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“I know of no pleasure deeper than that which comes from contemplating the natural world and trying to understand it”

David Attenborough, *in* Life on Air: Memoirs of a Broadcaster, 2002

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Resumo

A resistência a anti-helmínticos (RAH) de parasitas gastrointestinais de ruminantes e cavalos tem sido largamente descrita em todo o mundo nas últimas duas décadas. De modo a atrasar, ou até prevenir, o seu desenvolvimento, uma nova abordagem à desparasitação destes animais deve ser feita, considerando as ferramentas de diagnóstico disponíveis atualmente. Este estudo tem como objetivo evidenciar os desempenhos diferenciais de quatro métodos coprológicos comummente empregados, usando diferentes soluções de flutuação, e fazer algumas recomendações práticas sobre qual a técnica que parece ser mais adequada para enfrentar este problema emergente no maneio de equinos.

Esta tese divide-se em duas partes. Na primeira, três espécies de ungulados selvagens foram analisadas no ARTIS *Amsterdam Royal Zoo* para determinar a repetibilidade de quatro métodos coprológicos testados - flutuação simples (SF), flutuação com centrifugação (CF), McMaster (McM - sensibilidade de 50 ovos por grama [OPG]) e Mini-FLOTAC (MF - sensibilidade de 5 OPG) - em associação com três soluções de flutuação de diferente gravidade específica (GE): de sal (GE = 1,20), de sulfato de magnésio (MgSO4) (GE = 1,24) e de açucar (GE = 1,28) onde cada amostra foi analisada 10 vezes. Na segunda parte, foram colhidas 17 amostras fecais de equinos da raça Sorraia, em Abril de 2019 (12 fêmeas na pastagem e cinco machos estabulados), e analisadas com os mesmos métodos, mas apenas com as soluções de sal e açúcar. Cada amostra foi analisada em triplicado para SF e CF e em duplicado para McM e MF. Como parâmetro semi-quantitativo, o número de ovos foi contado em 10 campos de cada lâmina microscópica e a média foi obtida para cada réplica.

Na primeira parte, a CF foi capaz de evidenciar maiores contagens totais com menores coeficientes de variação (CV), principalmente com as soluções densas. MF apresentou muito boa precisão em todas as espécies, com diferentes soluções aparentemente melhores para cada uma. Na segunda parte, o SF_{sal} obteve os melhores resultados para a deteção de *Triodontophorus* spp., embora não significativos ($p > 0,05$). No entanto, teve um CV menor do que as de CF, o que a torna a técnica qualitativa mais precisa. Foram observadas altas cargas parasitárias (média de 1825 OPG para todas as técnicas) e $MF_{açúcar}$ foi a técnica mais consistente pela sua baixa variabilidade. Através de coproculturas confirmou-se a presença e prevalência de *Cyathostomum s.l.* tipo D (100%) e *S. vulgaris* (5,9%).

Os resultados obtidos aqui suportam o uso de CF e MF em parques zoológicos como ferramentas de diagnóstico válidas. Em suma, MF mostrou-se mais preciso em todo o estudo, exigindo a revisão das diretrizes atuais para o diagnóstico de RAH, nomeadamente em equídeos. Surpreendentemente, uma a solução de sal parece ser mais adequada para métodos qualitativos e a solução de açúcar mais adequada para os quantitativos.

Palavras-chave: Ungulados, zoo, cavalos de Sorraia, métodos coprológicos, estrongilídeos, sensibilidade, Mini-FLOTAC.

Abstract

Anthelmintic resistance (AHR) in gastrointestinal parasites of ruminants and horses has been continuously described all over the world in the last two decades. In order to delay, or even prevent, the further development of resistance to anthelmintics, a new deworming approach must be taken, considering the diagnostic tools available nowadays. This study aims to evidence the differential performances of four commonly employed coprological methods, using different flotation solutions, and make some practical recommendations on which technique seems better-suited to face this emerging problem in horse management.

This thesis contains two parts. In the first one, three wild ungulate species were analyzed at ARTIS Amsterdam Royal Zoo to determine the repeatability of four common coprological methods – simple flotation (SF), centrifugal flotation (CF), McMaster (McM - sensitivity of 50 eggs per gram [EPG]) and Mini-FLOTAC (MF – sensitivity of 5 EPG) – in association with three flotation solutions with different specific gravities: salt (SG=1.20), magnesium sulphate ($MgSO_4$) (SG=1.24) and sugar solution (SG=1.28); where each sample was analyzed 10 times. In the second part 17 fecal samples of Sorraia horses were collected in April 2019 (12 females on the pasture and five stabled males) and analyzed with the same methods but only with the salt and sugar solutions; each sample was analyzed in triplicates for SF and CF and in duplicates for McM and MF. As a semi-quantitative parameter, the number of eggs were counted in 10 fields of each slide and the mean was obtained for each replicate.

In the first part of the study, CF was able to evidence a higher amount of total egg counts, especially with the denser solutions, with lower coefficients of variation (CV). MF performed with very good precision across the species, with different solutions apparently better for each one of them. In the second part of the study, SF_{salt} obtained the best results for detecting *Triodontophorus* spp. eggs, although not significantly ($p>0.05$). Yet, it showed lower CV than CF techniques, which makes it a more precise and reliable technique. High parasitic burdens (mean of 1825 EPG for all techniques) were registered and MF_{sugar} performed more consistently as seen by its low variability. Through coprocultures, the prevalence of *Cyathostomum s.l.* type D (100%) and *S. vulgaris* (5.9%) were also detected.

The results obtained here support the use of CF and MF methods in zoos as valid diagnostic tools. In general, MF was shown to be more precise and reliable across the study, urging the need to review current guidelines for the diagnosis of AHR, namely in horses. Unexpectedly, a clear association was evidenced, as salt solutions seem to be better suited for qualitative methods, whereas the sugar solutions better suited for quantitative ones instead.

Keywords: Ungulates, zoos, Sorraia horses, coprological methods, strongyle, sensitivity, Mini-FLOTAC

Introductory note

The present research was carried out in ARTIS Amsterdam Royal Zoo and in the Laboratory of Parasitology and Parasitic Diseases of the Centre for Interdisciplinary Research in Animal Health, Faculty of Veterinary Medicine, University of Lisbon (CIISA-FMV-UL) as part of the author's training period during the 6th year of the Integrated Master in Veterinary Medicine. This work has already resulted in:

- A poster communication in the Proceedings of the 18th International Conference "Life Sciences for Sustainable Development", 26th to 28th of September, 2019, Cluj-Napoca, Romania (see Appendix A)

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List of Abbreviations and Symbols

cm – centimeter

mm – millimeter

μm – micrometer

AH – anthelmintic

AHR – anthelmintic resistance

BZ – benzimidazoles

CF – centrifugal flotation

EPG – eggs per gram

FEC – fecal egg count

FECRT – fecal egg count reduction test

IVM – ivermectin

LEV – levamisole

McM – McMaster

MF – Mini-FLOTAC

ML – macrocyclic lactones

MOR – morantel

MOX – moxidectin

PYR – pyrantel

SAT – selective anthelmintic treatment

SF – simple flotation

SG – specific gravity

1. Description of Internship Activities

The author's curricular internship took place at ARTIS, the Amsterdam Royal Zoo, between the 3rd of September and the 21st of December, consisting of a total of 640 hours. A description of the institution is given in the chapter 3.2 - ARTIS, Amsterdam Royal Zoo.

