspired by previous existing protocols, it is intended to be applied before, during and after unloading. The protocol is composed of both animal-based and management-based indicators, for a total of 46 indicators. The aim of the project was to provide a scientifically sound tool for the welfare assessment of horses travelling over long journeys.

Proposed solutions to improve horse welfare during transport include training to load (32,97,105), modification of the animal orientation inside the trailer (86,87), adjustment of stocking density (87,88), use of mirrors to reduce isolation-related stress (106), use of nutritional therapy (107), adjustment of lightening during loading and unloading (108). Nevertheless, further research is needed to develop a feasible on-farm procedure to prepare horses for transport. No scientific literature proposed solutions to enhance donkey welfare during transport.

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# 2. Objectives and approach

The overall aim of this scientific study was to experiment and provide applicable solutions to enhance equids welfare both on farm and during transport.

While protocols and methodologies to assess equids welfare are constantly improving and welfare data are increasingly collected on farm, improving animal welfare remains competence of the professionals, such as veterinarian, behaviourist, farrier, dental caregiver, etc.. Welfare assessment represents only the beginning of the journey towards the effective improvement of equids welfare. Owners and professionals need effective solutions to be applied in their daily activities, as feasible and beneficial as they can be. These solutions, nevertheless, must be scientifically validated, which is to say that they must be proved to actually enhance animal welfare.

In this work, scientific techniques were applied to test possible solutions, which may be implemented in daily practice.

The project focused on two different topics:

1) Evaluation of best management practices for equids on-farm;
2) Testing non-aversive training techniques to reduce stress during transport in meat horses and donkeys.

The research was designed in two parts, as described in table 1:

1) On-farm management of equids
2) Effect of training in reducing transport-related stress

## 2.1 On-farm management of equids

The aim of this section was to identify tools to improve welfare of horses and dairy donkeys on farm. Indeed, equids welfare could be affected by several factors, since horses and donkeys are involved in different activities and kept under different management system.

## 2.2 Effect of training in reducing transport-related stress

Aim of this section was to develop a non-aversive training method to teach meat horses and donkeys to load. Since animal welfare could be seriously compromised during transport procedures, and being loading on of the most stressful stage of transport, it has been hypothesized that effectively training equine to loading procedures before their first exposure to transport could mitigate the transport-related stress.

**Table 1.** An overview of the chapters included in the thesis

| | |
|---|---|
| Chapter 1 | General introduction |
| Chapter 2 | Objectives and approach |
| | On-farm management of equids |
| Chapter 3 | Dai F, Dalla Costa E, Burden F, Judge A, Minero M (2018) *The development of guidelines to improve dairy donkey management and welfare*. Italian Journal of Animal Science, 2019 18(1):189-193; doi: 10.1080/1828051X.2018.1503571<br><br>Dai F, Leach M, MacRae AM, Minero M, Dalla Costa E (2020) *Does Thirty-Minute Standardised Training Improve the Inter-Observer Reliability of the Horse Grimace Scale (HGS)? A Case Study*. Animals, 10: 781; doi:10.3390/ani10050781<br><br>Dai F, Dalla Costa E, Minero M, Briant C *Does housing system affect horse welfare? The AWIN welfare assessment protocol applied to horses kept in an outdoor group-housing system: the "parcours".* Animal Welfare, under review |
| | Effect of training in reducing transport-related stress |
| Chapter 4 | Dai F, Dalla Costa A, Bonfanti L, Caucci C, Di Martino G, Lucarelli R, Padalino B and Minero M (2019) *Positive Reinforcement-Based Training for Self-Loading of Meat Horses Reduces Loading Time and Stress-Related Behavior*. Frontiers in Veterinary Science 6:350; doi: 10.3389/fvets.2019.00350<br><br>Dai F, Dalla Costa E, Cannas S, Heinzl EUH, Michela M, Mazzola SM (2020) *May Salivary Chromogranin A Act as a Physiological Index of Stress in Transported Donkeys? A Pilot Study*. Animals, 10: 972; doi:10.3390/ani10060972<br><br>Dai F, Mazzola SM, Cannas S, Heinzl EUH, Padalino B, Minero M, Dalla Costa E (2020) *Habituation to Transport Helps Reducing Stress-Related Behavior in Donkeys During Loading*. Frontiers in Veterinary Science, 7: 593138; doi: 10.3389/fvets.2020.593138 |
| Chapter 5 | General conclusion |

# ON-FARM MANAGEMENT OF EQUIDS

# 3. On-farm management of equids

## 3.1 Brief Introduction to the Scientific Studies

Being horses and donkeys involved in different activities and kept under different management system, animal welfare could be affected by several factors. In order to enhance welfare, any information given to farmers to improve the management of their animals must be scientifically sound. Solutions must be proven to be effective in improving animal welfare in an on-farm setting.

The studies included in this section aimed at identifying tools to improve welfare of horses and dairy donkeys on farm.

The section includes three scientific papers:

1. In the first study guidelines to improve dairy donkey management and welfare are presented; the guidelines have been developed taking into consideration the existing scientific literature and are dedicated not only to farmers but also to any stakeholder of the sector.
2. In the second study, the focus has been posed on a tool for pain assessment in horses. Being pain an important welfare issue, a reliable assessment of existing pain conditions is a fundamental step to correctly treat horses. In this study, a standardized training method has been tested, to evaluate its efficacy in training new assessors.
3. In the third study, a different horse management system, namely on pasture group housing, has been evaluated from an animal welfare point of view, since this management system is anecdotally considered better than single-box housing for horse welfare.

## 3.2 The development of guidelines to improve dairy donkey management and welfare

Francesca Dai, Emanuela Dalla Costa, Faith Burden, Andrew Judge, Michela Minero

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Abstract. Donkey milk is a valuable product for babies suffering from multiple-allergies and cosmetic production; therefore, new dairy donkey farms are opening around Europe. Little information is available for farmers on sustainable production of donkey milk, including animal welfare, milk production, and processing. Targeted dissemination of information on appropriate animal management would assist dairy donkey farmers in preventing welfare problems. This research project aims to develop guidelines on good practice principles for sustainable donkey milk production. Different steps were followed to develop the guidelines:

1. identification of key issues for dairy donkey welfare, analysing the results of previous project and the available scientific literature;
2. systematic review research to select promising solutions for each issue included in the guidelines;
3. stakeholder consultation, in order to increase scientific soundness and to enhance their acceptability throughout the sector;
4. guidelines drafting and revisions by stakeholders;
5. guidelines launch.

The guidelines ‘Dairy donkeys: good practice principles for sustainable donkey milk production’ were launched in December 2017. They include suggestions derived from scientific literature and/or reported by internationally recognised experts. The guidelines provide clear and helpful advice on good animal management practices for anyone interested in donkey milk production. They comprise the following chapters: ‘Responsibilities’, ‘Feed and water’, ‘Housing and Management’, ‘Donkey health care’, ‘Humane killing’, ‘Appropriate behaviour’, and ‘Milking procedures’. The guidelines, translated in different languages (Italian, Spanish, Portuguese, French, Greek and Chinese Mandarin) are freely available online.

Keywords: Donkey; welfare; guidelines; milk; management

### 1. INTRODUCTION

The demand for donkey milk is increasing around Europe due to its unique characteristics: it is a valuable product for babies suffering from multiple-allergies (cow milk, hydrolysed cow milk proteins, goat milk, and soya) (1–6) and cosmetics production. The production systems adopted range from semi-intensive to semi-extensive systems.

Only a few Italian Regions have adopted specific legislation for producing and selling donkey milk (7); neither national consortia nor best practice guidelines exist (7,8). Furthermore, little information is available on sustainable production of donkey milk, including animal welfare, milk production and processing (9–11). Consequently, there is huge variability in the professionalism of different farmers (8,12). In 2017, Dai et al. highlighted that Italian dairy donkey farms do not follow uniform procedures for the management of animals and concluded that targeted dissemination of information about appropriate feeding, resources, hoof care and handling of dairy donkeys would increase awareness among farmers about donkey needs and assist them in preventing welfare problems (10,12). With this in mind, the University of Milan and The Donkey Sanctuary collaborated with industry stakeholders on the development of guidelines that take into consideration good practice principles for sustainable donkey milk production, and subsequently recommend practical solutions for their implementation.

## 2. THE DEVELOPMENT OF THE GUIDELINES

The guidelines ‘Dairy donkeys: good animal management practices for donkey milk production’ were developed according to the following steps: (1) identification of key issues for dairy donkey welfare; (2) systematic review search; (3) stakeholder consultation; (4) guidelines drafting and revisions; and (5) guidelines launch.

### *2.1 Identification of key issues for dairy donkey welfare*

The process was based on the results of a pilot research project titled ‘A pilot investigation to determine welfare standards on milk/meat donkey farms in Italy and potentially influence their main drivers’ (7,10,12). This preliminary investigation identified potential key issues for dairy donkey welfare. Following a face-to face meeting (see Stakeholder consultation section), scientists agreed on the selection of the key issues to be included in the guidelines.

### *2.2 Systematic review search*

A systematic review of the available relevant scientific literature was then conducted to select promising evidence for each key issue included in the guidelines. Scientific Databases consulted were Web of Science, CAB Abstracts, PubMed, Scopus. We searched the following keywords:

donkey* OR ass OR jenny OR jack OR Equus asinus OR equine OR equids OR equid

AND

welfare, identification, treatment*, therapy, hoof, hooves, nutrition, diet, management, foal management, stallion management, weaning, milking, milk, dairy, human-animal relationship, breeding, reproduction, selection, transport, slaughter, slaughterhouse, abattoir.

National and European Regulations and Best Practice Guidelines on Welfare were also considered. Authors evaluated each article for their scientific robustness (Table 1). The following aspects have been taken into consideration:

- Validity: concerns the extent to which a measurement actually measures those features the investigator wishes to measure and provides information that it is relevant to the question to be asked (13);
- Reliability/consistency: concerns the extent to which measurement is repeatable and consistent; that is free from random errors (13). The smaller the error component, the more reliable the measurement;
- Relevance: connected with the matter at hand, pertinent, of impact;
- Feasibility: practical likelihood of adopting the recommendation on-farm. It is a dynamic concept, dependent on factors such as the purpose of the recommendation and budgetary constraints. Together with farmers' and stakeholders' acceptance these comprise the main variables to be evaluated.

The scientists highlighted that certain issues were well investigated while others showed gaps in scientific knowledge.

**Table 1.** Symbols and definitions adopted for critical appraisal of scientific literature throughout the guidelines.

| | |
|---|---|
| ●●●●● | Evidence obtained from meta-analysis or systematic reviews of randomised controlled trials or at least one randomised controlled trial |
| ●●●●○ | Evidence obtained from at least one controlled study without randomisation |
| ●●●○○ | Evidence obtained from at least one other type of well-designed quasi-experimental study, without randomisation |
| ●●○○○ | Evidence obtained from well-designed non-experimental descriptive studies, such as comparative studies, correlation studies and case studies |
| ●○○○○ | Evidence obtained from expert committee reports or opinions and/or clinical experiences of respected authorities |

*2.3 Stakeholder platform consultation*

A multidisciplinary Stakeholder Platform was established. This included public authorities, civil society (NGOs and consumers association), farmers, industry (food processors and cosmetic industry), and academics. Stakeholders were selected for their acknowledged expertise in donkey management and welfare and (as for academics) peer-reviewed publications on relevant topics. Stakeholders were contacted by email to ask for their voluntary participation. A first platform comprised 11 stakeholders from four different European countries: three farmers, three academics, two public authorities (official veterinarians and ministry), two members of NGOs, one representative of food processor and cosmetic industry. They agreed to participate in a face-to-face meeting in Milan, in which the project and the aims were presented. Experts were asked to discuss on-farm welfare related aspects, in order to agree on a list of topics to be included in the guidelines. Possible solutions, described in scientific literature or derived from stakeholders' experience, were discussed in terms of the following parameters: importance (meaning the significance of the issue and the proposed solution for on-farm donkey welfare), appropriateness (meaning the relevancy of the proposed solution with the issue), effectiveness (meaning the ability of the proposed solution in solving the issue). The Stakeholder Platform was enlarged using a snowballing technique: members were asked to introduce two colleagues to be contacted by email to join the Stakeholder Platform. The final Stakeholder Platform comprised 29 European members: 5 farmers, 10 academics, 4 public authorities, 7 members of NGOs, 1 representative of food processor and cosmetic industry, 1 representative of consumers, and 1 farrier. Experts were asked to revise the guidelines drafts (see following paragraph). The stakeholders' involvement was intended not only to increase scientific information contained in the guidelines, but also to identify potential barriers to the practical application of the guidelines, and possible solutions, and enhance their acceptability throughout the sector.

*2.4 Guidelines drafting and revision*

After the meeting, a first draft of the guidelines was prepared based on the outcomes of the stakeholders' consultation. The draft guidelines were available online on a dedicated website (http://donkeynetwork.org.uk/) for a month to allow the Stakeholder Platform to revise them. Valuable feedback was obtained about suggestions for modification or requests for additional evidence, or alternative interpretation of evidence. Experts of specific sectors (i.e. reproductive medicine, nutrition, farriery, milking procedures, parasitology…) were asked to provide opinions on the topics not covered sufficiently in the scientific literature. The Stakeholder Platform was also able to contribute to and influence the graphic appearance of the final guidelines. Following the stakeholders suggestions, a second version was drafted and submitted for further revisions. The second draft was available online for 1 month. After the second revision, a final version of the guidelines was prepared and submitted for design and translation in to Italian, Spanish, Portuguese, French, Greek, and Chinese Mandarin.

*2.5 Guidelines launch*

The document 'Dairy donkeys: good animal management practices for donkey milk production' was firstly presented during the Intergroup 'Welfare and Conservation of Animals' meeting at European Parliament in Strasbourg on Thursday 26 October 2017. A face to face final meeting of the Stakeholder Platform was organised to present the guidelines and to draft a communication plan. The document was also made freely available online on the website http://donkeynetwork.org.uk/.

## 3. THE GUIDELINES

The guidelines ‘Dairy donkeys: good practice principles for sustainable donkey milk production’ are designed to provide clear and helpful advice on good animal management practices for anyone interested in sustainable donkey milk production. They can be freely downloaded at http://donkeynetwork.org.uk/. They contain the following chapters: Responsibilities, Feed and water, Housing and Management, Donkey health care, Humane killing, Appropriate behaviour, Milking procedures. Each section contains information about:

- Essential requirements. The essential requirements designated in this document must be met under law for livestock welfare purposes. Jurisdictions may vary in their definition of specific terms under their animal welfare legislation. Every endeavor has been made to adopt terms that have nationwide application. Readers are urged to check the relevant definitions under the relevant legislation to their jurisdiction.
- Additional practices. The additional practices to achieve desirable animal welfare outcomes are consistent with the recent scientific literature. They have no force of law, use the word ‘should’ and complement the essential requirements. Where appropriate science is not available, the additional practices reflect a value judgement that has to be made for some circumstances. Numbers in brackets refer to scientific papers reported in the References section at the end of the document.
- Warning. Take note topics, which could represent a serious issue for animal welfare.
- Further information. Additional material (such as pictures or tables) which can be a useful practical tool to ensure animal welfare.

Symbols have been used throughout the document in order to identify each section (Table 2).

**Table 2.** Interpretation of symbols used in the guidelines.

| | |
|---|---|
|  | Essential requirements |
|  | Additional practices |
|  | Warning |
|  | Further information |

## 4. REFLECTIONS AND FUTURE STEPS

This article presents the approach adopted to develop the guidelines 'Dairy donkeys: good animal management practices for donkey milk production'. The document is comprehensive and easy-to-use and includes suggestions derived from scientific literature and/or reported by internationally recognised experts. In order to raise awareness and encourage the use of the guidelines by those involved in the production of donkey milk, the Stakeholder Platform agreed on a communication plan. An endorsement at European Level is desirable in order to enhance the welfare of donkeys kept for milk production and it is hoped that European Union policy makers will use these guidelines as a basis for improving the welfare of dairy donkeys throughout Europe. The official veterinarians have been recognised as a main actor for the guidelines dissemination, since they have frequent contacts with the farmers and the civil society. Meetings will be organised in order to introduce them the guidelines and to ask their collaboration in the dissemination. Hard copies of the document will be delivered to farmers; specific events (such as fairs or farmers events) will be selected to present and distribute the document. Finally, the website will be advertised throughout social networks.

## 5. CONCLUSION

These guidelines, translated in different languages and freely available online, will permit a targeted dissemination of information about appropriate management procedures for dairy donkeys, increasing awareness among farmers about donkey needs and assist them in preventing welfare problems.

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## 3.3 Does Thirty-Minute Standardised Training Improve the Inter-Observer Reliability of the Horse Grimace Scale (HGS)? A Case Study.

Francesca Dai, Mattew Leach, Amelia MacRae, Michela Minero, Emanuela Dalla Costa

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Simple Summary. The recognition of pain in equine practice is highly dependent on the assessors' reliability in using pain assessment tools. The Horse Grimace Scale (HGS) is one such tool, a facial expression-based pain coding system able to identify a range of acute painful conditions in horses. This study aimed at evaluating the efficacy of a standardised HGS training program at improving the agreement of assessors without horse experience by comparison with an expert. The results suggest that 30-minute face-to-face training may not be sufficient to allow observers without horse experience to effectively learn about HGS and its consentient facial action units to then be able to effectively apply this scale. The training method applied could represent a starting point for a more comprehensive training program for assessors with no experience.