During his training period, the author was responsible for the diagnostic parasitology, analyzing over 150 fecal samples (Appendix B), while still accompanying the attaining vet in the routine rounds and check-ups. The parasitological work consisted of coprological analysis of animals of different classes, with two routine methods: a direct fecal smear to evaluate the presence of pathological protozoans; and a simple flotation method with a commercial magnesium sulphate solution to evidence any parasite eggs present. If any parasite infections were detected, the decision to deworm the animals was made by the veterinary team.

The clinical work at the zoo included: animals' chemical or physical immobilization and handling; primate vaccination and identification with microchip; ungulates, canids and primates contraceptive administration; wound cleaning and treatment of various mammals (tamarins, beavers and tapirs); and necropsies, among other routine activities. The student also participated in non-common procedures of zoological medicine, such as: health check-ups for transportation of African wild dogs (*Lycaon pictus*) and jaguars (*Panthera onca*); induction and monitorization of anesthesia of a chuckwalla (*Sauromatus ater*); clinical emergencies, including fractures in birds and primates and standing sedations in Asian elephants (*Elephas maximus*).

Besides these specific situations, there were some clinical cases that accompanied the author for the majority of its internship. Two clinical cases important to highlight were a Californian sea lion (*Zalophus californianus*) with corneal edema refractory to treatment and continuous pain management of a spinal hernia and a yellow-throated marten (*Martes flavigula*) with chronic hemorrhagic diarrhea refractory to treatment. Cases discussions with the chief veterinarian, Dr. Martine van Zijll Langhout, about these cases were common as changes in treatment of both cases were frequent. Euthanasia was also a topic of discussion in which the student was involved, when the well-being of the animals could not be sustained.

The author also had the chance to collaborate in a nature conservation project concerning the captive breeding and reintroduction of Polynesian tree snails (*Partula affinis*, *P. hyalina* and *P. nodosa*). In this project, he was involved in performing regular health check-ups of snails from the ARTIS collection and from St. Louis Zoo. These check-ups intended to screen the snails for *Cryptosporidium* spp. and heavy burdens of parasites to prevent the introduction of these pathogens in the wild. The importance of this preventive measure is that the

dissemination of these pathological agents could decimate other gastropods populations and lead to their extinction, as most of them are insular species.

In addition to following the daily work operations of the vet and zookeepers teams in ARTIS, the author was also able to go to other Dutch institutions, such as Wildlands Emmen Zoo and Gaia Zoo, to perceive other zoological realities. The opportunity to perform a necropsy on a Californian sea lion (*Zalophus californianus*) at the Faculty of Veterinary Medicine of Utrecht University, and the day spent at the primates rescue center Stichting AAP, must be highlighted, as they were pivotal moments for the student to contact with other areas of veterinary medicine, such as pathology and rehabilitation medicine of wild animals.

Figure 1 – Examples of internship activities. Left to right, top row: cardiopulmonary auscultation during hoof trimming of a red river hog (*Potamochoerus porcus*); collection of blood sample from a jaguar (*Panthera onca*); an X-ray of a bladder stone in a common chuckwalla (*Sauromalus ater*) prior to cystoliththotomy; corneal edema in a californian sea lion (*Zalophus californianus*). Bottom row: anesthesia reversal in an african wild dog (*Lycaon pictus*) after contraceptive injection; anesthesia of a yellow-throated marten (*Martes flavigula*) for a health check-up; gill probing of an ocellate river stingray (*Potamotrygon motoro*) (Originals).

2. Introduction

As part of veterinary medicine, diagnosing disease is probably the most important step to assure animal health and welfare, as in most cases, without it, no sort of treatment or action plan is implemented (Constable, Hinchcliff, Done and Grünberg, 2017). This is especially true when considering animals that are not as medically approachable as domestic and companion animals, such as livestock, due to their large individual numbers (Hoffman et al, 2012), or wild fauna, because of their habit to hide clinical signs of disease (Kouba and Willard, 2005), for example. Constant research on new diagnostic methods and evaluation of well-established methods, is then of great importance to guarantee the most accurate diagnosis.

In the past few years, an increase in the development of molecular-based methods, such as polymerase chain reaction (PCR) tests (Ryan, Paparini and Oskam, 2017) or enzyme-linked immunosorbent assays (ELISA) (Andersen, Howe, Olsen and Nielsen, 2013), to confirm the existence of pathogens and diseases is clearly noticeable. However, this type of techniques is off reach for a great part of the veterinary community, whether for practical, geographical or economic reasons, among others (Gonçalves et al, 2014). Thus, diagnostic methods that can be applied at the field level still have value and their development should not be discouraged, as they tend to be non-invasive, easier and cheaper to perform and their use more widespread as a consequence. Parasitology is probably one of the veterinary fields that most relies on these low technological methods, in order to easily study the presence of parasitic infections in animal populations worldwide and to rapidly make on-the-spot treatment-based decisions.

A perfect example of the reliance of veterinarians on diagnostic parasitology methods are the zoological institutions. Although zoos are perceived as prime places for the investigation of wild animals, the actual veterinary-related knowledge they produce is normally scarce and of difficult access. Among other reasons for this, the fact that zoos harbor large collections of endangered species threatened in their natural habitats invokes caution when handling them, limiting the collection of valuable data on behalf of the well-being of the animals. As a consequence, the greater part of the veterinary data collected from wild animals for diagnostic or research purposes comes from non-invasive methods, such as feces or urine collection or *post mortem* procedures like necropsies (Panayotova-Penecheva, 2013). Because of this, parasitology and pathology are two of the most routinely applied veterinary sciences in zoological institutions, as confirmed during the author's internship at ARTIS. However, despite necropsy procedures being fairly similar and well established worldwide, the parasitological methods applied lack that same consistency across institutions.

There is an immense variety of parasite types and stages that can be detected through parasitological methods (e.g. as blood smears for hemoparasites, muscle enzymatic digestion for the detection of *Trichinella* spp., urianalysis for *Stephanurus edentatus* in pigs, lymph node biopsy for evidencing amastigote forms of *Leishmania* spp., or necropsy for collection of adult parasitic forms from the pulmonary or digestive tract) (Foreyt, 2001; Bowman, 2014; Taylor, Coop and Wall, 2016). However, gastrointestinal parasites are more commonly diagnosticated, particularly in livestock, because of the morbility and production losses associated with them and probably due to the easy access to samples and user-friendly diagnostic techniques. Thus, analyzing feces by means of coprologic methods comprises the great majority of diagnostic logistics in parasitology, especially through flotation methods (simple floatation or McMaster) (Hendrix and Robinson, 2012; Ballweber, Beugnet, Marchiondo and Payne, 2014).

Furthermore, when considering the use of flotation methods to evidence the presence of eggs in the feces, for example, various factors become important to determine and evaluate, such as density of the flotation used, the density of the eggs, the straining mesh used and the time allowed for flotation (Vidyashankar, Hanlon and Kaplan, 2012). This has led to a great variability in the available proceedings of parasitological diagnostic methods, making it difficult to implement one as a gold standard. In the absence of this, it is then crucial that the performance of different processing steps and parasitological methods must be under continuous research, rendering a satisfactory assessment on the accuracy of each method's variation. Besides accuracy, the repeatability of each method, its precision, is also an important characteristic of a diagnostic test to determine its reliability (Gonçalves et al, 2014).

With recent reports of resistance to anthelmintics in all livestock species, this type of analysis on established parasitological methods is of extreme importance, taking into consideration that they are the basis of treatment-based decisions (Kaplan and Nielsen, 2010). The accuracy and reliability of these methods must be evaluated, in order to determine which methods should be used as trusted diagnostic and decision tools. Only then it will be possible to correctly tackle this emerging problem in animal management and prevent its further development.

3. Bibliography review

3.1. Parasitism and coprology

With the increasing concern for welfare and well-being of animals worldwide, the importance of applying non-invasive techniques of diagnostic and managing animal's health is also increasing. One of the veterinary sciences that uses this type of techniques routinely is parasitology, in particular coprology applied to it. The study of feces to evidence parasitism in animals has long been a reality in veterinary medicine. In the following bibliographical review, some of the methods and factors influencing coprological examinations will be addressed. For the purposes of this study, the concept of parasitism will be defined, according to Hendrix and Robinson (2012), as the relation established between two individuals of two different species, where one (parasite) lives on or within the other (host), for just a phase or its whole life. During this time, the parasite is metabolically dependent of the host and thus interferes with the metabolism of the latter, either positively or negatively. The latter defines parasitosis.

3.1.1. Collection and preservation of fecal samples

In order to perform an accurate coprological examination with any of the methods mentioned afterwards, a proper collection protocol must be carried out. Feces ought to be fresh for reliable results, either collected directly from the animal's rectum or from the field, preferably after witnessing the animal defecating, as long as they are not more than twelve hours old (Foreyt, 2001; Nielsen et al, 2010a). Samples should be collected individually, at least five grams each, with a minimum of 10 samples per herd or flock and put in a wide mouth plastic container duly identified with the animal's identification and sampling date (Taylor et al, 2016).

Unless examined in the same day of the collection, samples must be preserved according to the techniques to which they are intended. Cooling is the method of choice, being the easiest and allowing for later application of fixatives. Temperatures below 6ºC prevent development and hatching of the eggs, as does an anaerobic environment (Nielsen et al, 2010a). In fact, in 1986 Foreyt demonstrated that conserving samples at 4ºC allows for the recovery of up to 80% of the eggs, even after 50 days, setting the storage temperature traditionally used (Foreyt, 2001). On the other hand, temperatures below zero should be discouraged as they tend to result in egg rupture due to crystallization and, consequently, the inability to float.

Other preservation methods include the use of chemicals, depending on the diagnostic technique in view. Formalin is a very versatile and commonly used fixative considering it is readily available and preserves not only the helminth larvae and eggs but also protozoan cysts.

It consists of a diluted solution of formaldehyde at 37%[1] and thus it has carcinogenic potential, which implies some care while handling. The recommended concentrations to fixate the mentioned parasitic forms are 10% and 5%, respectively, with lower concentrations resulting in continued development of the eggs (Garcia, 2002). Despite that, a 2,5% concentration has showed higher egg recovery rates than 10 or 5% for at least one hundred days (Foreyt, 2001), making it a better choice for long-term studies. Unfortunately, fixation by formalin has been proved to alter eggs' density and, inevitably, altering those structures' floating properties (Smith, Wiles, Malone and Monahan, 2007).

3.1.2. Coprological Methods

A brief historical review of the coprological methods used in this study during author's internship is given below. Other methods are used in parasitology, such as the Baermann technique, which was not used and, therefore, not described in this thesis.

3.1.2.1. Direct Fecal Smear

As with many of the veterinary medical subjects, coprology, as the examination of stools for the study of parasitic infections, began gaining interest with human medicine developments. In this case, in 1878 when Grassi, Parona and Parona proved that parasite eggs could be evidenced in a direct fecal smear (Ballweber et al, 2014). Direct fecal smear is in fact the most basic technique for qualitatively assessing the presence of various gastrointestinal parasitic elements in an infected individual. The procedure comprises of mixing a very small amount of feces with a drop of saline solution on the slide, in a thin layer through which newspaper can be read, and observe with a coverslip afterwards (Hendrix and Robinson, 2012). The use of saline instead of water is to prevent any osmotic shock that may lead to distortion of trophozoites and amoebas, making them more difficult to identify (Bowman, 2014). Despite having a very low sensitivity due to the little amount of sample analysed (Zajac and Conboy, 2012), this technique is still in use nowadays because it is a very economic and time inexpensive procedure.

In spite of being crude, this method is valid to determine the main parasitic species in heavily infected animals. However, the main purpose of direct smears today is to examine the presence of protozoans like flagellates, ciliates or amoebas based on their motility, since the technique does not kill them (Bowman, 2014). The high amount of debris turns it difficult to identify helminth eggs, but it makes the protozoan movements easier to inspect, making this method a good routine procedure for the detection of protozoal infections. This takes particular

[1] A solution of formaldehyde 37% equals to formalin 100%;

importance when considering that this type of parasites ranks second in intestinal parasitosis' frequency reports in zoos (Panayotova-Pencheva, 2013).

However, this technique implies that the sample is analysed very shortly after being collected while it is still fresh. Ideally, samples should be no more than 5 minutes old (Broussard, 2003), although, according to the author's internship experience, positive results for motile protozoans can be found even after two to four hours, at room temperature. If distorted protozoans or loss of motility are suspected, a range of stains can be applied only to evidence their morphology, since all of them kill protozoans, with the most common in use being the Lugol's solution (Zajac and Conboy, 2012).