Abstract. The Horse Grimace Scale (HGS) is a facial-expression-based pain coding system that enables a range of acute painful conditions in horses to be effectively identified. Using valid assessment methods to identify pain in horses is of a clear importance; however, the reliability of the assessment is highly dependent on the assessors' ability to use it. Training of new assessors plays a critical role in underpinning reliability. The aim of the study was to evaluate whether a 30-minute standardised training program on HGS is effective at improving the agreement between observers with no horse experience and when compared to an HGS expert. Two hundred and six undergraduate students with no horse experience were recruited. Prior to any training, observers were asked to score 10 pictures of horse faces using the six Facial Action Units (FAUs) of the HGS. Then, an HGS expert provided a 30-minute face-to-face training session, including detailed descriptions and example pictures of each FAU. After training, observers scored 10 different pictures. Cohen's k coefficient was used to determine inter-observer reliability between each observer and the expert; a paired-sample t-test was conducted to determine differences in agreement pre- and post-training. Pre-training, Cohen's k ranged from 0.20 for tension above the eye area to 0.68 for stiffly backwards ears. Post-training, the reliability for stiffly backwards ears and orbital tightening significantly increased, reaching Cohen's k values of 0.90 and 0.91 respectively (paired-sample t-test; $p < 0.001$). The results suggest that this 30-minute face-to-face training session was not sufficient to allow observers without horse experience to effectively apply HGS. However, this standardised training program could represent a starting point for a more comprehensive training program for those without horse experience in order to increase their reliably in applying HGS.

Keywords: HGS, horse, pain assessment, training, welfare assessment

## 1. INTRODUCTION

Using valid assessment methods to identify pain in horses as a consequence of husbandry practices or in a clinical setting is of a clear importance (1,2). However, whatever assessment method is chosen, its reliability (repeatability in time and consistency within and between observers (3)) is highly dependent on the assessors' ability to use it. Several factors can complicate the recognition of pain in horses. They are a prey species and therefore may hide their pain (4); moreover, individual temperament has been shown to influence the intensity that pain-related behaviours are exhibited (5). A training program aiming to improve the accuracy of pain evaluation by new assessors should be developed in order to improve their inter-observer reliability (6,7). This would guarantee that the use of pain indicators by multiple individuals will provide reliable results, thus more consistently reflecting pain levels observed, and be applicable in daily clinical practice (8,9). Well-designed training programs are especially important for equine pain assessment, given the diversity observed in the horse industry, in terms of breeds, different housing systems, various disciplines, different professional levels (10) and the variability in background (i.e., experience, knowledge, etc.) of people involved in the sector (e.g., horse caretakers, veterinarians, owners, etc.).

The Horse Grimace Scale (HGS) is a facial-expression-based coding system, which can be used to recognise pain in horses (2,11–13). It includes six Facial Action Units (FAUs): stiffly backwards ears, orbital tightening, tension above the eye area, prominent strained chewing muscles, mouth strained and pronounced chin and strained nostrils. A score of 0 indicates high confidence of the observer that the action unit was absent. A score of 1 indicates either high confidence of a moderate appearance of the action unit or equivocation over its presence or absence. A score of 2 indicates high confidence of a marked appearance of the action unit. Facial expressions are particularly useful in pain assessment, as they cannot be completely suppressed by voluntary control, and importantly this is still evidenced in prey species (14,15). It has been shown that a short training period for new HGS assessors is sufficient to allow them to reliably apply this method with a good inter-observer reliability (11,13). However, in the above-mentioned studies, the new HGS assessors involved were experienced veterinarians familiar with normal species-specific behaviours. Untrained assessors with different backgrounds and experience could represent a possible bias in the evaluation of the efficacy of a training program (16). Therefore, the aim of a successful training program should ensure high reliability irrespective of the different background experience of the observer (17). No data are currently available regarding how observers without previous experience in either in pain assessment or horse behaviour can learn to apply the HGS reliably by comparison to HGS experts.

The present study aimed to evaluate whether a standardised face-to-face training program that combined theory and practical experience was effective at improving and ensuring the reliability of observers with no horse experience when utilising the HGS, measured in terms of inter-observer reliability.

## 2. MATERIALS AND METHODS

### *2.1 Ethic statement*

All the students were verbally informed about the methods and the objectives of the research and the data collection and entered the study on a voluntary basis. At any time, students could withdraw their consent. No sensitive data were collected, and it was not possible to identify the participants in the raw research data.

### *2.2 Students*

Undergraduate students (N=206) from five institutions voluntarily participated in the study (Table 1). Inclusion criteria were that participants had no direct horse experience and were unfamiliar with the Horse Grimace Scale scoring system.

**Table 1.** Number of recruited students from each institution.

| Course | Institution | N of students |
|---|---|---|
| Second year students in Veterinary Medicine | University of Milan | N=63 |
| Fourth year students in Veterinary Medicine | University of Teramo | N=31 |
| Third and fourth year students of Applied Biology | University of British Columbia | N=28 |
| Third year and MSc students in Animal Science | University of Newcastle | N=40 |
| Second and third year students in Animal Welfare and Husbandry | University of Milan | N=44 |

### *2.3 HGS standardised training program*

An HGS expert (an academic scientist renowned internationally for her expertise in horse welfare, who has previously scored over 200 pictures using HGS) provided a 30-min face-to-face training session. This training included: a presentation of the HGS scoring system, detailed descriptions of each Facial Action Unit (FAUs) with example pictures, and examples of images that had previously scored by the HGS expert. The students were encouraged to interact with the trainer, ask questions and actively discuss the method and the scoring of example pictures.

*2.4 Data collection*

Twenty previously scored pictures showing a profile view of the head of horses of different breeds and colours were selected (for an example see Figure 1). The pictures provided were collected from horses in pain due to acute laminitis (previously published data on the HGS (11)). High-quality pictures were selected with the aim of showing a wide range of FAU scores (balancing the number of pictures with scores of 0, 1 and 2 for the different FAUs). Pictures were projected on a screen one at a time. Data were collected in two phases: pre- and post-training. In the 'pre-training' phase students first received a brief lecture on the definition of pain and its effect on facial expressions in different species (e.g., mice, rats, rabbits) but not horses. They then were asked to score 10 pictures of horse faces. They were not introduced to the HGS in this phase. In the 'post-training' phase students received the HGS standardized training outlined in Section 2.2 and then scored a second different set of 10 pictures. All pictures were also scored by an HGS expert (E.D.C.).

**Figure 1.** Example of pictures scored by the students.

*2.5 Statistical analysis*

The Intraclass Correlation Coefficient (ICC) has been used in other studies to assess the reliability of grimace scales when scored by several observers with similar experience (interchangeable observers). However, the aim of the present study was to compare the HGS scores of an expert to those of observers (non-interchangeable due to the different experience) with no experience with horses. Therefore, Cohen's kappa coefficient was used to determine inter-observer reliability between each student and an HGS expert. The kappa statistic rages from 0 to 1 and can be interpreted as follows (18): agreement equivalent to chance (less than 0.10); slight agreement (0.10–0.20); fair agreement (0.21–0.40); moderate agreement (0.41–0.60); substantial agreement (0.61–0.80); near perfect agreement (0.81–0.99); perfect agreement (1). All statistical analyses were conducted using SPSS 25 (SPSS Inc., Chicago, USA). The data were tested for normality and homogeneity of variance using Kolmogorov–Smirnov and Levene tests, respectively. Paired-sample t-tests were conducted to determine if there was a significant difference in agreement between the students and the expert from pre- to post-training. Differences were considered to be statistically significant at $p \leq 0.05$.

## 3. RESULTS AND DISCUSSION

The training protocol presented in this paper was previously applied to a smaller number of trainees without horse experience to assess inter-observer reliability (19). It showed that reliability was excellent before training with an Intraclass Correlation Coefficient of 0.986, and then improved after 30 minutes of training to 0.992 (both high degrees of reliability). However, this study did not evaluate the agreement between observers with no horse experience with that of an expert, which is critical for determining the efficacy of training naive observers (16,20). The results of the present study showed a high variability of agreement between naïve observers and the expert for the different facial action units comprising the HGS: ranging from 0.20 for tension above the eye area to 0.68 for stiffly backwards ears (Figure 2). Only stiffly backwards ears (Cohen's kappa = 0.68) and orbital tightening (Cohen's kappa = 0.67) reached a substantial agreement before training, while all other FAUs only showed slight agreement or fair agreement. Following training, the agreement for stiffly backwards ears and orbital tightening significantly increased, reaching Cohen's kappa values of 0.90 and 0.91 respectively, indicating near perfect agreement (paired-sample t-test; $p < 0.001$); the agreement for prominent strained chewing muscles significantly increased to 0.28 indicating only a fair agreement (paired-sample t-test; $p < 0.05$). For the other FAUs, no significant modification of Cohen's kappa value was observed from pre- to post-training. Interestingly, stiffly backwards ears and orbital tightening were the same FAUs that showed the highest inter-observer reliability (ICC) in the previous studies that had a smaller number of trainees with and without horse experience (11,19). A possible explanation for this result is that these two FAUs seem rather easy to assess and robust.

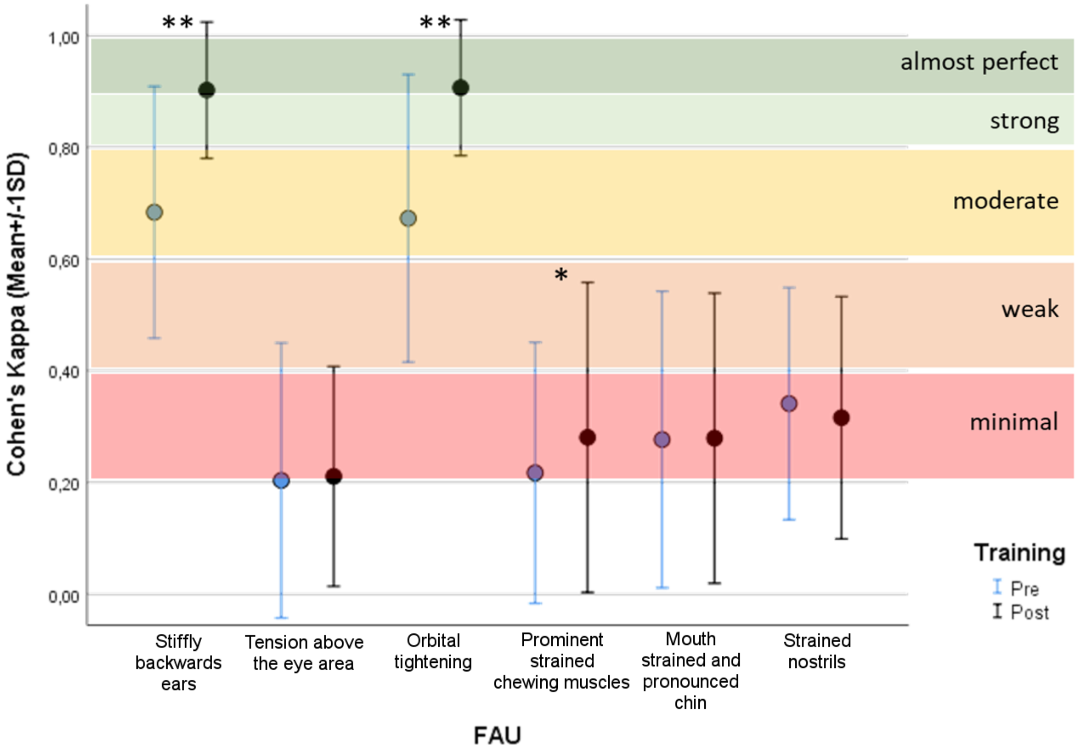


**Figure 2.** Mean ± SD of Cohen's Kappa values between students and an HGS expert pre- and post- training. Paired Sample T-Test, **p<0.001 *p<0.05

The training method utilised here obtained a significant improvement in the agreement between naïve observers and the expert for three out of six FAUs. A possible explanation for the lack of change in the remaining FAUs could be image quality, which can be defined as "the weighted combination of all of the visually significant attributes of an image" (22). High-quality pictures are required to more easily allow observers to identify the characteristics of each FAUs and detect differences between scores effectively; in our study, pictures were obtained from clinical settings with different lighting, sharpness, noise, contrast, artefacts and colour, so individual image quality varied. Due to the clinical setting, it was not always possible to capture each horse from the perfect angle to facilitate the most effective scoring, and this may influence the ability of the naive observers to recognise the different FAUs, in particular the above-eye area, the nostrils and the mouth. Another possible explanation is that the pictures were projected on a screen; this procedure was different from those reported in previous studies (11,13) where the pictures were presented on a monitor with high-quality resolution. In a previous study where the same images were scored by two trained veterinarians, lower ICC scores were recorded for the same FAUs (11), confirming that these FAUs could be more difficult to score. The FAU descriptions used to train the observers were those reported by Dalla Costa and colleagues (13), and so more detailed descriptions maybe needed to better clarify each FAU for a naïve assessor with no horse experience. Considering these results, including videos and live scoring could be a more effective training for improving the reliability of these FAUs. Vasseur and colleagues demonstrated that an in-depth description of each body condition score is needed to obtain a high inter-observer reliability, and that the use of a simple chart was not enough to assure assessor agreement (16). The same study also highlighted the need for observers to be exposed to “extreme” examples of the scores (e.g., Body Condition Score = 1 and Body Condition Score = 5) to allow the observers to differentiate extreme from normal conditions (16). In this study, we showed example pictures illustrating the different scores during the training; however, the number of pictures may have been insufficient for the naïve observers to clearly differentiate and memorize the different characteristics of each FAU. Since the goal of our study was to investigate the efficacy of a short face-to-face training, we chose only 30 minutes. This period may not have been long enough to allow observers without horse experience to effectively internalize the methods and efficiently apply them. In addition, the large number of observers per class did not allow a deep one-to-one exchange between each observer and the trainer. As a consequence, when using facial-expression-based scoring in a clinical situation, training should be planned in order to ensure new assessors’ competency in the field. As it has been demonstrated that the sole use of educational material (images) as a training tool is insufficient (16), mixed methods of training, using both pictures and live animals during the scoring process, may provide better results in term of inter- and/or intra-observer reliability (16,23,24). Gibbons et al. (23) highlighted that if trainees do not meet a target level of agreement, they should not be used for on-farm data collection, in research or in commercial farm evaluation. More needs to be done to design a training protocol for HGS, which could be applied to prepare new assessors without horse experience to ensure reliable assessment of the HGS and pain.

## 4. CONCLUSIONS

Our results suggest that the training program applied could represent a starting point for a more comprehensive training program for observers without horse experience in order to teach them how to reliably apply HGS. However, a dedicated picture collection composed of high-quality and uniform pictures, and a more extensive training program involving a lower number of observers per trainer, may be necessary. Finally, a session in which observers can practice scoring live animals seems fundamental for improving the accuracy of in-field pain evaluation.

**Author contributions:** Conceptualization, E.D.C and M.M.; Methodology, E.D.C and M.M.; Software, E.D.C. and F.D.; Formal Analysis, E.D.C.; Investigation, E.D.C., F.D., M.C.L. and A.M.R.; Data Curation, E.D.C.; Writing – Original Draft Preparation, E.D.C. and F.D.; Writing – Review & Editing, M.M., M.C.L. and A.M.R.; Supervision, M.M.; Project Administration, M.M.

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## 3.4 Does housing system affect horse welfare? The AWIN welfare assessment protocol applied to horses kept in an outdoor group-housing system: the "parcours".

Francesca Dai, Emanuela Dalla Costa, Michela Minero, Christine Briant

*Submitted to Animal Welfare on 04/09/2020*

Abstract. Outdoor-group housing is generally reported to be beneficial to the welfare of horses compared to single boxes as it is considered to be more similar to the living conditions of feral horses and it allows the full expression of behaviours such as grazing, social interactions and free movement. However, concerns regarding the ability to maintain a good nutritional state and the possibility of getting injured have been raised. No data reporting a comprehensive assessment of welfare for horses in outdoor group-housing systems is currently available. The present study aimed at applying a scientifically valid welfare assessment protocol to horses group-housed outdoor with the system "parcours", a particular management system used in the south of France. "Parcours" are semi-natural areas, grazed by domestic herbivores located in areas of lowland, mountain or marsh. 171 horses over 1 year owning to six farms and kept on "parcours" were evaluated by a trained veterinarian assessor using a modified version of the second level Animal Welfare Indicators (AWIN) welfare assessment protocol for horses. Results were considered in relation to a reference population of horses housed in single boxes evaluated with the same protocol. No major welfare issues were detected. Compared to single stabled horses, horses in "parcours" showed less abnormal behaviours such as stereotypies, could move freely for most of the day and could interact with conspecifics, maintaining at the same time a good nutritional state and a good relationship with humans. However, the main welfare concerns compared to horses in single boxes were related to the presence of superficial integument alterations such as alopecia, difficulty in reaching quality-controlled water sources and lack of shelter. The number of facilities involved in this study is relatively limited, further harmonised data collection should aim to enlarge the sample and allow comparison with different outdoor-group housing conditions.

Keywords. horse, welfare, parcours, AWIN, group-housing, management

1. INTRODUCTION

Thanks to their adaptability, horses are bred for many different types of activity (e.g. breeding, non-competitive recreational riding, leisure and sport, education) and kept in different housing and management conditions that could potentially influence their welfare (1–3).