3.1.2.2. Flotation

In 1906, the first scientific publication on a new coprologic method involving flotation of parasitic eggs appeared where Bass described a very simple flotation method using a solution of sodium chloride to evidence uncinariasis in humans (Faust et al, 1939). This new technique was based in the different densities of fecal debris and eggs of parasites causing them to have floating properties that allowed them to be separated in a liquid heavier than water. Because most fecal matter has a specific gravity (SG) of 1.3 g/mL or higher and some of the most studied helminthic eggs have a SG between 1.05-1.24 g/mL, using a flotation solution which has a higher density than the eggs, but lower than the fecal debris, can bring up a great amount of eggs present in the sample, while achieving clear slides to examine them on (David and Lindquist, 1982; Hendrix & Robinson, 2012). According to the solution used, the results may vary, as different parasites have different egg densities (David and Lindquist, 1982; Norris et al, 2018).

The principle of flotation can also be applied when using a centrifuge to concentrate eggs at the top of a denser solution. This technique was first described by Lane in 1924 (Lane, 1924 as cited by Ballweber et al, 2014) and has since then suffered many modifications to increase its analytical sensitivity (Stoll, 1930; Egwang and Slocombe, 1982). However, factors such as centrifugation time, rotations per minute (RPM), rotor radii and, consequently, relative centrifugal force (RCF) influence results and are important in the comparison of different studies (Ballweber et al 2014).

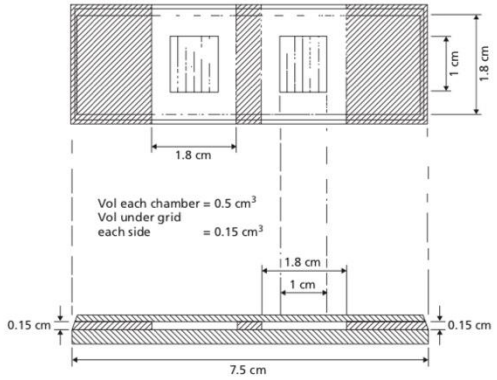
3.1.2.3. Egg-counting Techniques

The amount of eggs in the feces can also be measured by techniques that use the principle of flotation of eggs in denser solutions. Fecal egg counts (FEC) are obtained from

these methods and they are usually described as the amount of eggs per gram (EPG) in the feces. A brief description of the two egg-counting techniques used in this study is given next.

3.1.2.3.1. McMaster

Figure 2 – McMaster slide scheme, evidencing each chamber's volume and dimensions (source: Taylor, Coop and Wall, 2016)



The most used method to determine EPG of infected animals is the McMaster technique, created in the McMaster Animal Health Laboratory in Sydney (Gordon and Whitlock, 1939), which gives it the name. This technique uses a reading slide with two chambers, each with a counting grid in the undersurface of the top glass. When fully filled, each chamber has the volume of 0.5 cm^3, but only of 0.15 cm^3 under the counting grid, as seen in fig. 2. This method is usually performed with a detection limit of 50 EPG (Taylor et al, 2016). Several modifications have also been described concerning the flotation solution used, the ratio of feces to fluid, amount of reading chambers and, consequently, its analytical sensitivity, as reviewed by Ballweber et al (2014). The main advantages of this method are: the waiting time to read the slide is very short, due to the low height of the chambers (0.15 cm); and that the slides can be washed and reused, making them ideal for field assessments.

3.1.2.3.2. Mini-FLOTAC

Over the years, many variations of the McMaster technique have been presented. The most recent one is the Mini-FLOTAC method, which uses a mixing chamber, the Fill-FLOTAC, and a reading disk, Mini-FLOTAC (Cringoli et al, 2013). The Fill-FLOTAC is a unique apparatus in the sense that it facilitates the sample collection, weighting, homogenization and filtration all in one equipment. It includes a sampling kit, a mixer and a 250 μm filter built in (fig. 3). This is one of the main advantages of this method, as it can be applied virtually anywhere, without the need of any other laboratory materials. The other main advantage is its detection limit, being able to go as low as 5 EPG. This is mainly due to the high volume of fecal suspension analysed with the reading disk (fig. 3), as each of the two gridded chambers have a reading volume of 1 mL. Because of these reasons, this technique is becoming of increasingly common use in human and veterinary medicine (Cringoli et al, 2013). The recommended floating solution to be used with this method by its developers is saturated saline, but recent studies achieved good results with other sensitive solutions for different human, domestic and wild animals'

parasite eggs (Barda et al, 2014; Maurelli et al, 2014; Noel, Scare, Bellaw and Nielsen, 2017; Alvarado-Villalobos et al, 2017; Bortoluzzi et al, 2018; Dias de Castro et al, 2017).

Figure 3 – Fill-FLOTAC with its sampling kit, filter and container and the reading disk, Mini-FLOTAC (Original)

3.1.2.4. Fecal Cultures

Nematode eggs recovered from fecal samples, namely strongyle type eggs, can be hatched in the laboratory using a hot air oven or incubator. This method is particularly useful when trying to determine the exact genus/species of parasite infecting animals, by obtaining their infective stage larvae. These are normally the third stage larvae, in the case of strongylids, and they are mainly distinguished by the number of intestinal cells present in their bodies, among other characteristics, such as body length and oesophagus type (Bowman, 2014; Taylor et al, 2016). Various identification keys have been proposed over the years, concerning all types of parasites, focusing on these characteristics (Madeira de Carvalho, 2001; Santos, Madeira de Carvalho and Molento, 2018).

3.2. ARTIS Amsterdam Royal Zoo

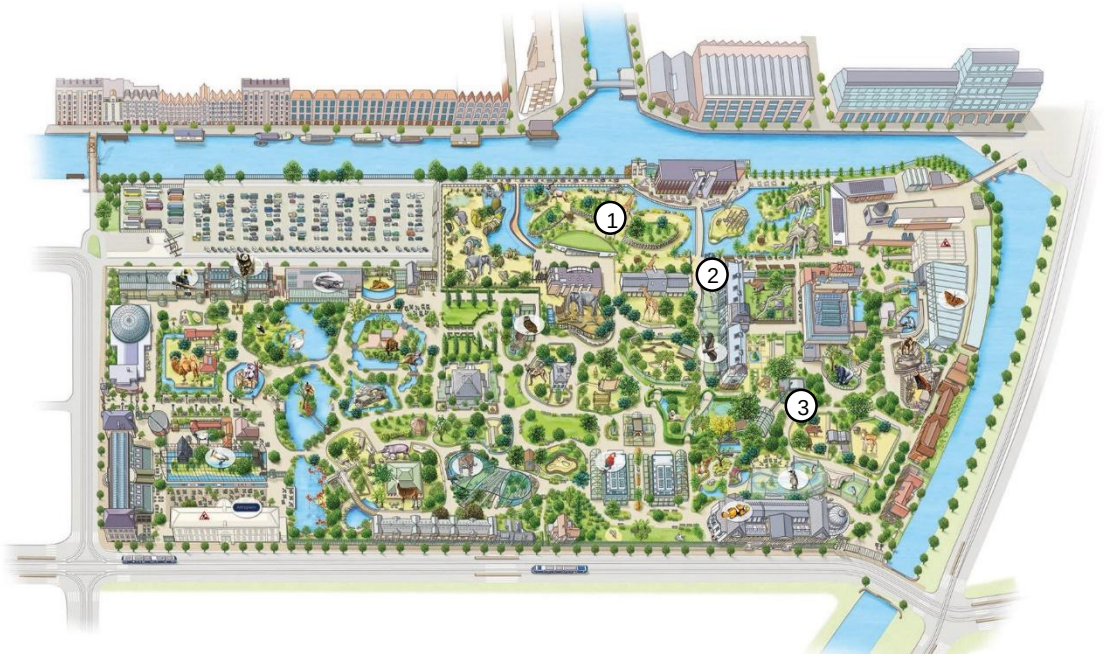
3.2.1. History and context

ARTIS is the oldest zoo in the Netherlands and it is located in Amsterdam. Its full name, *Natura Artis Magistra*, means “Nature is the teacher of the Arts”, motto of its original owners, Messrs Westerman, Werleman and Wijsmuller. They intended to create a publicly accessible zoo, unlike most zoological institutions at that time. Due to risk of bankruptcy in 1939, ARTIS was transferred to the City of Amsterdam, making it publicly owned until today (ARTIS, 2017).

Even confined in the city centre, the zoo extends for 14 hectares in the Plantage neighbourhood and comprises, among other enclosures, an aquarium, a pheasentry and a planetarium (fig. 4). Moreover, ARTIS also includes Micropia, the only microbe museum in the world. With almost 800 species and over 8000 specimens (World Association of Zoos and Aquariums, 2016), the zoo is listed as a full member of the European Association for Zoos and Aquariums (EAZA) and participates in various conservation projects such as reintroduction of

jaguars in Argentina, vultures in Sardinia and snails in Polynesia. It receives over a million visitors every year and is considered the 14th best zoo in Europe (Sheridan, 2016).

Figure 4 - Map of ARTIS, evidencing the Grevy's zebra and greater kudus' enclosure (1), the laboratory of the veterinary department (2) and the red river hogs' enclosure (3) (source: ARTIS, 2019).



3.2.2. Animals used as experimental models

A description of the animals used in ARTIS as experimental models is given below along with a brief clinical history of their parasitology. All the information was obtained from the Zoological Information Management Software (ZIMS) from Species360©.

3.2.2.1. Greater Kudu

Greater kudus (*Tragelaphus strepsiceros*) are one of the largest antelopes in Africa and span over most of southern Africa and, in lesser numbers, in the eastern side of the continent. Its most distinguishable characteristic is their spiral horns with an average length of 120 cm (fig 5.), making them the biggest of the bushbuck family (Estes, 1991). They inhabit mostly shrublands and are currently considered as a species of least concern in the Red List by the International Union for Conservation of Nature and Natural Resources (IUCN) because of their stable population

Figure 5 – Greater kudu in ARTIS with its spiral horns (Original).

numbers and their only threats being competition for natural resources with humans (IUCN, 2016).

The three male greater kudus studied were all born around September 2014 and arrived at ARTIS in May 2015. As the group shared the same interior enclosure, individual sample collection was not possible, thus the animals' general clinical history was considered the same for the three of them. At arrival, a *Trichuris* spp. and a *Cryptosporidium* spp. infections were detected and treated with febantel (Rintal®) and toltrazuril (Baycox®), respectively. The febantel treatment was repeated three more times with an interval of three months. After that, a deworming program with ivermectin (IVM) (Eraquell® and Equimectin®) was started with an interval of 6 months. In November 2018, a *Nematodirus* spp. infection was detected and treated with IVM. In December 2018, strongylid eggs were found in fecal samples and a dose of IVM and praziquantel (Iverpraz®) was given.

3.2.3.2. Grevy's Zebra

The Grevy's zebra (*Equus grevyi*) is the largest of the three species of zebra and is distinguishable from the other two by its white belly, stripeless area around the base of the tail and narrow stripes (fig. 6). Their habitat consists of semi-arid grasslands or scrublands with very little pluviosity, making them the zebra species less dependent on water, not having the need to drink every day (Rubenstein, 2010). Having been extinct in former territories, nowadays the Grevy zebra's range is confined to Kenya and a small area in Ethiopia (Rubenstein et al, 2016). The significant decrease observed throughout the years for these populations is not only due to anthropomorphic actions, such as trophy hunting and competition with livestock and local communities for water sources, but natural causes have also been important, as it is shown by the last anthrax outbreak in Kenya (Muoria et al, 2007).

Figure 6 – Grevy's zebras grazing in ARTIS (Original).

The female zebra studied was born in ARTIS in September 2017. In April 2018, it had a *Parascaris* spp. infection. The treatment for it consisted in dosages of IVM in April and July (Ivomec®), August and December (Eraquell®) and a pyrantel (PYR) treatment (Strongid®) in September. As of the ending of the traineeship in December 2018, the zebra had negative results in coprology. However, this infection was present throughout the whole year and even during the author's curricular internship.

3.2.3.3. Red River Hog

Red river hogs (*Potamochoerus porcus*) are an African swine species distributed across west and central sub-Saharan Africa inhabiting its forests. What differentiates this species from other swine species is its reddish colour, dorsal white strip and characteristic hair tuffs pending from its ears (fig. 7). Both male and female present big tusks as in other wild pigs (Nowak, 1999). Although these hogs are considered as a least concern species by IUCN, its population numbers are decreasing due to hunting and trapping of terrestrial animals in those areas (Reyna, Jori, Querouil and Leus, 2016).

Figure 7 – Red river hog evidencing its hair tuffs pending from its ears (source: www.arkive.org).

Three adult males, two born in 2011 and one in 2013 comprised the group of studied red river hogs. As of August 2014, all three specimens were at ARTIS and were dewormed with IVM (Eraquell®). In December 2014, treatments with febendazole (Pigfen®) and levamisole were given. In 2016, after adult worms were found in feces, three treatments with rotational anthelmintics (AH) were performed: one in January with praziquantel; in June with febantel; and in December with IVM (Eraquell®). In 2017, three doses of febendazole were given, with a 6-month interval. In addition, in November 2017, a treatment with praziquantel and pyrantel were given. Also, in this month, two treatments with febendazole, with an interval of two weeks, were performed. In 2018, five treatments with febendazole were given, with an average interval of 2 months. Three treatments with IVM (Eraquell®) were also given, in February, November and December, after *Ascaris* spp. eggs were found in fecal samples each time. A treatment with flubendazole (Flubenol®) was also made in May. As of January 2019, the hogs were negative for worm ova in coprology.