The most common housing system is single boxes (4,5); it is reported that the proportion of sport horses stabled in single boxes ranges from 32 to 90% in different

Countries (3,6–9). It is remarkable that single-box housing is reported by welfare scientists to be detrimental for horse welfare (10) as confinement prevents horses from satisfying highly motivated behaviours such as movement (11) and social relationships (12). As herbivores, grazing occupies up to 16 hours of the feral horse's day (13–15), while in box housing systems the daily ration given can be consumed in less than 3 hours and often contains few forages (16); a decreased exposure to pasture is reported to be a risk factor for the onset of colic (17). Horses are a social species (18), but single boxes prevent them to freely interact with conspecifics so they cannot form cohesive social bonds. Dalla Costa and colleagues (2017) highlighted that only 9.8% of horses in Europe have the possibility to nibble and partly groom with conspecifics and 22.3% do not have any possibility of social contact, neither visual nor olfactory (1). As a consequence of frustration of fundamental needs imposed by these housing conditions, a high proportion of horses develop some kind of undesired behaviours (16,19): the reported prevalence of stereotypies in horses kept in box ranges from 14.4% (10) to 32.5% (16).

Outdoor group housing (e.g. paddock or pasture) could be seen as more similar to feral horse living conditions. Many authors state that the more the housing system is similar to an animal natural environment the more the animal would enjoy good welfare (see (20) for a review). In fact, housing conditions similar to those enjoyed by feral conspecifics allow animals to perform species-specific behaviours freely, but, on the other hand, could threaten their welfare by enhancing the possibility to develop injuries and illnesses (20,21) and reducing human-animal bond. As for horses, outdoor group housing is generally considered less practical for the caretaker, and potentially dangerous for horse health (2): by stabling their horses, owners perceive to better manage nutrition, parasite control, coat care, protection from atmospheric agents, while reducing the risks of aggressive interactions with other horses and the need for the horse to work for food (2). However, to date no scientific data reporting a global assessment of welfare for horses in outdoor group-housing is available. In south of France, a particular outdoor group-housing system named “parcours” is traditionnaly adopted. “Parcours” are semi-natural areas, grazed by domestic herbivores; they are spontaneous plant formations of lawns, moors and woods located in areas of lowland, mountain or marsh. Here horses can eat grass, but also leaves or tree branches. Therefore, horses can contribute to the maintenance of these uncultivated areas and can participate in the prevention of vegetation fires. Thus “parcours” are considered environmentally sustainable, but an evaluation of the welfare of the horses kept in this management condition is necessary.

Horse welfare assessment could be based on the collection of animal-, resource- and/or management-based indicators. Animal-based indicators relate directly to the animal itself rather than to the environment in which the horse is kept (22), therefore these indicators can be collected in different housing conditions and used to infer how the animal is affected by external factors such as housing system. The AWIN welfare assessment protocol for horses, based on the Welfare Quality® principles and criteria, includes 25 animal-, resource- and management-based indicators (23). Some adaptation of the AWIN protocol were suggested by the authors for assessing the welfare of horses kept in groups, however, to authors knowledge, no specific data collection using the AWIN protocol on horses group-housed outdoor was published.

The aim of the present work was applying a complete and comprehensive welfare assessment method to horses housed in a particular outdoor group-housing system, the so-called “parcours”.

## 2. MATERIAL AND METHODS

### *2.1 Horses and facilities*

Six farms in Région Sud Provence-Alpes-Côte d'Azur (France) were visited between June and November 2019. Selection criteria for the facilities were: being located in the Région Sud of France, adopting an outdoor group-housing system like a “parcours” all the year-long, keeping at least ten horses older than 1 year. All the selected facilities were contacted over the phone and participated in the study on a voluntary basis. On each farm, all the horses older than 1 year were included, for a total of 171 assessed horses. When foals (<1 year old) were kept together with adults, they were not evaluated. Assessed animals were 1-25 years old (mean=8,95±6,65), of both sexes (M=61; F=86; G=61; Fpr=10), of different breeds (Arabian=117, Angloarabian=1, Merens=12, Camargue=37, NA=4) and kept for different purposes (endurance=102, leisure=1, breeding=52, retired=6, NA=10). Horses were in groups of varying sizes (mean 5,76±3.62 individuals per group) and on areas of varying sizes (from less than 1 ha to more than 500 ha). A total number of 33 groups was assessed.

### *2.2 Welfare assessment*

The second level of the AWIN Welfare assessment protocol for horses (24) was adopted. In order to adapt the assessment protocol to the outdoor group-housing system, the assessment protocol was modified: a total of 22 animal-based indicators and 4 resource-based indicators was included (table 1). A veterinarian, experienced in horse behaviour and welfare evaluation, performed all the assessments.

### *2.3 Statistical analysis*

Data collected on-farm were reported in an Excel file and subsequently analysed using SPSS statistical package (IBM Corp., 2019). Descriptive statistics were performed; the proportion of satisfactory or unsatisfactory scores for each welfare indicator was calculated.

**Table 1.** Welfare assessment protocol applied (modified from (24)). *Results of these indicators are not presented in the paper

| Welfare principles | Welfare criteria | Welfare indicators |
|---|---|---|
| Good feeding | Appropriate nutrition | Body Condition Score<br>Management based: forage availability |
| | Absence of prolonged thirst | Resource-based: clean water availability |
| Good housing | Comfort around resting | Resource-based: shelter availability, bedding, turnout time |
| | Thermal stress | Signs of cold stress (shivering, apathy, huddling) or hot stress (increased frequency/depth of respiration, flared nostrils, profuse sweating, apathy)* |
| Good health | Absence of physical injuries | Integument alterations, swollen joints, lameness, prolapse |
| | Absence of disease | Hair coat condition, discharges, abnormal breathing, coughing |
| | Absence of pain and pain induced by management procedures | Horse Grimace Scale (HGS), signs of hoof neglect, lesions at mouth corners |
| Appropriate behaviour | Expression of social behaviour | Positive and negative social interactions*<br>Resource-based: possibility of social interaction |
| | Expression of other behaviours | Stereotypies |
| | Good human-animal relationship | Human-animal relationship tests (Avoidance Distance Test, Forced Human Approach test) |
| | Positive emotional state | Qualitative Behavioural Assessment (QBA)* |

*2.4 Ethics*

This study was conducted in compliance with the Directive 2010/63/EU of The European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and followed the requirements of the International Society for Applied Ethology (ISAE). The study received approval from the Comité d'éthique de Val de Loire (number CE19-2020-1908-1). No animals underwent more than a minimal distress; all the procedures were conforming to a routine assessment as in good farm practices.

Verbal informed consent was gained from the farmers prior to taking part in this research. Written consent was deemed unnecessary as no personal details of the participants were recorded.

3. RESULT AND DISCUSSION

The results of the welfare assessment will be reported and discussed for each welfare principle ("good feeding", "good housing", "good health" and "appropriate behaviour"), and compared to those already collected using the AWIN welfare assessment protocol in 355 single stabled horses in Italy and Germany and published by Dalla Costa and colleagues (1).

*3.1 Good feeding*

As regards the principle "good feeding" (figure 1a), most of the assessed animals enjoyed appropriate nutrition (body condition score=3, 59.6%). Extremes were rare (BCS=1; 1.17% and BCS=5; 1,17%). While not perfectly fit, most of the horses were overweight (BCS>3; 31%), rather than underweight (BCS<3; 9.4%). Most of the overweight horses had a BCS=4 (29,82%). Our results are in line with what previously observed in single-box housed horses (1,3), suggesting that group-housing in semi-extensive condition like "parcours" does not represent a risk factor for poor nutrition. This result confirms what was suggested by Souris and colleagues (13), who observed that horses released in a natural environment with temperate climate are able to adapt their daily intake according to pastures availability and climate changing, maintaining a good body condition score or improving it. However, as reported by Dalla Costa and colleagues (5) "excellent body condition in a horse does not necessarily mean that foraging need is fulfilled", which is not the case of group-housing at pasture. In fact, this housing condition allows horses to express natural grazing behaviour, satisfying the behavioural need to forage (25). The restriction of this behavioural pattern and the reduced time dedicated to feeding imposed by box-housing are considered to be risk factors for stereotypies (see (26) for a review) and colic development (17). While avoiding the risk of under-nourishing horses, it is important to keep in mind that excessive body fat is responsible for both health problems (such as insulin resistance, colic, laminitis) and loss of performance (27–30). Five over six farms in our study also gave horses access to hay, in addition to pasture.

This may represent the reason for the high percentage of overweight horses in our sample. Owners may desire to administer hay to guarantee an adequate food intake; however, when grazing is permitted, this supplementation may not be necessary and, in fact, put the animal at risk of increasing weight.

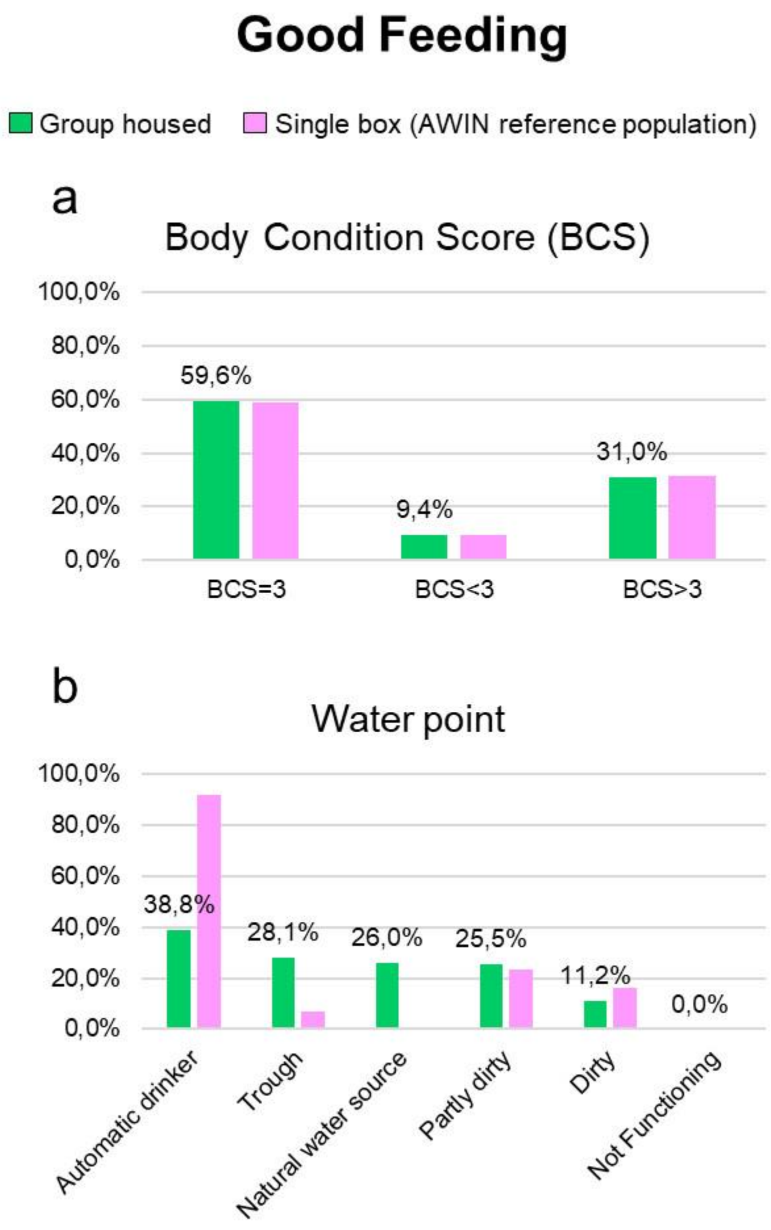


**Figure 1.** Results of the welfare assessment (% of horses) related to the principle “good feeding” in group housed horses and horses in the AWIN reference population. a) Body condition score; b) water availability

Horses had free access to a water point (figure 1b), which could be an automatic drinker (38,78%), a trough (28,06%) or a natural source of water (26,02%). In 7,14% of cases it was not possible to find and check the water point, probably due to the large dimension of the pasture. When a water point was available, 27,55% of horses enjoyed clean water, while 25,51% had access to a partially dirty water point (water point dirty but water clean) and 11,22% to a dirty one (water point and water dirty). These results are comparable to those of single stabled horses, where partially dirty and dirty were 24.5% and 17.5%, respectively (1). It is important taking into consideration that the daily inspection and cleaning of water points in very large pastures may be challenging, while boxes are easily accessible. An important aspect is drinkability and accessibility of water points, especially when the only source of water provided is a natural one. The water quality in this case should be checked, to ensure that it has appropriate standards of drinkability, also in terms of chemical, physical, and biological characteristics. Cleanliness of water is of paramount importance, since it is well known that horses tend to refuse to drink dirty water (31); furthermore, water troughs and buckets should be cleaned regularly because shared water sources are a common means of spreading diseases (32). Another aspect to take into consideration when dealing with horses kept on pasture is the water temperature in the through: cold water in winter and warm water in summer can cause a decrease in water consumption (33), which is reported to be the primary predisposing factor for impaction colic (34).

*3.2 Good housing*

As regards the principle "good housing", the situation observed in the present study is obviously very different from the one observed in the AWIN population. All the evaluated horses had the possibility to move freely during the entire day, while 48% of the horses in the AWIN population had less than 2 hours a day of turnout time (figure 2a), meaning that they spent almost the entire day inside the box with no possibilities to freely exercise. The limitation of freedom of movement is detrimental for horse welfare, since movement is a highly motivated behaviour (11). One possible concern for horses kept on pasture is the possibility to shelter from bad weather conditions (35). In our sample, 10.7% of horses had no access to a shelter (figure 2b). The absence of a shelter represents an important risk factor for horse welfare: thermo-neutral zone for horses is estimated to be in a range from 5° to 25° C (36), when environmental temperature differ from this range, thermoregulation is achieved by changes in behaviour, including shelter-seeking (35,37). Several studies demonstrated the need for horses to access a shelter during raining or windy days (38–42). Shelter seeking is observed also in hot sunny days (43,44). Thermoregulation is not always the main motivation factor for sheltering: horses prefer to use a shelter also to reduce the insect harassment (45,46). The majority of horses in the present study had access to a shelter: natural, such as trees (83.3%), or artificial (1%). Although one publication (35) reported that when having the possibility to choose horses prefer artificial shelters over natural ones, particularly in cold and rainy conditions, it seems difficult to provide artificial shelters when horses move over large areas, like in our study. It has to be taken into consideration that natural shelters are more similar to natural environment and that when only natural shelters are available, horses prefer to spend time under dense vegetation (47).

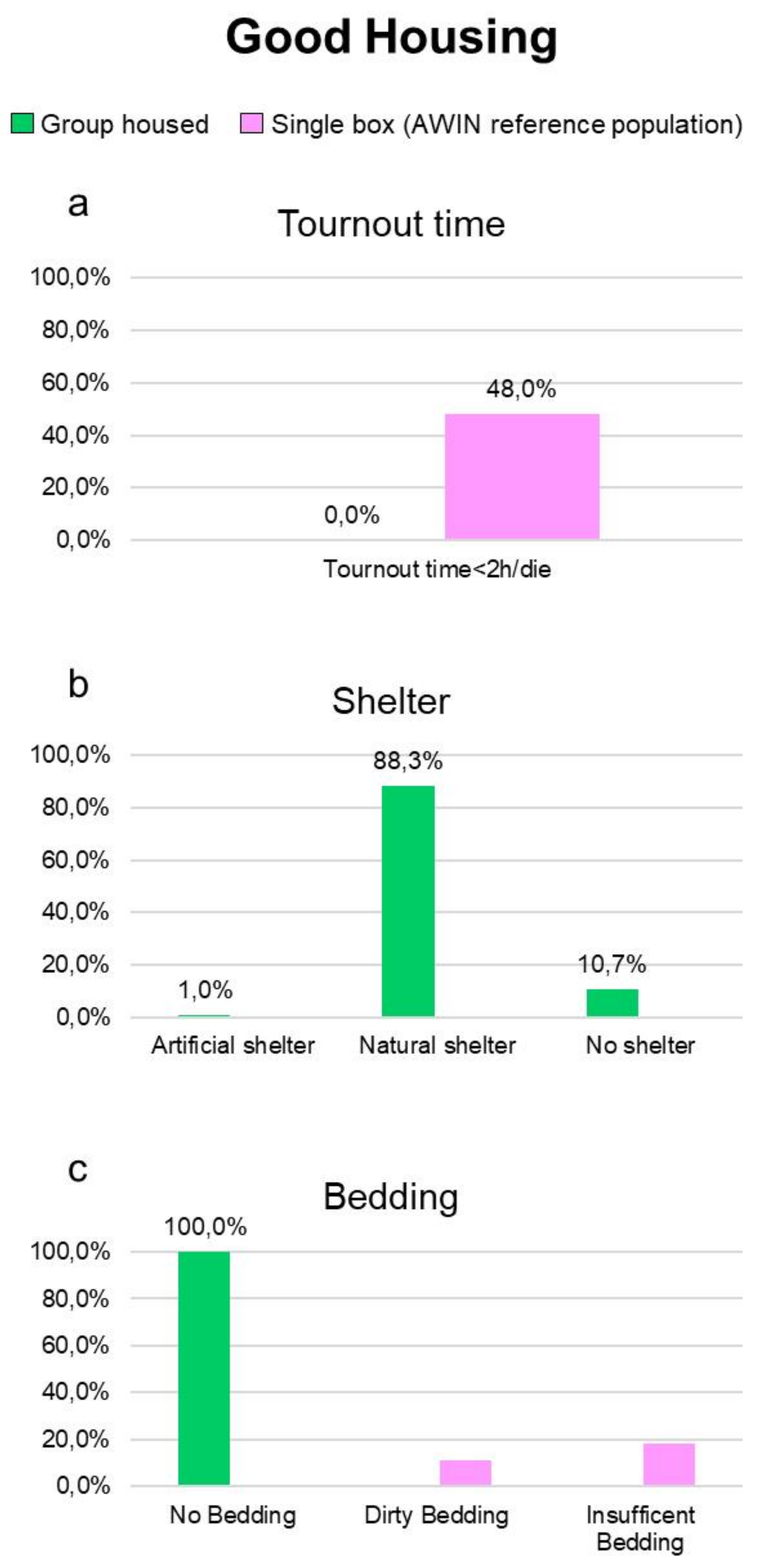


**Figure 2.** Results of the welfare assessment (% of horses) related to the principle "good housing" in group housed horses and horses in the AWIN reference population. a) turnout time; b) shelter availability; c) bedding

None of the visited farms used bedding, while only 0.3% of horses kept in box in the AWIN population had no bedding (figure 2c). It could be hypothesized that owners of horses kept on “parcours” did not consider useful providing a bedding, since horses had the possibility to choose the more comfortable place to lie down. It is worth noticing that bedsores were not observed (see paragraph “good health”).

*3.3 Good health*

The evaluated horses generally enjoyed good health and none of them presented severe health conditions; results are analogue to those found in single box housed horses (figure 3).

While we did not perform any clinical examination on assessed horses, indicators such as coughing, abnormal breathing, nasal discharge and ocular discharge were chosen since they are well recognised symptoms of diverse respiratory problems (48). Respiratory problems are reported to be common in horses kept in single boxes with a prevalence that range between <3% and 16.9% (3,6,49). In “parcours” housed horses, we found that 1.8% (3 out of 171) of horses showed dyspnoea, 1.2% (2 out of 171) coughing, and 6.4% (11 out of 171) showed clear serous nasal discharge, while none presented purulent or hematic discharge. Even though this prevalence is higher than what found in the reference population, it is lower than the ones reported for stabled horses by other authors (3,6,49). Since, respiratory problems have been associated with the housing system, stable hygiene practices and bedding choice (48,50), our results can be explained in the light of the low ammonia levels, dust concentration and fungal presence in an open-air environment. Similarly, we observed a low prevalence of ocular discharge (10.5% of horses, 18 out of 171) and no individuals had a thick, purulent or hematic discharge. Visser and colleagues (2014) reported a prevalence of 20% of ocular discharge in stable housed horses and identified as risk factors the number of horses housed in the same stable and the absence of an air outlet.

Lameness is generally considered to be a common cause of welfare impairment in horses: the reported prevalence of lameness in stabled horses ranges between 13% and 33% (3,51–53). In the present study, we identified a much lower prevalence of lameness (2.3%). The cause of lameness was not investigated, but it is worth noticing that 1 horse showed also swollen joints and 3 horses had some signs of hoof neglect, which can be responsible for the lameness. Several risk factors have been recognised for lameness: age (older horses are more at risk of lameness), current use of horse (being used for riding school or recreation increases the risk of lameness), back pain caused by inappropriate saddle, foot problems, training regimen (using only one surface for training increase the risk of lameness) (3,19,51). It could be hypothesised that horses kept in “parcours”, having more possibility to exercise freely on different grounds, developed a musculoskeletal system better adapted to different surfaces and exercises, consequently reducing the risks of injuries during sport activities. Murray and colleagues (51) also identified the lack of warm up before exercise as a risk factor for musculoskeletal injuries and consequently lameness. Horses kept on “parcours”, having the permanent possibility to move, could perform a “natural warm up”, decreasing the risk of lameness.

While in the AWIN population the majority of horses had no skin lesions, in the present study only 29.8% of horses presented an intact skin. Noteworthy, most of the horses (53.8%) had alopecic areas, while 23.4% presented superficial lesions and 0.6% deep wounds. Causes of skin lesions were not investigated, but alopecia is often related to itch which may be caused by insect biting, ectoparasites or allergic reactions. In fact, 18.8% of horses presented swellings on the skin, probably related to insect biting. Flies control at pasture horse may represent a challenge for owners; some management practices (such as the use of repellents, fly traps, protective masks and/or rugs, and the frequent removal of dung) may help in reducing flies’ bites (46). However, it should be noted that only a limited number of products are currently approved for treatment of ectoparasites in horses, meaning that these products should be used judiciously with special emphasis on the safety of these products for horses, people and the environment (54). Superficial lesions may be caused by scratches on branches or rocks, or by aggressive interactions with other horses. One of the major concerns which prevent owners from keeping their horses in group is the possibility of aggressive behaviours, causing lesions or restricted access to fundamental resources (2). However, several studies demonstrated that the level of aggression significantly decrease with the increasing of group membership stability (55–57) and with increasing the available area/horse to at least 300 $m^2$ (58). Indeed, group stability, similarly to what happens in feral horses (59,60), allows stable dominance relationships and stable networks of friendships, reducing the number of aggressive interactions among members (55,56,61–63). Vice versa, when changes in group composition are recurrent, the number of interactions, mainly agonistic ones, is high (62,64). This is explained by the fact that in stable groups each individual is aware of the social network and, consequently, the aggression is ritualized (56,65,66). Therefore, the risk factor for injuries should not be considered the group housing per se, but the lack of group stability (62). In our study the number of aggressive interactions/horse/hour was 0.76+1.2 (data not presented) which seems rather low compared to data reported for horses observed in semi-natural conditions (58) and the average area/horse was much greater than 300 m2. Therefore, these two factors do not seem to be the main causes of the many observed skin lesions. It could be hypothesized that, compared to owners who keep their horses in boxes, owners keeping horses at pasture are less attentive to hoof care, especially when horses are not used on a daily basis for sport activities. However, only 5.8% of horses (10 out of 171) in our sample presented some signs of hoof neglect; this result is comparable with the prevalence observed in the AWIN reference population (3.1 %). Regardless of housing system, daily care and regular routine farriery are fundamental, since neglect of these practices predisposes to the development of foot problems (67–69).


**Figure 3.** Results of the welfare assessment (% of horses) related to the principle “good health” in group housed horses and horses in the AWIN reference population. a) cough and dyspnoea; b) nasal and ocular discharges; c) coat condition; d) lameness; e) signs of hoof neglect and swollen joints; f) HGS; g) skin lesions

Horse Grimace Scale is a facial-expression-based pain coding system (70) which can be considered a specific tool to assess pain in horses (71) and easily applicable by non-expert observers (72). An HGS value⩾2 is considered to be an indicator of pain (73). In the present study, the HGS score was ⩾2 in 2.3% of cases (figure 3f), this is similar to horses kept in single boxes; thus, confirming that horses were regularly checked for possible pain-related conditions. It is important to underline that in 4.6% of cases, it was not possible to assess HGS (NA), meaning that horses were not close enough to permit an accurate scoring or they were wearing masks that partly covered horse's head.

*3.4 Appropriate behaviour*

Figure 4 reports results regarding the principle "appropriate behaviour". Regarding social interaction (figure 4b), horses were kept in groups of different dimensions (mean 5,76±3.62 conspecifics); ten groups included foals and nine groups included stallions. Only one stallion (0.6%), used for reproduction, was kept alone; he had possibility for visual and olfactory contacts with other horses. In the AWIN population, it was reported that 22.3% of stabled horses had no possibilities for visual or physical contact, while Hockenhull & Creighton (8) reported that in UK it is 3%. Being horses a social species, social interaction with conspecific is a behavioural need. The restriction imposed by housing conditions is deemed responsible for the development of a range of abnormal behaviours, such as stereotypies (16,19). Previous studies reported a prevalence of stereotypies in horses kept in box ranging from 14.4% (10) to 32.5% (16), while in the present study we observed only 1.2% of stereotypies (2 horses out of 171) (figure 4a). A recognised risks factor for stereotypies development is the frustration of fundamental needs (e.g. grazing, movement, social relationship) (16,19); being housed in group and having permanent access to pasture can therefore explain the low prevalence of stereotypies observed in the present study.

A possible concern preventing owners from keeping their horses at pasture is the difficulty in catching them (2). Differently from what expected, 64,3% of horses in our sample showed no avoidance reactions when approached by the unknown assessor, similarly to the box-housed horses included in the AWIN population (figure 4c). Moreover, 48.5% of horses showed positive responses to the Forced human approach test. While unwilling to interact with a stranger, 32.2% of horses showed avoidance reaction, only 2.3% showed some aggressive behaviours; only on for 17% of horses it was not possible to perform the test, because they went away from the observer during the Avoidance distance test. While a larger proportion of horses in the AWIN population exhibited positive reactions to the test (figure 4d), it is worth noticing that the horses included in the present study preferred to move away from the observer, when not willing to interact, instead of being aggressive, thus potentially reducing the risks for human injuries. Similar results were obtained in a previous study comparing two groups of ponies, kept in group on a pasture or housed in individual boxes, in restricted conditions (74). Avoidance from an undesired stimulus is a natural behaviour for a prey species and suggests that observed horses had the perception of being able to control their own environment, deciding when to interact instead of feeling forced to interact with humans. Interactions with owners were not formally noted, however the assessor observed that most of the horses were more friendly with the owner and showed less frequently avoidance reactions. To overcome the catching difficulties potentially perceived by owners (2), a specific training to teach the horse to come when called using non aversive methods may be useful (75). Training could also help in simplifying horses' daily inspections.

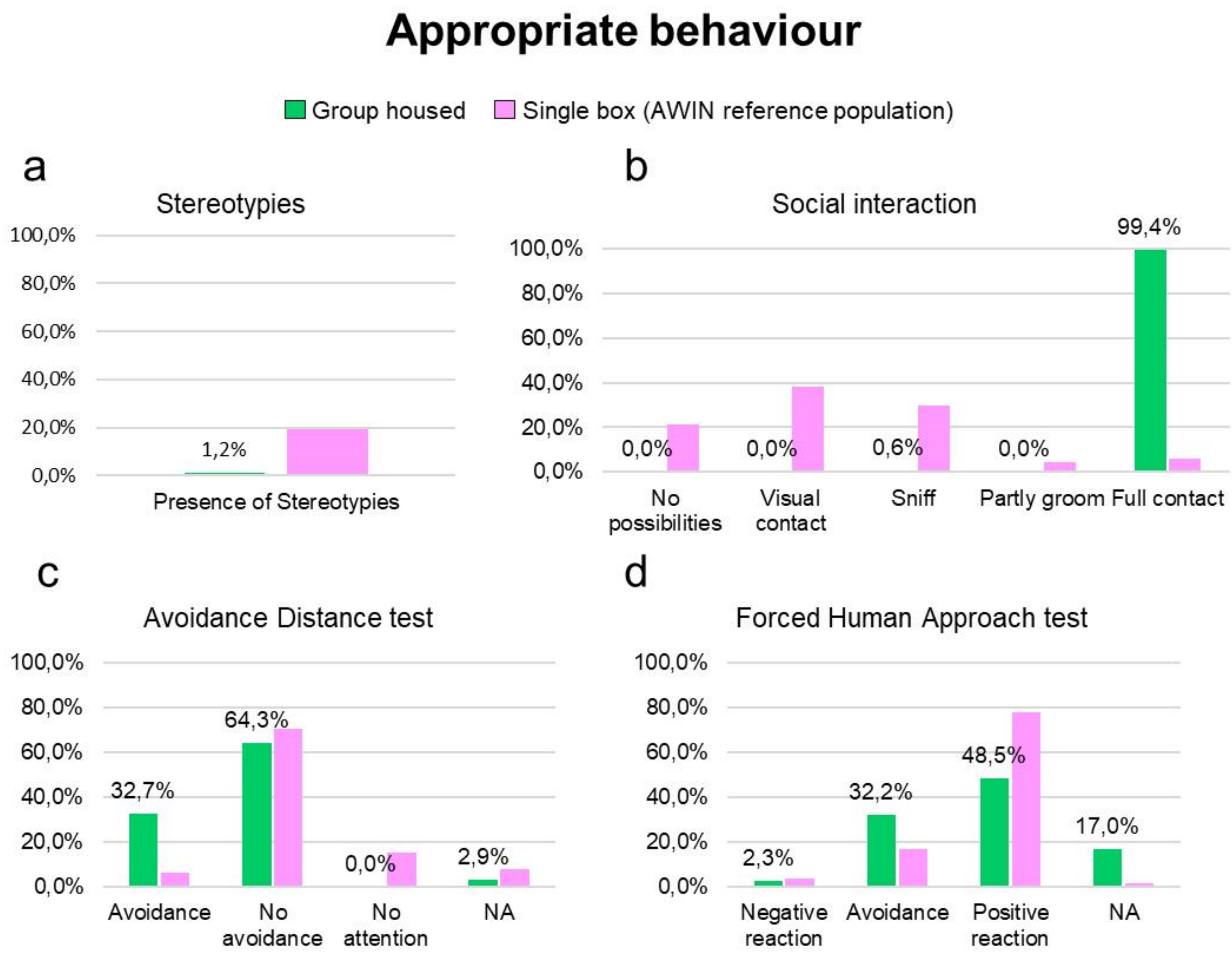


**Figure 4.** Results of the welfare assessment (% of horses) related to the principle "appropriate behaviour" in group housed horses and horses in the AWIN reference population. a) stereotypies; b) possibility of social interaction; c) Avoidance Distance test; d) Forced Human Approach test

## 4. ANIMAL WELFARE IMPLICATIONS

Single box is reported to be the most common housing system for horses, even though it prevents horses from satisfying highly motivated behaviours such as free movement and social interactions. While outdoor group housing could be seen as more similar to feral horse living condition, it is considered to increase the possibility to develop injuries and illnesses. This study reports, for the first time, results from a comprehensive welfare data collection carried out on group-housed horses on “parcours” and comparisons with data collected on single-stable horses. The reported outcomes can help in creating a common database on horse welfare status and understanding underlying relations with housing conditions and management.

## 5. CONCLUSIONS

The application of a complete and comprehensive assessment method to evaluate the welfare of group housed horses kept on “parcours” was proved to be useful and gave the possibility to compare results to those collected on single stable horses. The findings reported confirmed that the overall welfare was good; in particular, group housed horses showed less abnormal behaviours such as stereotypies, could move freely for most of the day and could interact with conspecifics, but at the same time they maintain a good relationship with humans. The main welfare concerns compared to horses in single boxes were related to water source, lack of artificial shelter and presence of superficial integument alteration such as alopecia, probably linked to difficulties in external parasites control. As in single boxes, overweigh was observed in a significant proportion of horses (especially in those facilities where hay was administered in addition to natural resources).The facilities involved in this study were limited in terms of numerosity and geographical location; for this reason, the sample does not necessarily represent the welfare status of all horses kept on “parcours” or in pasture. Following the same approach, further harmonised data collection is forecast to enlarge the sample and may be include other housing conditions.

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# EFFECT OF TRAINING IN REDUCING TRANSPORT-RELATED STRESS

# 4. Effect of training in reducing transport-related stress

## 4.1 Brief Introduction to the Scientific Studies

Equids are among the most transported animals in Europe. Animal welfare could be seriously compromised during transport procedures. With this in mind, the studies included in this section aimed at developing a non-aversive training method to teach meat horses and donkeys to load. The hypothesis was that effectively training equine to loading procedures before their first exposure to transport mitigates the transport-related stress. Proposed methods have been discussed in term of on-farm feasibility, efficacy in training the animals and efficacy in reducing transport stress using both behavioural and physiological indicators.

The section includes three scientific papers:

1) In the first study, a positive reinforcement-based training for self-loading has been applied on meat horses; comparing trained horses with a control group, stress-related behaviour during loading procedures and time needed to complete loading was evaluated to judge the efficacy of the training.
2) In the second study, a new physiological indicator of stress response (Chromogranin A) has been evaluated in donkeys, to assess its efficacy in detecting transport-related stress in a non-invasive way.
3) In the third study, the focus has been posed on habituation, proposed as a more feasible training method on farm, being relatively effortless for the farmer. Habituation to transport has been assessed for its ability to reducing transport-related stress in donkeys.

## 4.2 Positive Reinforcement-Based Training for Self-Loading of Meat Horses Reduces Loading Time and Stress-Related Behavior

Francesca Dai, Alessandro Dalla Costa, Lebana Bonfanti, Claudia Caucci, Guido Di Martino, Roberta Lucarelli, Barbara Padalino, Michela Minero

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Abstract. The present work aimed to evaluate the efficacy of a self-loading training using positive reinforcement on stress-related behaviors shown by meat horses during loading procedures into a truck. Thirty-two meat horses (M = 18; F = 14; 6 month-old) were included in the study. All horses had limited interactions with the farmer and were not used to be restrained nor lead by halter. Horses were divided in two groups: Control Group (C; N = 14) and Training Group (T; N = 18). T horses were trained to self-load: in order to teach the horses to enter into the truck, a targeting training technique throughout a shaping process was applied. Training sessions were performed three times a week, from 9:30 a.m. to 1:30 p.m. and from 2:30 p.m. to 4:30 p.m., for 6 weeks; training was then repeated once a week to maintain the memory until the transport toward a slaughterhouse. The loading phase was video-recorded and loading time was directly recorded using a stopwatch. All horses were transported to the same slaughterhouse in 14 different days using the same truck. Behavior was subsequently analyzed with a focal animal continuous recording method. Loading time was shorter in T horses (mean ± SD = 44.44 ± 47.58 s) than in C horses (mean ± SD = 463.09 ± 918.19 s) (T-test; p = 0.019). T horses showed more forward locomotion toward the truck than C horses (T-test; p = 0.029). Our preliminary findings suggest that self-loading training may be useful tomitigate loading-related stress inmeat horses,minimally socializedwith humans.