3.3. Horse as the subject host

3.3.1. Sorraia breed

The Sorraia breed is one of the four Portuguese horse breeds and is considered one of the most threatened of extinction. Due to its low national breeders and population numbers, it is currently considered as a particularly rare breed in Portugal (Carolino, Afonso and Calção, 2013) and as a critical-maintained breed by the Food and Agriculture Organization (FAO) (FAO, 2000). As of the last survey in 2016, according to *Sociedade Portuguesa de Recursos Genéticos Animais* (SPREGA), 152 females and 149 males were registered in Portugal, with

15 national breeders in regions of Ribatejo and Alentejo (SPREGA, n.d.). Moreover, since this breed was recovered from only 12 specimens (7 females and 5 males), the inbreeding coefficients in the existing horses are concerningly high, being higher than 25% with a rate of inbreeding of 5.2% per generation (Pinheiro, Kjöllerström and Oom, 2013). This fact only underlines even more the risk of extinction that this breed is under and the need to take concrete steps in the conservation of this genetical resource.

Figure 8 – Herd of Sorraia horses with their white-fringed hair (source: www.autoctones.ruralbit.com).

With an average height of 1.44m for females and 1.48m for males, this breed is considered a small sized horse and is commonly used as a working or riding breed (Pinheiro, 2008). Sorraia horses are described as having a subconvex body shape and a long convex profile head with a dark muzzled area. However, its most distinguishable characteristics are its dun or grullo color, darken extremities (ears, hooves and muzzle), black dorsal stripe, sometimes zebra stripes on the legs and two-colored hair in the mane and tail, mainly dark and fringed with light-colored hair (Pinheiro et al, 2013), as depicted in fig. 8. All these characteristics point to the primitive origin of this breed, as they look very similar to the Paleolithic representations of pre-historic horses. Further evidence can be found on genetic studies, suggesting that the Sorraia breed is the most likely ancestor of all the Southern-Iberian horse breeds (Luís, Bastos-Silveira, Cothran and Oom, 2006).

3.3.2. Parasitism in Sorraia horses

Very few studies concerning the gastrointestinal parasites of Sorraia horses have been performed. To the author's knowledge only three were developed, one in the horse stud farm of Alter do Chão, in Alentejo, and two in the Escola Superior Agrária de Santarém, in Ribatejo. In the first one, *Cyathostomum* spp. and *S. vulgaris* were detected (Osório, 2011), whereas the other two detected a larger variety of gastrointestinal nematodes. *Cyathostomum* spp. was present in all analysed horses, followed by *Triodontophorus* spp., *Strongylus* spp., *Trichostrongylus axei*, and *Oesophagostomum* spp., in decreasing order of prevalence (Crespo, Rosa e Ferreirinha, 2003; Crespo, Tagarroso, Rosa, Vicente e Borges, 2004). The FEC for these two studies were initially of about 740 EPG and 500 EPG, respectively.

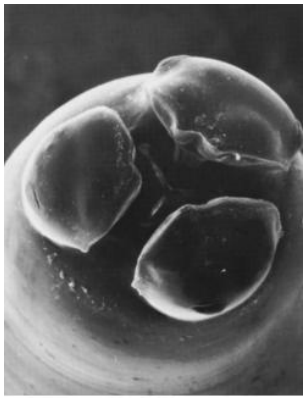
3.4. Parasites used as experimental models

In this study, six genera of parasites belonging to three families were found in the analysed fecal samples. Their morphology, epidemiology and ecology are briefly addressed below.

3.4.1. Family Ascarididae

Ascarids are some of the largest nematodes found in animals and have a very distinct pathogenesis from other nematodes. The two species mentioned in this study share most morphological traits and pathogenicity so a joined description of *Ascaris suum* and *Parascaris* spp. will follow. After that, an individualized short review of their epidemiology, ecology and control will be given.

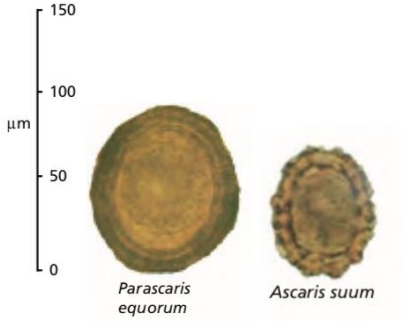
Figure 9 – *Ascaris suum* evidencing the characteristic three lips surrounding the stoma of ascarids (source: Bowman, 2014).



As a main morphological characteristic, ascarids share three lips surrounding their mouth, making them very distinct from other nematodes (fig. 9). The reason for these lies in their form of feeding, as they use the serrated teeth in the lips to cut the intestine mucosa and obtain blood. Their adult forms are rigid parasites and are also very recognizable by their size. *Ascaris suum* is the largest intestinal nematode of swine species worldwide and *Parascaris* spp. is one of the biggest in horses, with females from both species reaching lengths of about 40 cm (Bowman, 2014).

Their life cycle is direct and does not differ in-between these species and is presented in section 3.4.1.2.. Their eggs are also similar with the ones from *Ascaris suum* being ovoid (50-75 x 40-55 µm) and the ones from *Parascaris* spp. being spherical (85-100 x 80-90 µm), depicted in fig. 10 (Taylor et al, 2016). They are easily identifiable under the microscope as they are of medium size with a very thick and multilayered shell, with an outer layer that is irregularly mammilated (fig.10).

Figure 10 – *Parascaris equorum* and *Ascaris suum* egg showing its multi-layered appearance and thick walls. Adapted from Taylor, Coop and Wall, 2016.



Once ingested, the infective eggs hatch in the stomach and the third-stage larvae (L3) enters the portal vein via the large intestine walls, causing some mild hemorrhage. In the liver, the migration of the larvae through the parenchyma will lead to fibrosis and the formation of “milk spots”, discovered in slaughterhouses. However, these lesions will self-cure after a few weeks and, when present, only indicates recent infection by these ascarids. After the liver, the larvae migrate to the lungs

via the vena cava and the pulmonary artery, where they will penetrate the alveoli and continue to the trachea. This may be accompanied with coughing and nasal discharges. It's in this phase that L3 moults to a fourth-stage larva (L4) and is swallowed to enter in the digestive system again, maturing into a fifth-stage larva (L5) in the small intestine and completing the liver-lung-tracheal-enteric migration, characteristic of these parasites (Cooper and Figueiredo, 2013; Bowman, 2014; Reinemeyer and Nielsen, 2018). The prepatent periods of these parasites are also similar, being 7-9 weeks for *A. suum* and 10 weeks for *Parascaris* spp. (Taylor et al, 2016).

The effect of these migrations through various organs is massive, but the most important pathogenic effect of ascarids is the interference with normal nutrition and growth and the risk of peritonitis associated with small intestine obliteration and rupture, which emphasizes the economical need to prevent this type of parasitosis, either in pigs or horses.

3.4.1.1. *Ascaris suum*

Being one of the most important helminths in swine, *A. suum* is the most prevalent intestinal species worldwide in younger pigs. In developed countries, prevalence ranging between 15-35% has been registered, depending on the production phase and age. These prevalence rates are also greatly influenced by the production systems in place, as in intensive systems, prevalence as low as 11% has been detected and in extensive/organic systems, as high as 73% (Thamsborg, Nejsum and Mejer, 2013). Nonetheless, age must be one of the predominant factors in *A. suum* infection, since natural resistance to this parasite has been showed to increase with multiple infections (Cooper and Figueiredo, 2013).

Because of their thick shell, the infective eggs are very resistant to desiccation and disinfectants and can survive for long periods in the environment (up to 4-8 weeks), depending on sun exposure (Murrell, 1986; Gaasenbeek and Borgsteede, 1998), making this the biggest problem to prevent the transmission of *Ascaris suum*. Furthermore, due to the foraging behavior of pigs and their natural resting position laying on the ground, the highest prevalence of this parasite is detected in early age piglets that suckle in the mammary skin of sows highly contaminated with *A. suum* eggs (Taylor et al, 2016). Hygiene of the environment is thus the most important factor in preventing its transmission (Murrell, 1986). As seen from the prevalence of this parasite in intensive production systems, hygiene does not exclude it from pig farms. However, the great majority of AHs used in pigs are effective against *A. suum* (fenbendazole, levamisole, ivermectin, among others), with only pyrantel tartrate being effective against infective L3 in the intestines (Bowman, 2014). Resistance of this parasite to AH drugs may be rising as some reports suggest it, in particular to levamisole (Zhao, 2017).

3.4.1.2. *Parascaris* spp.

It is commonly accepted that *Parascaris equorum* is the model for its genus. However, recent studies have suggested that the majority of *Parascaris* spp. eggs found nowadays are in fact from *P. univalens*. Nonetheless, *P. equorum* is used as the model species of these parasites in the following characterization. The distinction between *P. equorum* and *P. univalens* can only be made through molecular methods, such as PCR (Nielsen et al, 2014a; Reinemeyer and Nielsen, 2018). Since in the present study none of these techniques were perfomed, the ascarid eggs found in horses were registered as being *Parascaris* spp., in order to be more scientifically correct.

In similarity to *A. suum*, *P. equorum* is also a widespread parasite in all equids and the most pathogenic to foals of under one year old. The larval migration through the intestinal wall and liver, evidenced in fig. 11, lead to a state of malabsorption and hypoalbuminemia, compromising the normal growth and leading to an oedematous appearance (Taylor et al, 2016). In spite of this, hosts infected with this parasite demonstrate the same immunological behaviour, as the ones with *A. suum*, since infected foals tend to develop immune resistance to the parasite larvae after one year of age (Reinemeyer, 2012; Bowman, 2014).

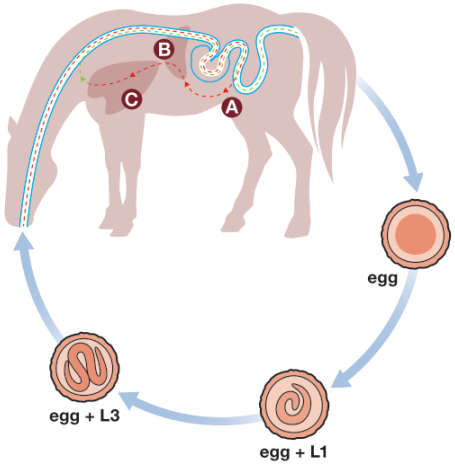


Figure 11 – *Parascaris equorum* life cycle: (A) Hatching of third stage larvae (L3) in the stomach and small intestine, penetration of intestinal veins; (B) larvae reach liver via portal vein, migration through liver tissue and penetration of liver veins; (C) Larvae reach lung via vena cava and right heart, penetration into lung alveoles and migraton via trachea and pharynx to small intestine (moulting to L4 and L5 prior to development into adults). Adapted from ESCCAP, 2019.

Due to differences in livestock management of pigs and horses, hygiene is not so easily obtained with foals as with suckling pigs and prevention relies only on eliminating the contact of foals with fecal matter. The widespread of this parasitosis reflects the frequency of these contacts and so deworming is often used as a prevention tool. Nowadays, piperazine compouds, fenbendazole, pyrantel, ivermectin and moxidectin, among other equine AHs, are effective against *P. equorum* (Bowman, 2014). However, lower rates of efficacy have been reported in the past few years, as will be discussed further. One thing to consider is that, because of the rigid nature and size of this species, deworming infected animals with adult

parasites may pose an increased risk of impaction and complete bowel obstruction, as dead parasite plugs may form in the intestinal lumen (Owen and Slocombe, 1985; Bowman, 2014).

3.4.2. Family Molineidae

3.4.2.1. *Nematodirus* spp.

Being a part of the Trichostrongyloidea superfamily, this genus differs considerably from the rest of the parasites discussed in this thesis. These parasites are identifiable by a dorsal triangular tooth in the stoma, anterior longitudinal cuticular ridges and a vesicle in the cephalic region. They tend to be of small size, with females reaching 25 mm and a spine in the end of the tail, and males of the different species of *Nematodirus* spp. are differentiated by the morphology of their two spiculae, which tips are fused together (Bowman, 2014; Taylor et al, 2016). Another thing that separates this genus from the other trichostrongylids, is their large-sized eggs, as seen in fig. 12, which can be up to twice the size of the normal trichonstrongylid egg, becoming instantly recognizable under the microscope (Taylor et al, 2016).

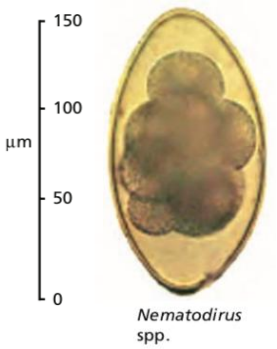


Figure 12 – *Nematodirus* sp. egg, one of the largest nematode eggs of ruminants. Adapted from Taylor, Coop and Wall, 2016.