Keywords: horse, transport, welfare, training, behavior

### 1. INTRODUCTION

Loading is considered one of the most stressful stages of animal transport (1, 2), involving new experiences such as being handled by humans, being mixed with unfamiliar animals and entering a novel environment (the vehicle) (3). Several studies highlighted the relationship between loading and transport-related problems in sport horses: many horses exhibit strong reactions during loading, which can lead to injuries to the handlers (including rope burns, lost fingers, broken bones, or bruises, and bleeding) and to the animal (including lacerations to the head from banging into the trailer, scrapes and cuts on the legs, broken legs from falling, or even a broken back if the animal falls backwards) (4). Horses subjected to transport stress can be more susceptible to a number of disorders, such as pneumonia, diarrhea, colic, laminitis, injuries, and rhabdomyolysis 5), which, not only severely affect their welfare, but can also be costly for the owner. Whilst only few studies concentrated on the incidence of transport related stress on equine meat quality (6, 7), a large body of research describes how transport stress negatively affects meat quality in several other species [see for review (8, 9)].Not only loading problems are a source of stress for the animals, but also costly in time for the personnel involved in the loading, endangering the economic benefit for the owner (10, 11). Preparation of animals to transport through the adoption of appropriate management measures plays an important role in mitigating transport-related stress (3). Reducing pre-transport stress decreases the probability of compromising animal welfare during the transport phase (3). Among suggested pre-transport measures reported in the literature, we can find: adequate route planning (12), proper evaluation of animal-related factors, such as species, breed, age, temperament, behavior, and health status (3), appropriate handling during loading and unloading (i.e., collection of animals, weighing, loading, penning should be done in calm and gentle manner to minimize stress) (3). Rearing conditions and previous experiences, both with handling and with transport procedures, have a high impact on the stress response of animals during handling at loading (13–15). In sport horses, studies suggest that habituation to loading and traveling significantly reduces the likelihood that horses develop transport related behavioral problems and injury (16, 17). Loading training using positive reinforcement [consisting of reward delivery in response to the desired behavior (18)] also seems to reduce loading time and stress during loading (14, 19). Finally, it has been proven that self-loading techniques reduce the likelihood of horses showing behavioral problems (such as attempting to escape, rearing, kicking, pulling back, standing still, pawing) at loading (16, 17). While there is a body of literature reporting about the effect of training to load in sport horses [i.e., (4, 11, 14, 16)], nothing has been published on meat horses completely naïve to transport. This study population deserves great attention because horses kept for meat production are generally transported to the slaughterhouse without any training [see (20–22) for a review], with adverse effects on their welfare (7, 23, 24). It was consequently hypothesized that self-loading training would reduce the time to load, stress-related behavior, and behavioral problems during loading in meat horses. The present work aimed to evaluate the efficacy of a self-loading training technique on stress-related behaviors and loading problems in meat horses loading into a truck.

## 2. MATERIALS AND METHODS

### *2.1 Farm and Animals*

The study was conducted at a meat horse farm located in North Eastern Italy. Thirty-two Spanish Breton meat horses of both sexes (M=18; F =14), aged 15±2.79months (min=12month; max = 24 months), were randomly selected to be included in the study. Horses were originally imported from Spain at 6 months of age and remained at the farm for fattening until the age of 12–24 months. At the beginning of the experimental procedures, horses had been on farm for 4 months. Upon arrival to farm, animals were randomly divided by the farmer into pens balanced for gender. Two pens were randomly selected by the researchers to enter the study (pen 1: 14 horses; pen 2: 18 horses; density = 2 m2 per horse). Groups were kept stable throughout the fattening period and the experiment. The stable had deep litter bedding and, when climatic conditions were favorable, the horses had access to an outdoor area with concrete floor (128 m2 per group). The outdoor area was connected to a load lane (14.5m long), leading to a concrete ramp (6m long, 8% of slope) and the trailer (trailer ramp: 1.5m long, 10% of slope). Horses had ad libitum access to total mixed ration and all pens were equipped with automatic drinkers. All horses had limited interactions with the farmer and were not used to be restrained, lead by halter or head collar nor transported. Horse interactions with the farmer included daily check of the animals from outside the pens and feeding using a truck. No physical interaction normally occurred.

*2.2 Training Protocol*

Animals in pen 1 were categorized as Control Group (C; N = 14) and animals in pen 2 were categorized as Training Group (T; N = 18). Horses in the Training Group were subjected to a non-aversive training to self-load. A target training using operant conditioning [a learning method occurring through rewards and punishments for behavior (25)] and positive reinforcement (a nibble of flaked corn) was applied. Target training (4) consisted of training the horses to follow the target (a yellow stick) that was progressively moved toward the truck (IVECO EUROCARGO 75E17). Training was subdivided into two phases; the first inside the stable and the second in the outdoor area. Horses were firstly habituated to receive food from the trainer’s hand, then they were reinforced when touching the target placed in front of their nose. After the horses touched the target every time for five repetitions in a row, the target was moved 50 cm in front and slightly laterally to their nose; horses were reinforced when touching the target after having followed its movement. When all the horses in the pen were able to follow the target for five steps inside the stable, they were allowed to access the outdoor area. In this second phase, a shaping technique [the differential reinforcement of successive approximations toward a target behavior (25)] was applied to train the horses to load into the truck. Horses were firstly reinforced when following the target in the load lane connecting the outdoor area to the truck; then they were reinforced only when following the target up to the start of the vehicle loading ramp. Finally, the truck was positioned at the end of the ramp with opened doors and handfuls of flaked corn on the floor. Horses were led to the ramp with the help of the target and were then left free to load; three horses at a time were trained together in order to take advantage of their gregarious behavior. When loading on the truck, horses were left free to explore it, eat the food located inside the truck and unload to return back to the outdoor pen with the other horses. The training was considered successful when the horse entered the trailer each time it was allowed to do so for 1 week (i.e., three times). Each of the two phases lasted 3 weeks; training sessions were performed three times a week, from 9:30 a.m. to 1:30 p.m. and from 2:30 p.m. to 4:30 p.m.; training was then repeated once a week to maintain the memory until the transport to the slaughterhouse. All training procedures were performed by the same experimenter (FD).

*2.3 Loading to Transport*

Horses were transported to the slaughterhouse according to the farm's ordinary routine. Two to four horses at a time were transported with the same truck used during the training phase. Transport procedures took place in the afternoon (~4 pm) on different days from April to October 2018, for a total of 14 transports. The farm manager conducted all the transport phases (loading, travel, and unloading) following the usual farm procedures. Usual loading procedures adopted on the farm involved minimal handling of small groups of horses, in order to exploit their gregarious behavior: moving fences to let horses enter the loading lane, inciting horses from behind using voice and moving a stick only when they refused to move.

*2.4 Behavioral Evaluation*

For each horse, the loading phase of the transport to the slaughterhouse was video-recorded using a digital video camera (Canon Legria HFR88), controlled by the experimenter. The ethogram used for behavior analysis was adapted from Yngvesson et al. (10) (Table 1).Horse behavior was analyzed with a focal animal continuous recording method, using the software Solomon Coder beta 17.03.22. Duration of different behaviors was recorded. The latency to load (from the beginning of the procedures until the horse had all four feet on the trailer) was directly recorded using a stopwatch.

*2.5 Statistical Analysis*

Behavioral data were analyzed with SPSS Statistic version 25 (IBM Corp.). Walk and Trot were considered as Forward Locomotion. Based on the total length of the observation of the video recordings, durations of behaviors were calculated as percentage of total observation time (proportional duration time). Descriptive statistics [median, mean, and standard deviation (SD) of proportional durations] was performed. Data were tested for normality and homogeneity of variance using Kolmogorov–Smirnov and Levene test, respectively. Behavioral data were not normally distributed; therefore, a log transformation was applied to approximately conform them to normality. A two-tailed t-test was applied to identify differences in duration of different behaviors between Control Group and Training Group. Differences between groups in latency to load were analyzed with a two-tailed t-test. As basic assumptions for the t-test (equal standard deviations) were not met for the comparison of latency to load between control horses showing stress-related behaviors with other horses of the control group, a Mann–Whitney test was used. Differences were considered to be statistically significant if $p < 0.05$.

**Table 1.** Ethogram for the evaluation of horse behavior during loading [modified from (10)].

| **Behavior** | **Description** |
|---|---|
| Forward walk* | The horse walks toward the trailer |
| Forward trot* | The horse trots toward the trailer |
| Forward gallop* | The horse gallops toward the trailer |
| Backwards§ | The horse moves away from the trailer |
| Standing§ | The horse stands on the four legs |
| Turn back§ | The horse tries to turn all its body in the opposite direction of the trailer |
| Still§ | The horse stops moving, digging in its heels, refusing to proceed |
| Rear§ | The horse rears with its front legs |
| Kick§ | The horse kicks, one or two legs is lifted and moved rapidly and forcefully |
| Mount§ | The horse mounts the horse in front of him/her |
| Paw§ | The horse rises a foreleg and scrapes the floor |
| Sniffing§ | The horse sniffs the ground |
| Defecate§ | The horse drops manure |
| Urinate§ | The horse drops urines |
| Other | Any other behavior |

**High frequencies of forward locomotion behaviors were considered to be associated to low stress.*

*§High frequencies of these behaviors were considered to be stress-related.*

## 3. RESULTS

No horses showed intense fearful reactions toward the trainer, the target nor the trailer. All horses in the training group reached the success criterion (i.e., entering the trailer each time they were allowed to do so for 1 week) at the end of the sixth week of training. Time needed to load was significantly different between the two groups (T-test; $t = -2.472$; $p=0.019$); trained horses needed significantly less time to load (min 6.4 s, max 172.8 s, median 34.3 s, mean 44.4 ± 47.6 s) than control horses (min 17.4 s, max 262.3 s, median 50.7 s, mean 463.1 ± 918.2 s) (Figure 1A). Horses in the Training group showed more forward locomotion toward the truck (walk and trot) than control horses (T-test; $t = 2.299$; $p = 0.029$) (Figure 1B). Duration of other behaviors did not differ between the two groups. However, it is worth noticing that some stress-related behaviors were manifested nearly exclusively in control group: rear (one horse in the control group, for 0.06% of the time), kick (one horse in the control group, for 0.02% of the time), mount (one horse in the control group, for 1.02% of the time), paw (four horses in the control group, for 1.39 ± 0.92% of the time, and one horse in the training group, for 7.64% of the time), defecate (three horses in the control group, for 1.71 ± 0.49% of the time). Mean loading time for horses showing stress-related behaviors was significantly higher than for the other subjects of the control group (1234.08 ± 1258.44 vs. 34.76 ± 15.83 s; Mann–Whitney Test p = 0.012).

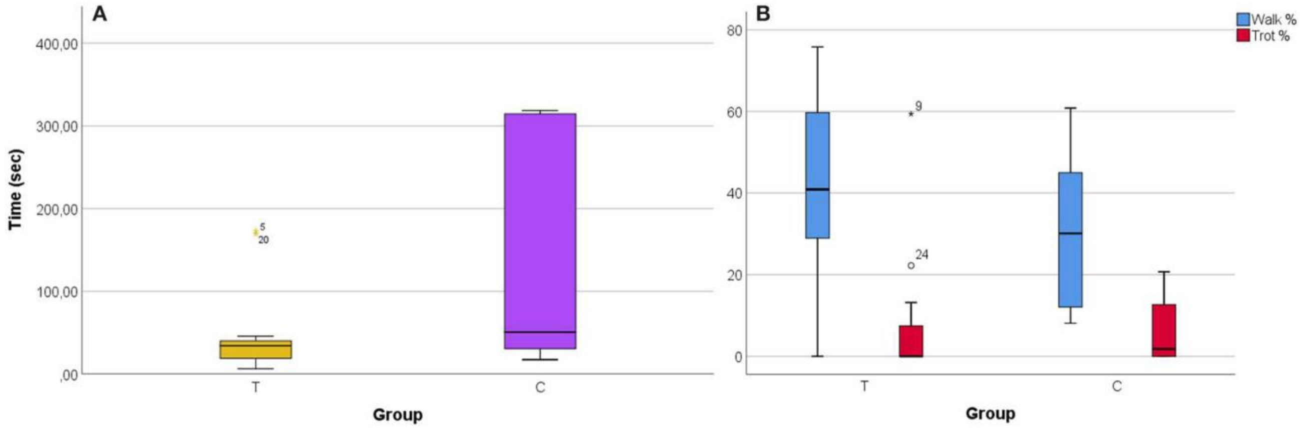


**Figure 1**. Boxplots reporting data distribution of: (A) time needed to load in Training Group (T) and Control Group (C); (B) Percentage of time spent walking and trotting in Training Group (T) and Control Group (C). The band inside the box represent the median; the whiskers represents the lowest datum still within 1.5 interquartile range of the lower quartile, and the highest datum still within 1.5 interquartile range of the upper quartile; mild outliers are presented as ◦, while extreme outliers as *.

## 4. DISCUSSION

This study evaluated the efficacy of a self-loading training using positive reinforcement on behavior and loading duration in meat horses. The results supported partially our hypothesis. Self-loading training significantly reduced the time to load and increased the percentage of forward movement (walk/trot), but we did not find a difference between the frequencies of the stress related behavior. The latter finding may be due to the fact that the horses were not completely naïve to transport, since they experienced the travel from Spain to Italy, and consequently had already been exposed to at least one loading procedure. It has been reported that the first loading experience is the most stressful and that the time to load and stress-related behavior frequencies dramatically reduce between the first and the second loading (26). Further studies should be carried out comparing self-loading trained horses with a control group of horses with no experience at all. Other limitations of this study should be taken into account while interpreting our results. As the interaction with humans is reported to be, per se, a stressful event in not accustomed animals [see (27) for a review] the different time of interaction with humans between the two groups should be considered as a limitation of this study. It would have been appropriate to include in our study a sham control group of horses exposed to the same contact time with a person as the trained horses, but without being trained. Due to time and economic constraints, this was not possible and therefore we cannot exclude that being exposed to human contact per se might have affected stress-related behaviors during loading procedures. The duration of training (lasting 6 weeks, 3 days per week) was one of the major constraints to the on-farm applicability of the outcomes of the current study, consequently our findings are applicable only to the tested protocol. Future work should envisage shorter yet effective training protocols. Finally, our sample size was relatively small, only one farm was included and there were no repetitions, thus this work should be repeated on a larger number of horses and facilities, and possibly considering additional stress indicators, to ascertain our preliminary results and to compare different loading protocols. Learning is the relatively permanent change in an animal behavior due to experience (28) and here we hypothesized that, similarly to what was found in the literature on sport horses (11, 16, 17), meat horses trained to load showed different responses to loading procedures compared to untrained control horses. The training protocol applied in this study effectively reduced the time needed to load the horse onto the truck and therefore the entire duration of the loading procedure. This result has practical implication, considering that horses that refuse to load add considerable delays in travel and increase the risk of injuries both for the horses themselves and the personnel (10, 11). Moreover, the variability of the loading duration reflected a higher inter-individual variability in the control group, confirming that trained horses responded to the situation in a more similar manner while untrained ones showed more variable responses. This result could be interpreted in the light of a reduction of the stress caused by loading procedures following training. We found relatively small differences between trained and control horses regarding exhibited behaviors. In addition to the consideration that it was not their first experience, it is worth noting that the usual loading procedures adopted by the farmer, involving minor handling and taking advantage of gregarious behavior of the horses, most likely had a positive effect in keeping the loading-related stress at a minimum in all the considered subjects, minimizing the differences between groups. However, it is also worth to highlight that some behaviors (such as rear, kick, mount, paw, defecate) recognized to be stress related (10) were displayed almost exclusively by horses of the control group. Even a very low frequency of those behaviors should be considered a risk, since they are associated with injuries both to horse and horse handlers (17). Therefore, habituation to load and self-loading training using positive reinforcement should be practiced as early as possible in meat horses, as already suggested in sport horses (11), in order to reduce the stress which the horses are subjected during transport procedures and the related risks. Notwithstanding all the limitations aforementioned, to the authors' knowledge, this is the first study documenting the effects of self-loading training in meat horses. Our results are useful to enhance the welfare of meat horses confirming what reported by a growing number of research focused on the association between training for loading/traveling and transport related behavioral problems in performance horses. Our study provides the basis for future larger-scale studies assessing the impact of loading training on the welfare of horses during the entire transport and at slaughter. It would be relevant to include the assessment of additional animal based indicators such as heart rate, eye thermographic evaluation, cortisol levels.

## 5. CONCLUSION

Self-loading using positive reinforcement seems to be effective in reducing loading time and the occurrence of behaviors indicative of loading problems in meat horses. Further studies, conducted with a bigger sample size and on several different facilities are needed in order to confirm these results. Our findings provide the basis for future studies looking into streamlining of a feasible protocol to ensure that these principles of loading meat horses are used, which will help improve the welfare of meat horses.

**Ethics statement:** This study was conducted in compliance with the Directive 2010/63/EU Of The European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and followed the requirements of the International Society for Applied Ethology (ISAE). Ethical Guidelines; no animals underwent more than a minimal distress. No animals were transported in order to record data for the purposes of this study. Transports were conducted in compliance with Council Regulation (EC) No 1/2005 of 22 December 2004 on the protection of animals during transport and related operations. Verbal informed consent was gained from the farmer prior to taking part in this research. Written consent was deemed unnecessary as no personal details of the participants were recorded.

**Author contributions:** FD, LB, GD, and MM contributed conception and design of the study. FD, CC, AD, and RL performed the experiment. FD and MM performed the statistical analysis. FD wrote the first draft of the manuscript. FD, LB, CC, AD, GD, RL, BP, and MM wrote sections of themanuscript. All authors contributed tomanuscript revision, read, and approved the submitted version.

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

## 4.3 May Salivary Chromogranin A Act as a Physiological Index of Stress in Transported Donkeys? A Pilot Study

Francesca Dai, Emanuela Dalla Costa, Simona Cannas, Eugenio Ugo Luigi Heinzl, Michela Minero, Silvia Michela Mazzola

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Simple Summary. Transportation is recognized as a stressful animal husbandry procedure, inducing both short term and prolonged effects on welfare. Donkeys are transported for several purposes; therefore, validating a reliable and non-invasive stress indicator is pivotal. This study aimed to investigate whether salivary chromogranin A (CgA) concentration, known as an index of stress in humans and pigs, could represent a novel index of transportation-induced stress in donkeys. The research involved the measurement of salivary CgA in 19 donkeys, 15 min before and 15 min after two transportations. The transportation, which took place on two consecutive days, followed the routine procedures of the farm. The analysis of salivary CgA was carried out by an enzyme-linked immunosorbent assay test. Results showed that CgA salivary levels significantly decreased after both transportations. The physiological mechanisms underlying this result may be related to catestatin acting as an inhibitor of catecholamine release; however, due to the limited number of subjects involved, this hypothesis requires further investigation.

Abstract. Road transport is known to be a stressful animal husbandry procedure as it induces the activation of two main physiological stress-related pathways: the hypothalamic-pituitary-adrenal cortex axis and the sympathetic-adrenal medulla axis. This preliminary study aimed to investigate whether salivary chromogranin A (CgA) concentration, known as a biomarker of the sympathetic activity system during psychological stress, may represent a novel physiological index of transportation-induced stress in donkeys. Nineteen Romagnolo donkeys, raised in groups on paddocks, were subject to two transportations, following the farm's routine procedures, for a mean duration of 64 min each on two consecutive days. Salivary samples were gently collected 15 min before and 15 min after each transportation. Salivary CgA was measured by a commercially available enzyme-linked immunosorbent assay test. Results showed that CgA salivary levels significantly decreased after both transportations. The physiological mechanisms underlying this result may be related to catestatin activity, a bioactive product of the proteolytic cleavage of CgA, that acts as an inhibitor of catecholamine release. This hypothesis requires further investigation, particularly considering the limited number of subjects involved in this preliminary study. The identification of a reliable and non-invasive

stress-marker would represent a useful tool for improving farm animals' welfare in transport conditions.
Keywords: donkeys; transport; stress; welfare; chromogranin A

## 1. INTRODUCTION

Worldwide, it is estimated that there are approximately 50 million donkeys (1); across Europe, donkeys are kept for a variety of purposes, such as pets, leisure activities, therapy programs, or milk and meat production (2). Therefore, they can be transported not only for reaching the slaughterhouse but also for sport activities or reaching people in need of therapy, changing owner, reaching veterinary clinics, breeding, small fair events, or sale (3). Transportation is one of the leading husbandry practices identified as a cause of stress that may produce changes in animals' clinical and biochemical parameters (4–8). Several potential stressors are intrinsically related to transport, including loading, unloading, and confinement in unfamiliar environments; overcrowding; mixing different age groups; vibrations; changes in temperature and humidity; inadequate ventilation; noise; poor vehicle design and road conditions; duration of the journey; and often deprivation of food and water (9–11). Under such circumstances, the stress response has the function of providing the energy needed in order to cope with these challenges through the activation of two main physiological pathways: the hypothalamic-pituitary-adrenal cortex axis (HPA) and the sympathetic-adrenal medulla axis (SAM). Once the HPA and SAM axes are activated, they trigger specific physiological changes that can be measured to assess the degree of activation (3). The activities of the SAM system can be biochemically evaluated as an objective index of stress, but to obtain reliable data, it is crucial to have an accurate and non-invasive sampling method. The use of saliva rather than blood has the advantages of non-invasiveness, rapidity of sample collection, ease of collection, and the ability to maintain subject mobility during collection (12). Catecholamines are commonly used as a sensitive biochemical index of stress-induced SAM system activation, but they are difficult to measure in saliva due to low concentration and rapid degradation (13,14). Little research has been carried out on donkeys' responses to transportation related stress, mainly focusing on adrenocorticotropic hormone and cortisol level variations (10,15) or free iodothyronine (8). Recently, during an investigation of the surrogates of catecholamines that are detectable in saliva, chromogranin A (CgA) was determined to be a useful index of psychological stress (16). CgA is an acidic glucoprotein that is co-localized with catecholamines in the secretory granules of a wide variety of endocrine structures and neurons (17). CgA and catecholamines are co-released into an extracellular environment when the SAM system is stimulated. The adrenal medulla is the primary source of circulating CgA, while adrenergic nerve endings and neuroendocrine cells secrete CgA in peripheral tissues (16). In humans, CgA concentration is used as an index of stressed conditions. Compared to catecholamines, CgA has greater stability in the circulatory system and therefore may be a more accurate index of sympathetic activity system during the stress response (18,19). Detection of salivary CgA levels may have a higher analytical and diagnostic performance, as salivary sampling is non-invasive and, unlike the circulating form, CgA in the saliva is not bound to other proteins (20). Moreover, equine CgA expression has been confirmed and described (21). These findings led us to hypothesize that salivary CgA concentrations, known as a biomarker of sympathetic activity system, may represent a novel physiological index of transportation-induced stress in donkeys. This preliminary study aimed to investigate this hypothesis since, to the best of our knowledge, the scientific literature contains no readily-available information about this topic.

## 2. MATERIALS AND METHODS

The study included nineteen Romagnolo donkeys (M = 15; F = 4), aged 443.4 ± 148.4 days, kept for meat production on a farm situated in Northern Italy. No donkeys were transported only for data collection for the purposes of this study; all transportations were part of the farm's breeding procedures. Since the farm was limited in size, all planned transportations were monitored. Despite this, a small number of animals were available, unbalanced by gender. In the territory considered, there were no donkey farms with similar management; therefore, for this pilot study, the number of subjects observed was not integrated with others from different breeding systems, in order to avoid data bias.

The donkeys were kept on pasture with their mothers and other donkeys of different ages. Animals were free to graze and, when needed, received hay and mixed feed; both automatic drinkers and buckets were used to provide fresh water ad libitum. All donkeys were used for human contact.

All donkeys were transported twice, on two consecutive days, from January to July, using the same truck, in small groups (two to four donkeys per transportation, coming from the same familiar group). The truck's internal dimensions (therefore the space available for the animals) were 2.50 by 6.50 m, with mobile partitions which were kept open. A spotlight illuminated the van, and the vents were kept open, compatibly with the weather conditions. In the first transportation (Trip 1), animals left the pasture to reach the farm, where they were housed in a shelter with straw bedding and provided with hay ad libitum and access to clean water. The transportation started at around 4:30 pm and its duration ranged from 50 min to 88 min (mean 64.69 ± 14.57 min). The day after, at around 4 am, donkeys were transported to the slaughterhouse (Trip 2). This transportation lasted from 60 to 75 min (mean 64.75 ± 5.54 min). Animals were always transported with conspecifics from the familiar group, with two to four donkeys per transportation. The farm manager and groom performed all the transportation procedures (loading, travel, and unloading) according to the on-farm routine. They conducted the donkeys with a lead rope, which they were accustomed to, gently encouraging them to get on and off the truck, and they also offered them food. Transportations were conducted in compliance with Council Regulation (EC) No 1/2005 of 22 December 2004 on the protection of animals during transport and related operations. Verbal informed consent was gained from the farmer prior to taking part in this research. Written consent was deemed unnecessary as no personal details of the participants were recorded.

Saliva collection is recognized to be non-invasive, and no animal underwent more than minimal distress. Saliva samples were collected using SalivaBio Children's Swabs (Salimetrics®, Carlsbad, CA, USA) 15 min before loading when donkeys were at pasture, and again at the end of the transportations, 15 min after unloading from the truck. The swab was inserted into the donkey's mouth, while the animal was gently restrained with a head collar, without isolating it from the rest of the herd; the donkey was free to chew the swab for 1–2 min without constraint. The sampling procedure lasted no more than 5 min for each donkey, the animals being accustomed to the halter and to being handled by the groom. After the sampling, the swab was put in the device tube, which was closed with a plastic stopper to prevent evaporation and placed in ice and stored at −20 °C immediately after it arrived at the laboratory. The temperature was maintained until analysis. At the time of analysis, the samples were thawed at room temperature and centrifuged (3500 rpm for 15 min, at 4 °C) according to the protocol for salivary samples described by the kit producer. The analysis was performed using a commercially available enzyme-linked immunosorbent assay (ELISA) sandwich kit for the accurate quantitative detection of horse chromogranin A (Li StarFish Srl, Milan, Italy), validated for saliva samples. Samples were aliquoted into wells in duplicate (40 μL) and, at the end of the procedure, performed as indicated by the kit producer manual, the absorbance was measured using a wavelength of 450 nm in a microplate plate reader (Multiskan EX, LabSystem, Thermo Fisher Scientific, Milan, Italy). The average intra- and inter-assay coefficients of variation, respectively, were 6.5% and 8.3%. The assay sensitivity was 0.67 ng/mL. Mean intra-assay coefficient of variations for the final samples were 4.56%. The laboratory researcher was blinded to the hypotheses and conditions.

Data were entered into Microsoft Excel (Microsoft Corporation, 2010, Washington, DC, USA) before being analyzed with the SPSS statistical package (SPSS Statistic 25, IBM, Armonk, NY, USA). A descriptive analysis was first performed to determine the mean and standard deviation of CgA concentration. A match-paired Wilcoxon's test was used to compare concentrations from pre- to posttransportation in both transportations (Trip 1 and Trip 2) and pre- and post-transportation CgA concentration between transportations. p-values ≤ 0.05 were deemed statistically significant.

## 3. RESULTS

Our results showed that, in donkeys, chromogranin A levels significantly decreased after transportation (match-paired Wilcoxon's test, $p < 0.05$) (Figure 1). CgA values during the first transportation (both pre- and post-transportation) were not significantly different from those of the second transportation (match-paired Wilcoxon's test, $p > 0.05$).

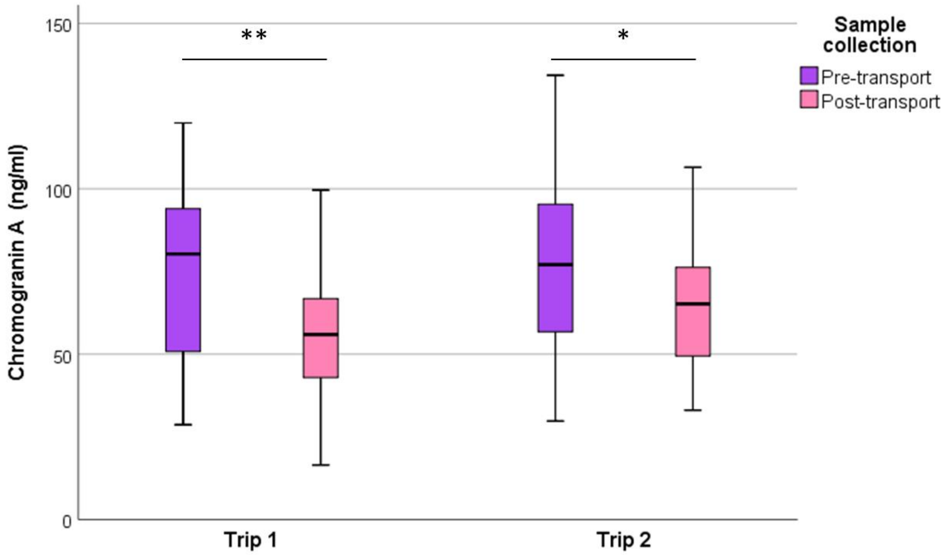


**Figure 1.** Chromogranin A (ng/mL) pre- and post-transportation drawn in a box plot for both trips; lines extending from the whiskers indicate the variability outside the upper and lower quartiles. (match-paired Wilcoxon's test ** $p < 0.01$; * $p < 0.05$).

## 4. DISCUSSION

To the authors' knowledge, chromogranin A salivary concentration has not been measured before in donkeys. The primary structure of the equine nucleotide sequence encoding CgA was determined by Sato et al. (22), which also compared the aminoacidic sequence of equine CgA with those of human, porcine, bovine and other species, showing high conservation of the NH2 terminal- 1-177 and COOH terminal 314–430 aminoacidic regions. This result made us hypothesize that the commercially available CgA ELISA kit for horses, tested for the accurate quantitative detection of chromogranin A in serum, plasma, urine, saliva, and tissue homogenates, had adequate sensitivity for CgA titration in donkeys. In horses, road transportation is known to activate the hypothalamicpituitary-adrenal cortex and the sympathetic and adrenal medullary systems (5,6,10,20,21,23), but, to the best of our knowledge, the change in CgA has not yet been tested. Differently from what could be expected, in our study, the salivary concentration of CgA decreased after each of the two considered transportations. Although, in the present study, all possible measures were taken to minimize stressors (e.g., not mixing the animals with unknown conspecifics, using the same truck, transporting them for short distances, gentle handling by known stockman), at the end of the transports, we expected an increase in salivary CgA concentration as a reflection of sympatheticadrenal medullary axis activation. Our results could be partially related to what was observed by Fazio et al. (24), who investigated the effects of different human handling on young horses in stabled conditions and during the short-transport road. They demonstrated that the quality of handling to which horses were subjected could affect the stress response produced by transportation. The role of salivary CgA as a marker of stress is described in the literature in pigs (25), dogs (13), and calves, with results not always consistent. Escribano et al. (25) described the validation of a time-resolved immunofluorometric CgA assay and its application as a marker of acute stress in porcine saliva in a model of acute experimental stress, in which animals were immobilized for 3 min with a nose snare. The authors evidenced a significant increase in salivary CgA levels at 15 min post-stressor stimulus. Since the time course of the changes of CgA salivary levels post-stress is not yet known, in the present study, we opted to utilize the timing suggested by Escribano (25). Ott et al. (26) investigated salivary CgA as a biomarker for pigs' stress evaluation, and their results indicated that salivary CgA reacts differently to different types of stressors. They exposed pigs to stressors that cause significant social and physiological changes: the mixing of unfamiliar animals (a procedure associated with the increase in stress metabolites, the suppression of the immune function, growth retardation, and skin lesions), and fasting and refeeding. The mixing of unfamiliar pigs induced a decrease in CgA salivary concentrations which conversely showed a significant elevation after the feed deprivation period (26). In Escribano et al. (27), the CgA levels in pigs' saliva showed an increase both during transportation and housing in the slaughterhouse and after applying a psychosocial stress model, where finishing pigs were mixed with familiar animals after a previous period of isolation. More recently, Escribano et al. (28) evaluated salivary CgA concentration at weaning, known to induce acute stress in pigs. They observed a highly significant increase in CgA in saliva one day after weaning, compared to the pre-weaning levels. The authors hypothesized that weaners, subjected to mother–young detachment, undergo neuroendocrine and behavioral consequences and are subject to other stressors like uncertainty, social hierarchy re-establishment, and fear of the novel environment. These stimuli have demonstrated the induction of the "flight and fight" response in pigs which is associated with the activation of the SAM system (28). In a 2010 study, Ogino et al. investigated the influence of social isolation and transportation on CgA salivary levels in calves. They evidenced that social isolation induced a significant decrease in the salivary CgA concentration, while transport evoked a rise, even if not statistically significant. In humans, CgA is considered a sensitive and reliable index of psychological stress. Nakane et al. (19) showed that salivary CgA levels were elevated before subjects gave an oral presentation and then decreased immediately after. Fujimoto et al. (29) measured salivary CgA levels in two groups of people (young and elderly) in order to evaluate mental stress during upper gastrointestinal endoscopies. They compared CgA salivary levels at rest, before endoscopy, and during endoscopy and highlighted that, in both groups, CgA levels decreased significantly during endoscopy, more so in the elderly patients. Our results in donkeys seem consistent with what has been shown in the study by Yamakoshi et al. (30). They evaluated salivary CgA in a stressful situation created by simulated monotonous driving and evidenced that CgA levels fell gradually in accordance with the gradual increase of the subjective rating of stress and significantly decreased over the two hours of simulated monotonous driving, in which the driver felt considerably stressed (30). A possible hypothesis explaining why CgA levels can be affected differently according to different stressors and exposure times is related to catestatin action (31). Catestatin is a bioactive product of the proteolytic cleavage of CgA that acts as a potent inhibitor of catecholamine (CA) nicotinic cholinergic stimulated secretion, desensitization of catecholamine release. This peptide exhibits potent catecholamine release-inhibitory activity by acting on the neuronal nicotinic acetylcholine receptors and transcription of chromogranin A gene (32,33). CgA and CA are co-released into the extracellular environment, and CA mediates sympathoadrenal activity on cardiovascular target ceIls in order to increase blood pressure. Yamakoshi et al.'s findings demonstrated that sympathetic-induced vasomotor constriction was stimulated during the two hours of monotonous driving simulation: peripheral vessel constriction induced a significative increase in total peripheral resistance, a significative decrease of normalized pulse volume, and an increase in blood pressure, which are associated with an increase in the subjective rating of stress (30). The stimulus analyzed in Yamakoshi's research could probably be compared to the 50–80 min journey, without particularly acute stressful conditions, to which the donkeys of the present study were subjected. Yamakoshi's results indicate that the two hours of monotonous driving evoked considerable stress in drivers, resulting in a gradual rise in blood pressure caused by an increase in sympathetic-induced vasoconstriction, rather than by sympathoadrenal activity, that is not activated in the monotonous situation (30). It is known that the increase in blood pressure is correlated with catestatin activation and that catestatin is associated with augmented baroreflex sensitivity (33,34). Moreover, catestatin acts as negative feedback on CgA secretion, which is blocked by this peptide fragment (32). This mechanism could explain why CgA levels decreased during the simulated monotonous driving in Yamakoshi's research, although CgA is a feasible marker of stress. We hypothesize that this physiological mechanism could be the basis of the decrease in the salivary concentration of CgA observed after transportation in the donkeys. If confirmed in future studies, this hypothesis would further prove what has been recently found by Srithunyarat et al. (35) in a study on dogs, when they reported that canine psychological stress was associated with an increased level of salivary catestatin.

## 5. CONCLUSION

Since this study involved only a limited number of donkeys, additional research considering a larger animal sample is needed to confirm ELISA's reliability for donkeys' salivary CgA levels and establish its physiological range in resting conditions. Measuring salivary CgA concentrations in donkeys may represent a promising tool for obtaining information about their stress response through a non-invasive technique that is not influenced by sampling and can be easily carried out on many animals directly in on-farm conditions.

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## 4.4 Habituation to Transport Helps Reducing Stress-Related Behavior in Donkeys During Loading

Francesca Dai, Silvia Mazzola, Simona Cannas, Eugenio Ugo Luigi Heinzl, Barbara Padalino, Michela Minero, Emanuela Dalla Costa

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Abstract. Adopting proper animal management strategies, including training, might reduce to a substantial extent the adverse effects of transport-related stress in animals. The aim of this study was to evaluate the effect of habituation to transport on stress-related behaviors and physiological indicators during loading and unloading in donkeys. Fourteen donkeys were recruited and divided in two treatment groups: Habituation (H; M=5, F=2) and Control (C; M=5, F=2). H donkeys were gradually habituated to be transported, traveling together with their mothers and other adult donkeys well-accustomed to transport, while C donkeys had never been transported before. Loading and unloading phases were video recorded and behavior was analyzed. Saliva samples for cortisol levels determination were at rest and after unloading. Latency time to load was significantly shorter for H donkeys than C donkeys (Mann-Whitney; p=0.004). C donkeys also showed significantly more stress-related behaviors (Mann-Whitney; p=0.026) and required a higher but not statistically significant number of human interventions to load. Cortisol concentration increased in both groups, but no differences were found between them (Mann-Whitney; p>0.05). These results suggest that habituation to transport could mitigate stress during loading procedure in donkeys reducing loading time, frequency of stress-related behaviors and diminishing the need of human intervention.

Keywords: donkey, transport, welfare, behavior, stress, habituation

1. INTRODUCTION

Throughout Europe, a population of about 395.910 donkeys is estimated (1), of which 93.468 are registered in the Equine Italian Database (2). Donkeys in Italy can be kept as pets, or used for leisure activities, therapy programs, or milk and meat production (3).

Transport is part of the management for the majority of pets and farm animals, including donkeys, having different purposes, such as reaching slaughterhouse, moving to a different farm, breeding, competitions and fairs, and medical procedures (4). Transport procedures are known to be stressful for animals, having both short term and prolonged effects on their welfare (4–7). It is also known that transport-related stress could influence meat quality in a variety of species (5,8–10), thus potentially reducing profits derived from animal farming for meat production. When transported, several potential stressors can impact animal welfare, including interaction with humans, loading, unloading, and penning in a new, unfamiliar environment, and confinement with and without motion, vibrations, changes in temperature and humidity, inadequate ventilation, and often, deprivation of food and water (11). In particular, loading is considered to be one of the most stressful components of transport for most animals, including equines (12–17), and it is reported to be the phase with the higher number of transport-related injuries particularly in horses which show transport related behavioral problems or have been trained with inappropriate training methods (18,19). Several stressors are involved in pre-loading and loading procedures: separation from a familiar environment and social group (6), interactions with humans (14,20), walking on the ramp (21), entering the trailer (22). Stress and fear during loading are also reinforced by recollections of previous unpleasant travelling experiences (11,23–25).

The reduction of adverse effects of pre-transport factors decreases the probability of compromising animal welfare during the transport phase itself (7). The pre-transport preparation of animals plays an important role: it is reported that adopting proper management measures might reduce to a substantial extent the adverse effects of loading on stress, improving animal welfare (7). Habituation, in particular, is known to lead to decreased behavioral reactions to a previous novel situation (26), and habituation to loading as a foal, a yearling or an adult horse was reported to make loading behavior become as normal as walking into a stall (23). As highlighted by Padalino and Riley, people involved in equine transport should apply best practices, such as training of animals using evidence-based methodologies (4). In order to evaluate the efficacy of transport training methods, transport-related stress should be evaluated using both behavioral and physiological indicators. In horses, stress-related behaviors during loading include pawing, kicking out, bolting, head-shaking, and avoidance reactions, such as rearing, pulling away sideways, or backwards (13,16,17,26,27). During transport, reported stress-related behaviors are vocalizing, head tossing, pawing, scrambling, head-turning, kicking at the vehicle, biting and kicking at other horses, and reduced feeding/drinking (27–31). Reported unloading stress-related behaviors are a reluctance to exit the vehicle, prolonged immobility, and running off (21). To the authors' knowledge, no research has been conducted on donkeys to assess stress-related behavior during transport. Several physiological indicators have been proposed to evaluate transport related stress, both in horses and in donkeys, such as cortisol (11,32–36), ß-endorphin (11,36,37), adrenocorticotropic hormone (32) and chromogranin-A (38), and infrared thermography (39).

The aim of this study was to evaluate the effect of habituation to transport procedures on stress related to loading and unloading, using behavioral and physiological indicators, in donkeys.

## 2. MATERIAL AND METHODS

### *2.1 Ethics statement*

This was an opportunistic study, no animals were transported to record data for the purposes of this study, no farm routine management has been modified for the purpose of the study. To obtain the best from each pasture, donkeys were routinely moved from one pasture to another. Therefore, no extra work was required to the farmer. No animals underwent more than a minimal distress. Transports were conducted in compliance with Council Regulation (EC) No 1/2005 of 22 December 2004 on the protection of animals during transport and related operations. Verbal informed consent was gained from the farmer prior to taking part in this research. Written consent was deemed unnecessary as no personal details of the participants were recorded.

### *2.2 Animals and facility*

All the donkey kept on farm were health checked and handled daily by the farm manager and/or the groom, so they were used to human contact. Fourteen healthy Romagnolo donkey foals (M=10; F=4; 1.2±0.4 years) intended for meat production were included in the study. Only healthy foals born in 2018 were included in the study. Donkeys were born and raised on the same farm located in Northern Italy. Animals were group-housed at pasture with access to clean water *ad libitum* with both automatic drinkers and buckets. Donkeys were free to graze; pastures were managed to guarantee adequate nutrition in terms of quantity and quality of grass. If needed, depending on the season, weather, pasture conditions, and donkey growth rate, hay and mixed feed were provided.

### *2.3 Treatments*

Donkeys were randomly divided in two sex-balanced groups of seven subjects each: Control (C; M=5, F=2) and Habituation (H; M=5, F=2). All the animals were used to be handled and cared for by the same handlers. Foals in the H group were gradually habituated to be transported over short distances (from one pasture to another, about 30 minutes journeys), travelling together with adult donkeys with travel experience, including their own mothers. This habituation training started when the donkeys aged 6 months and lasted until they were taken to the slaughterhouse (1.2±0.4 years). During the habituation, transport procedures were always performed using the same truck and by the same stockmen people familiar to the donkeys (farm manager and groom). Foals were left free to load following other donkeys, taking advantage of their gregarious behavior, so they were not led by handlers or pushed by them in anyway. Donkeys in the H group were subjected to a minimum of 5 transports and no injuries were reported in both donkeys and donkey handlers. Donkeys in the C group were naïve to transport since they were housed together in a pasture, different from one of the H group, from birth. All the animals were used to the handlers: while donkeys in the C group were not used to be loaded nor to travel, animals were used to be handled and cared for by the same handlers.

*2.4 Data collection*

Data were collected during the transport from the pasture (where they were kept) to the main. For C donkeys, this was the first transport of their life. All donkeys involved in the study were transported in small groups (two to four donkeys, coming from the same familiar group) with the same truck, for a total of six transports. The transports started at around 4.30 pm, and their durations ranged from 50 min to 88 min (mean 64.69±14.57 min). All transport procedures (loading and unloading) were performed by the stockmen according to the usual farm routine. Donkeys were conducted with a lead rope and gently encouraged to move by handlers, also offering food. Animals were loaded in group (two to four donkeys at a time) in order to take advantage of their gregarious behavior. At arrival, the truck door was opened, and the donkeys were left free to unload without leading or encouraging them.

*2.5 Behavioral analysis*

The loading and unloading phases were video recorded using an HD digital video camera (Canon Legria HFR88) controlled by the researcher. The loading time (from the procedures beginning, with the donkey being in front of the ramp, until the donkey had all four feet on the trailer) and time to unload (from the trailer doors opening until the donkey had all four feet on the ground) was directly recorded using a stopwatch. Donkey behavior during loading and unloading was separately analyzed by a treatment-blind animal scientist, experienced in equine behavior analysis. A focal animal continuous recording method was applied, using the software Solomon Coder beta 17.03.22. The frequency and duration of different behaviors were recorded. Since no literature is available on donkey behavior during transport, the ethogram was adapted from the one used for horses by Dai et al. (13) (Table 1).

Furthermore, each interaction between the handlers and the donkeys was noted from videos. Any interactions to facilitate loading was considered (pulling the rope, pushing the donkey from the back, inciting the animal, offering food).

*2.6 Salivary cortisol evaluation*

For cortisol levels determination, saliva samples were collected using SalivaBio Children's Swab (Salimetrics®, Carlsbad, CA, USA) in the pasture with donkeys at rest immediately before starting loading procedures and immediately after unloading. In order to minimize the impact of the circadian pulsatile cortisol release pattern (40), for each donkey two more samples were taken under control conditions (at the pasture), on the days immediately prior to transport, in the same time slot in which the transport was scheduled (between 4 pm and 5 pm). The swab was inserted in donkey's mouth, gently restraining the animal with a head collar; the donkey was left free to chew the swap for 1-2 minute, then the swab was put in the device tube, closed with a plastic stopper to prevent evaporation, placed in ice and then stored at −20 °C immediately after it arrived at the laboratory. The temperature was maintained until analysis. At the time of analysis, the samples were thawed at room temperature and centrifuged (3,500 rpm for 15 minutes, at 4°C) according to the protocol for salivary samples. Analysis was performed using a commercially available multispecies cortisol enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, Farmingdale, NY,USA), following previously validated protocols (41). Samples were aliquoted into wells in duplicate (100 µL), and absorbance measured using a wavelength of 405 nm in a microplate plate reader (Multiskan EX, LabSystem, Thermo Fisher Scientific, Milan, Italy). A recovery test was applied to determine if the value obtained from our samples were accurate (e.g., no interferences with the measurements due to the presence of undesired factors in the sample matrix). The mean recovery was 109.1% ± 8,4, while the average intra- and inter-assay coefficients of variation, respectively, were 3.9% and 7.8%. The assay sensitivity was 56.72 pg/ml (range 156 - 10000 pg/ml). The laboratory researcher was blinded to the hypotheses and conditions.

**TABLE 1** Ethogram for the evaluation of donkey behavior during loading and unloading [modified from (13)]. *Behaviors that were not observed were not considered for the statistical analysis.

| **Behavior** | **Description** | **Category** |
|---|---|---|
| Walk | The donkey walks toward the trailer | Forward locomotion |
| Trot | The donkey trots toward the trailer | Forward locomotion |
| Gallop | The donkey gallops toward the trailer | Forward locomotion |
| Backwards | The donkey moves away from the trailer | Stress-related behavior |
| Standing | The donkey stands on the four legs | Stress-related behavior |
| Turn back | The donkey tries to turn all its body in the opposite direction of the trailer | Stress-related behavior |
| Refuse to walk | The donkey stops moving, digging in its heels, refusing to proceed | Stress-related behavior |
| Rear | The donkey rears with its front legs | Stress-related behavior |
| Kick | The donkey kicks, one or two legs is lifted and moved rapidly and forcefully | Stress-related behavior |
| Mount | The donkey mounts the donkey in front of him/her | Stress-related behavior |
| Defecate | The donkey drops manure | Stress-related behavior |
| Urinate* | The donkey drops urines | Stress-related behavior |
| Paw* | The donkey rises a foreleg and scrapes the floor | Stress-related behavior |
| Sniffing* | The donkey sniffs the ground | Stress-related behavior |

*2.7 Statistical analysis*

Behaviors of the categories forward locomotion and stress-related behaviors were considered together for the statistical analysis (table 1). Based on the total length of the observation of the video recordings, durations of behaviors were calculated as percentage of total observation time (proportional duration time). Behaviors that were not observed (urinate, paw, sniffing) were not considered for the statistical analysis. Cortisol variations (delta) for each subject of the two groups were calculated. Statistical analysis was performed using SPSS 25 (SPSS Inc., Chicago, IL, USA). Data were tested for normality and homogeneity of variance using the Kolmogorov-Smirnov and Levene test, respectively. Mann-Whitney test was used to investigate differences between groups in behavior during loading and unloading, time to load and unload, human intervention and cortisol concentration (delta). Statistical significance was accepted at $p \leq 0.05$.

## 3. RESULTS AND DISCUSSION

*3.1 Behavior analysis*

Results of the behavioral analysis showed that latency to load was significantly shorter in H donkeys (mean 7.97±4.62 sec) than in C donkeys (mean 83.23±143.84 sec) (Mann-Whitney test; p=0.004) (Figure 1). H donkeys showed more forward locomotion toward the truck than C donkeys (87,89±20,48 % and 41,71±33,51 %, respectively; Mann-Whitney test; p=0.026). Furthermore, C donkeys showed significantly more stress-related behaviors than H donkeys (58,29±33,51 % and 12,11±20,48 %, respectively; Mann-Whitney test; p=0.026) (Figure 2). These results are similar to those reported in trained horses (13, 19). However, positive and negative training reinforcements might require more time than farmers will dedicate (13);for this reason, theproposed and tested habituation training, including the foal following the mother and other known conspecifics, seems instead to be effective in donkeys and may prove to be more feasible when introduced in an on-farm routine as the trailer could be left in the pasture so that the animals can explore it and get habituated to load and unload. Habituation has been strongly recommended for horses (22) and was proven to minimize the incidence of transport related behavioral problems and subsequently injuries (18). In the latter study, self-loading also was found associated with a reduction of loading problem behavior and injuries, however, it is worth to know that self-loading require lots of time, effort, and training skills. Even though, habituation requires some time, Houpt et al. (22) clearly tested that when a foal is habituated to load into a trailer following the mare, loading into a trailer becomes easy as walking into a box for both the foal and its handler. This is the first study where this training to transport procedures using habituation with the foal following conspecifics was tested in donkeys.

Group C donkeys required a higher but not statistically significant number of human interventions to load compared to H's (H: mean 1.29±0.95; C mean: 7.43±14.03, Mann-Whitney test; p = 0.32). The lack of statistically significant difference may probably be due to the high individual variability observed in C subjects and it needs to be ascertained with further studies. However, it is worth noting that this result has interesting practical fallouts considering both animal welfare and human safety. Indeed, interactions with large animals may become dangerous for handlers, especially when animals are stressed and/or frightened: several studies conducted in sport horses with behavioral problems related to transport highlighted the high occurrence of injuries in humans during loading, such as rope burns, lost fingers, broken bones, or bruises, and bleeding (17,18,42). Not only loading may be risky for the handlers, but inappropriate animal management in this transport phase has been reported to be a risk factor also for horse injuries (19,42). The adoption of an adequate training for loading has been deemed useful in increasing human safety by reducing horse-related injuries among handlers (18).

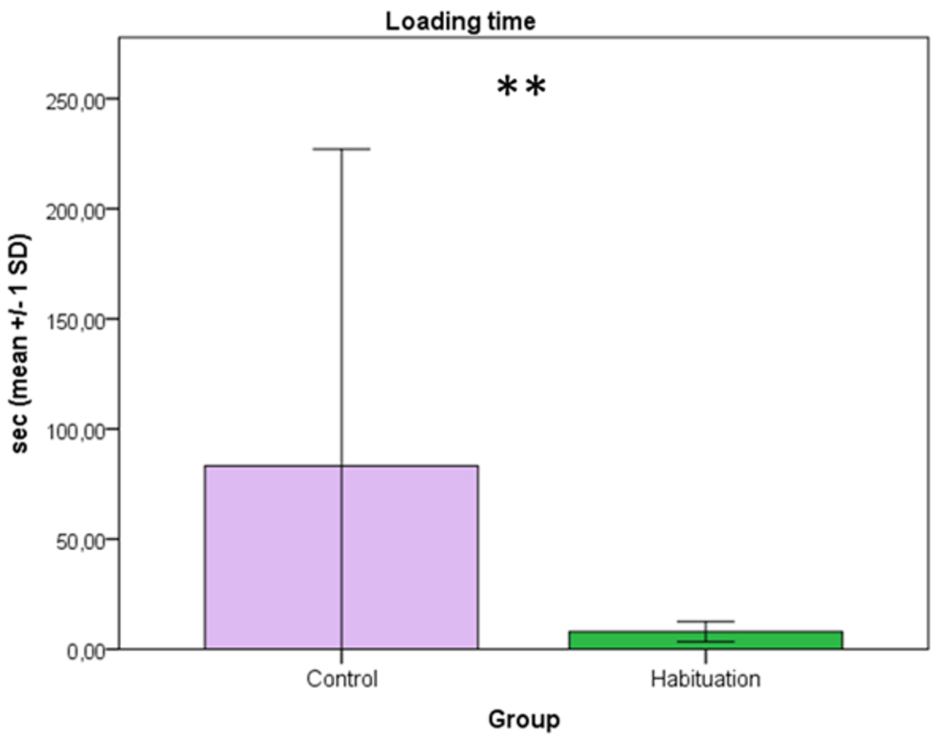


**Figure 1.** The time (sec) for loading is presented on the y-axis (mean ± 1 SD) with the groups (control VS habituation) on the x-axis (Mann-Whitney test; **p=0.004).

Even if loading represents the most stressful stage of animal transport (7,12,43), also unloading may be challenging. Critical factors are steepness and slipperiness of the ramp, and the novel environment the animals are required to enter (6). Consequently to stress and/or anxiety related to unloading, horses have been observed freezing inside the vehicle or performing flight responses (21). In the present study, donkey did not exhibit abnormal behavior during the unloading phase, and no differences between groups were found in the unloading time (Mann-Whitney test; $p>0.05$), with C group unloading in 48.9±32.4 sec (mean ± 1 SD), while H group unloading in 71.0±31.2 sec. Besides, the behavior of the donkeys in the two groups was similar during the unloading procedure

(Mann-Whitney test; p>0.05). Besides, the behavior of the donkeys in the two groups was similar during the unloading procedure (Mann-Whitney test; p > 0.05). Having traveled with other members of the social group could have contributed to attenuating the stress at the time of unloading. Taking advantage of the donkeys' gregarious attitude, the animals got out of the truck without showing behaviors attributable to stress.

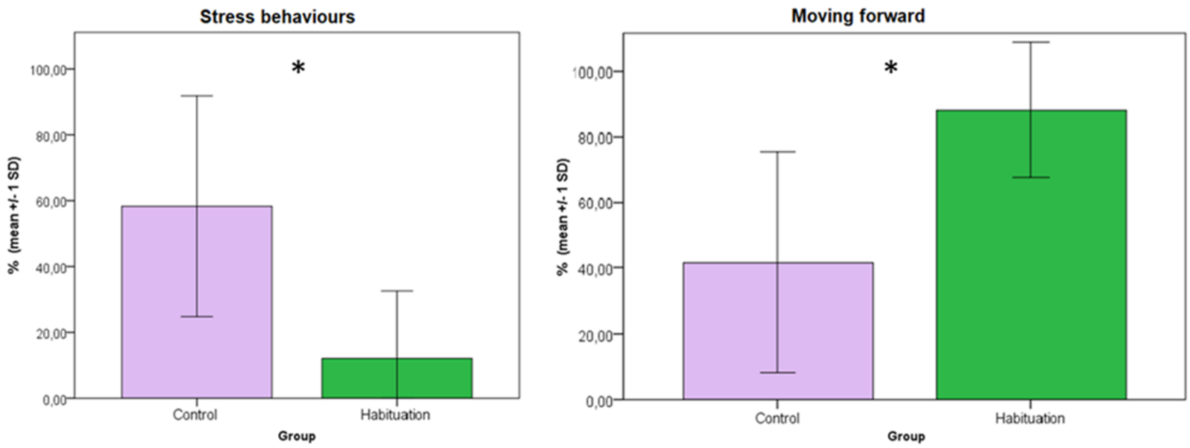


**Figure 2.** Mean percentages of time (+/-1 SD) of stress related and moving forward behaviors of donkeys in the two treatment groups during loading procedure. (Mann-Whitney test; *p<0.05)

*3.2 Salivary Cortisol Evaluation*

No differences were found in delta cortisol concentration between groups (Mann-Whitney test; p>0.05) (Figure 3). From Figure 3, it is evident the great variability of group C data, much greater than those of the group H. In case of acute stress, cortisol secretion increases significantly, with a secretion level that varies from individual to individual, depending on the individual perception of the stressor, but described as correlated with the intensity of the stress (44). These results highlighted the subjectivity of the activation of the hypothalamic-pituitary-adrenal cortex axis induced by the stressogenic stimulus: this variability, associated with the small number of donkeys, may be the basis of the lack of significance of the data. The great variability may be also related to the different space allowance and conditions (group of 2 or 4) during the different journeys tested.

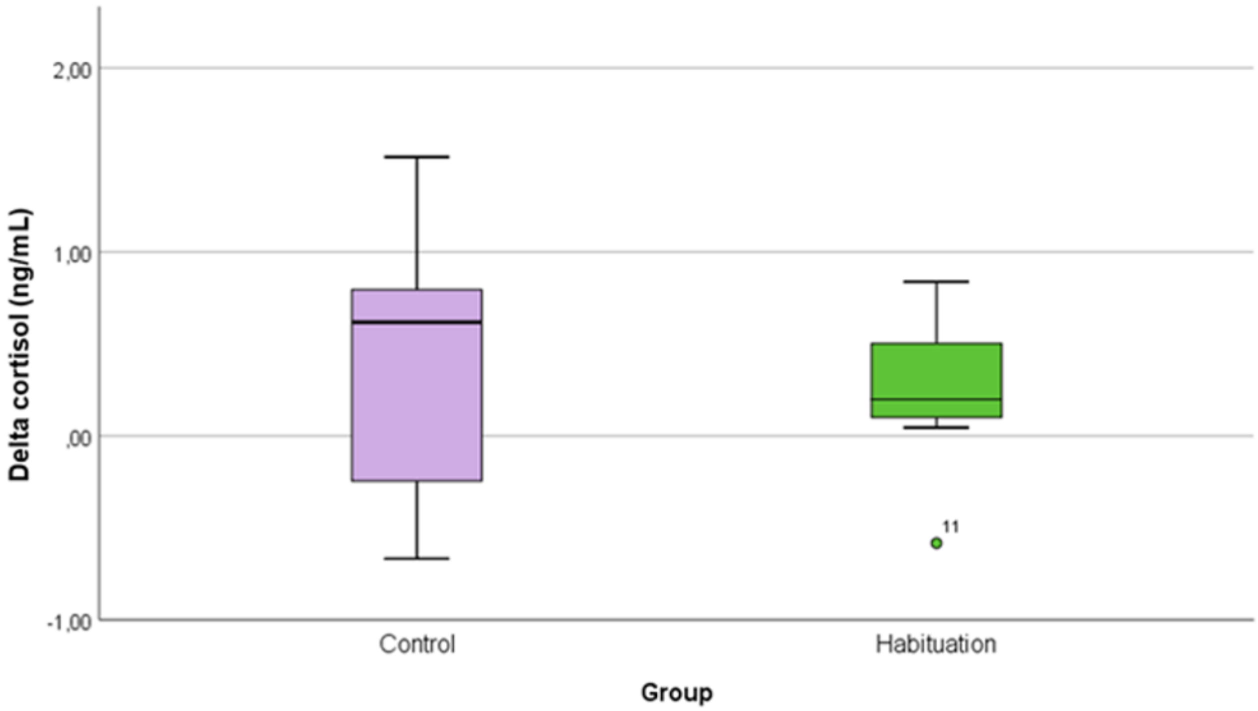


**Figure 3.** Box plot of the transport-related salivary cortisol variation (delta) in the two groups of donkeys. Outliers are represented with dots.

*3.3 Limitations and future perspectives*

In this study, no donkeys were transported only for the purpose of data collection: all transports were part of the farm's management procedures. The limited size of the farm has led to a small number of donkeys available. In a convenient geographical location, there were no donkey farms with the same donkey breed with similar management: therefore, to avoid data bias (especially considering physiological data), the number of subjects observed was not integrated with those of other farms. It clearly appears that the sample size and the unique provenience of the evaluated donkeys represent limitations for the generalization of our results. Further studies applying training to transport procedures through habituation with the foal following conspecifics are foreseen to generalize the results to donkeys kept for other purposes and subjected to different management. As stress related to transport could be affected by several potential stressors (11), future studies should also consider to evaluate different habituation protocols such as load on a trailer without movement and habituation to loading on a trailer and the vehicle movement.

Donkeys were not habituated to saliva sample. Even if the method is reported to be non-invasive and should not induce a significant stress to the animal, being rapid and permitting animal mobility (38,41), the procedure represented a novel stimulus for the animal. Although the time required for sampling was not sufficient to allow the presence of free cortisol in the saliva (45), it may not be excluded that the procedure induced a certain degree of stress in donkeys, therefore influencing our result. However, the applied methodology was the same for H and C groups, eliminating any potential bias between treatment groups. Cortisol is released from the adrenal glands in pulses controlled by the hypothalamus's paraventricular nucleus, which receives circadian pulses from the suprachiasmatic nucleus of the hypothalamus and integrates information from cognitive processes and emotional and physical stress reactions (46). The cortisol secretory pulses' variations result from the ultradian rhythm: the secretory episodes occur at a relatively stable frequency, with variable amplitudes, responsible for the typical circadian rhythm. Over 24 h, between 15 and 22 secretory cortisol pulses are expected, with an early morning peak and a nadir by the first half of the night. In the present work, we tried to minimize the impact of this pulsatile circadian release of cortisol: all transport took place in the afternoon, and, for each animal, salivary sampling was at the transport and also the day before, the same time as transport was planned.

Measuring other physiological indicators, such as heart rate variability (HRV), respiratory parameters, beta-endorphin, catecholamines or glucose levels, would have increased the scientific robustness of results, however, it would have also decreased the study's feasibility increasing the invasiveness of the data collection.

As one of the reasons for breeding donkeys is meat production and numerous studies highlighted how transport stress negatively affects meat quality in several species [see (5) for review], in future studies, it would be interesting to analyze the effect of transport related stress on donkey meat. As a matter of fact, only few studies described the incidence of transport related stress on equine meat quality (47, 48).

Regardless of the above-mentioned limitations, to the authors' knowledge, this is the first study documenting the effects of habituation to transport in meat donkeys. In the present study, meat donkeys were taken only as a model, as two groups of animals of the same breed, balanced for sex and age, with the same management and handling from the same stockmen could be subject to the different training procedure. As donkeys are frequently transported for several purposes, including changing ownership, leisure activities, therapy, sport activities, habituation following conspecifics could be helpful in reducing stress related to transport. This result has a practical fallout, since habituation with the foal following the conspecifics could be more feasible, easier and should be recommended for donkeys.

## 4. CONCLUSIONS

These results, although preliminary, suggest that habituation to transport following conspecifics could mitigate stress responses during loading in donkeys, reducing loading time, the frequency of stress-related behaviors and the handler's intervention. Further research, conducted on a larger donkey population on several farms, is needed in order to confirm these results.

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

**Author contributions:** Conceptualization, F.D., E.D.C, M.M.; methodology, F.D. and B.P.; formal analysis, E.D.C.; investigation, F.D., E.H., S.M.M.; resources, M.M.; data curation, F.D.; writing—original draft preparation, F.D., S.M.M., E.D.C.; writing—review and editing, M.M., S.C., E.D.C., E.H., B.P.; visualization, E.D.C.; supervision, M.M.; project administration, M.M. All authors have read and agreed to the published version of the manuscript.

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# 5. General conclusion

Equids sector represents an important socio-economic sector, involving, at different levels, 896.000 people in Europe only, and producing an exchange of 100 billion euros per year. Indeed, equids welfare is still a hot topic in Europe: scientific studies published in the last 20 years demonstrated how horses and donkeys are still affected by several welfare problems. Routine on-farm procedures, resource availability, facilities design, sport and work activities, and transport can represent potential threats to equids welfare. In order to improve animal welfare, a first, fundamental, step is to evaluate it in a reliable way. A variety of studies have been conducted to develop scientifically sound and feasible welfare assessment protocols to be applied on-farm, both for horses and for donkeys. A large number of indicators have also been investigated to evaluate equids welfare during the diverse phases of transport. However, welfare evaluation only represents the first step in a longer voyage towards the improvement of equids welfare. To sustain an effective change in management conditions, facilities features, working conditions and transport procedures to embrace more sustainable practices, it is necessary to scientifically prove the efficacy of any proposed modification in improving animal welfare. Moreover, changes need to be feasible and acceptable by the stakeholders.

With this in mind, this scientific thesis aimed to experiment solutions to enhance equids welfare both on farm and during transport, providing sustainable and applicable suggestions to be implemented in daily practices. Therefore, the project focused on two different topics: the evaluation of best management practices for equids on-farm and the testing of non-aversive training techniques to reduce stress during transport in meat horses and donkeys.

Chapter 3 firstly presented the process towards the development of the guidelines ‘Dairy donkeys: good animal management practices for donkey milk production’, including the best management practices to breed dairy donkeys. Thanks to the extensive use of symbols and images, the guidelines are not only comprehensive but also easy-to-use by a non-academic public. The development of such a document represents the first step for a targeted dissemination of information about appropriate management procedures for dairy donkeys, with the goal of increasing awareness among farmers about donkey needs and assist them in preventing welfare problems.

Secondly, the focus has been put on the development of a standardised training method for a facial expression-based pain coding system, the Horse Grimace Scale (HGS). The results of the study suggest that the training method applied could represent a starting point for developing a wider training program for observers without horse experience. Some drawbacks have been identified, such as the need for a dedicated picture collection of high-quality and uniform pictures, and the necessity of a more extensive training program involving a lower number of observers per trainer. Moreover, a session in which observers can practice scoring live animals is deemed essential for improving the accuracy of in-field pain evaluation.

Finally, Chapter 3 presents the results of the welfare evaluation of horses kept in group in semi-extensive conditions, conducted in France in collaboration with INRA. Our results suggest that outdoor group housing can represent a valuable alternative for horse management. While effectively permitting highly motivated behaviour, such as grazing and social contact, attention needs to be posed on feed management to avoid obesity, provision of adequate shelter to avoid thermal stress and insect harassment, and group management to avoid mixing incompatible subjects or frequent changes in group composition, which can increase aggressions. Further studies on a wider population may be useful to fully prove the beneficial effect of outdoor group housing on horse welfare.

Chapter 4 investigates the effects of training in reducing transport-related stress in horses and donkeys kept for meat production. As for horses, a positive reinforcement-based training has been applied to teach foals to self-load. The comparison between trained horses and a control group of animals revealed that such a training can be effective in reducing stress related behaviours during loading procedures and time needed to complete loading. However, it remains to be investigated if training is able to alleviate the overall impact of pre-slaughter transportation. It was not possible, for practical and economical constraints, to habituate the horses to vehicle movements and noises simulating a travel: this remains an open question for future studies. In addition, the feasibility of a positive reinforcement-based training remains to be discussed. Since it required several weeks to be completed, it may not be applicable in daily practice on a meat farm. As for donkeys, Chapter 4 presents the impact of habituation to transport procedures in reducing transport-related stress. The results suggest that habituation could mitigate stress during loading in meat donkeys, reducing loading time, the frequency of stress-related behaviours and the interaction between donkeys and handlers. However, since no differences have been found between the two groups after transport, we failed in demonstrating the efficacy of habituation in reducing stress related to travel and unloading.

Despite the described limitations, it is worth remembering that loading is considered the riskiest transport phase, both for the animals and the handlers; therefore, the reduction of undesired behaviours (such as trying to escape, kicking, rearing…) together with the reduction of number of human interventions needed, both for horses and for donkeys during loading procedures, should be considered a positive result of the presented studies. Moreover, problems during loading could largely increase the total time needed for transport, increasing the costs for the transporter. Future researches should try to improve the training methods proposed, including effective training to travel and unloading phases, with the aim of enhancing the welfare of equids during the entire transport.

In addition, Chapter 4 introduces a new physiological indicator of stress (i.e. Chromogranin A), used for the first time to evaluate transport-related stress in donkeys. This measure could represent an improvement in transport stress evaluation, since it can be detected in saliva, whom collection not only is considered to be non-invasive and little stressful for the animal, but also does not require specifically trained staff and can be easily carried out on many animals directly on-farm. Our results showed a decrease of Chromogranin A after transport. Even if our preliminary study involved only a limited number of donkeys, it can be considered as a starting point for future research involving a larger animal sample.

In conclusion, this thesis represents a starting point in addressing important questions for equids welfare “from science to practice”. Finding practical solutions, such as training animals to loading procedure or finding new indicators to evaluate stress response, still represent important challenge and should be considered for future scientific research.

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# 7. List of publication of the Author

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