These parasites are typically found in domestic and wild ruminants, such as cattle, small ruminants, camelids and deer, with one species specific of rabbits. From the eight recognized species of *Nematodirus* spp., *N. battus* and *N. filicollis* are the most important and prevalent in domestic ruminants in temperate climates (Michel, 1969; Taylor et al, 2016). Nonetheless, all species have direct life cycles and they are very similar in-between species but greatly different from other trichostrongylids. Whereas in eggs from *Trichostrongylus* spp. or *Ostertagia* spp. a first-stage larva (L1) is hatched, the eggs of *Nematodirus* spp. hatch an already infective L3, depending on extrinsic stimuli (Bowman, 2014). The level of dependence on these external factors, especially temperature thresholds, is particularly high for *N. battus*, since it needs a prolonged period of chill (11°C) followed by a warmer climate (17-20°C) to hatch. This justifies the occurrence of clinical disease in spring in the northern hemisphere and, normally, with only one generation of parasites per year. It may also be the reason for the presence of *N. battus* to be geographically limited to temperate areas, when compared to other more widespread and less demanding *Nematodirus* spp., such as *N. filicollis*, *N. spathiger* and *N. helvetianus* (Michel, 1969; Van Dijk and Morgan, 2009). These species also have different prepatent periods, being around 2 weeks for *N. battus* and 3 weeks for *N. filicollis* and *N. helvetianus* (Taylor et al, 2016).

After ingestion of the infective L3 on the pasture, the larvae reach the small intestine where it penetrates the mucosa and moult into L4. This larval stage is responsible for the major pathogenic effects in nematodiriosis as L4 are the cause of severe damage and mucosal erosion, with consequent villous atrophy. Haemorrhagic diarrhoeas are common clinical signs, as well as low body condition scores, because of the destruction of the intestinal wall and its diminished absorption capacity. Despite the severe damage caused by these parasites, most AHs used in livestock are effective against them, such as levamisole, macrocyclic lactones and benzimidazoles (Bowman, 2014; Taylor et al, 2016)

3.4.3. Family Strongylidae

This family includes most of the common and important equine nematodes (64 of 83 parasite species) and are generally described as stongylids (Lichtenfels, Kharchenko and Dvojonos, 2008). All the parasites in this family share characteristics such as a well-developed buccal capsule surrounded by leaf crowns and morphologically identifiable copulatory bursas in the males. These characteristics are important traits for hematophagous parasites such as these that, unlike the rest of the parasites addressed in this thesis, inhabit mainly the cecum and colon (Taylor et al, 2016). They can be divided in two general subfamilies: Cyathostominae containing small to medium-sized parasites with a cylindrical buccal capsule, commonly named cyathostomins or small strongylids; and Strongylinae, containing medium to large-sized parasites with a globular or funnel-shaped buccal capsule, called strongylins or large strongylids (Bowman, 2014).

3.4.3.1. Subfamily Cyathostominae

According to Lichtenfels et al (2008), around 50 species of this subfamily were identified as equid parasites worldwide and most individuals can carry thousands of adult nematodes of about 5 to 10 different common species. The 10 most common cyathostomins found are *Cyathostomum catinatum, C. pateratum, Coronocyclus coronatus, Cylicostephanus longibursatus, C. goldi, C. calicatus, C. minutus, Cylicocyclus nassatus, C. leptostomum* and *C. insigne* (Lyons, Tolliver and Drudge, 1999; Madeira de Carvalho, 2001; Corning, 2009; Kornaś, Basiaga and Kharchenko, 2011). Despite this relatively low variability in individual hosts, these parasites can make up more than 90% of the parasite population of its host (Kooyman et al, 2016) and represent 95-100% of all strongyle eggs shed in the feces of horses (Kaplan, 2002; Bello and Allen, 2009; Bowman, 2014). Because of its abundance,

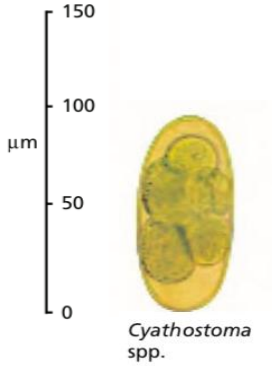


Figure 13 – A cyathostomin egg, typically strongylid. Adapted from Taylor, Coop and Wall, 2016.

prevalence, pathogenicity and growing resistance to AHs, this group of parasites is now considered one of the most important in equine management (Love, Murphy and Mellor, 1999; Corning, 2009).

As seen, this group of parasites has a lot of species diversity but the *Cyathostomum* spp. genus has been considered the type genus of the small strongylids (Lichtenfels et al, 2008). Although some cyathostomins can reach up to 25 mm, *Cyathostomum spp.* are normally smaller with 12 mm in length (Taylor et al, 2016) with a visible dorsal gutter and a wide buccal capsule with absent teeth. Their eggs are typically strongylid (fig. 13) with an oval shape and thin walls (32-56 x 53-105 µm) (Lichtenfels et al, 2008).

As seen in fig. 14, evidencing the direct life cycle of this species, after the embryonated eggs are expelled, the eggs hatch a L1, moult to L2 and a L3 can develop in just 3 days (Corning, 2009). This is the long-lasting infective stage for cyathostomins and as larvae are ingested from herbage and reach the cecum and colon, they lose their protective sheath from the last moult while penetrating the mucosa. In this stage, these larvae are considered early third stage larvae (EL3). While some species stay in the mucosa, in the Lieberkühn crypts, others may infiltrate even further into the submucosa. Here, the inflammatory response to their presence leads to the formation of a capsule surrounding the larvae, where they will moult to L4. As soon as these larvae reach a considerable size, the capsule is ruptured along with the mucosa (excystation), releasing them into the intestinal lumen where they will mature into adults (Bowman, 2014; Reinemeyer and Nielsen, 2018; Martins et al, 2019).

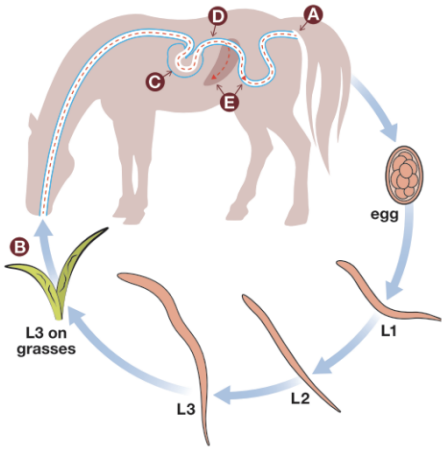


Figure 14 – Life cycle of small strongyles: (A) egg shedding; (B) oral uptake of third stage larvae (L3) with grass; (C) exsheathment through gastric fluids; (D) passage of exsheathed L3 through small intestine; (E) invasion of mucosa/submucosa of colon and caecum, moult to fourth stage, return to intestinal lumen and final moult before development to adult stage. Adapted from ESCCAP, 2019.

The prepatent period for cyathostomins is about 2-3 months (Taylor et al, 2016). However, unlike other intestinal nematodes, small strongylids larvae can pause their development if environmental conditions are not suitable for their survival in the free-living phase. They can then enter in hypobiosis, a state of latency when their development is inhibited. Some encysted larvae have been registered to be in hypobiosis for as much as 2.5 years (Gibson, 1953 cited in Reinemeyer and Nielsen, 2018). In temperate climates, such as

in Portugal, this tends to happen during the winter and as the temperatures rise in the spring so does the development of the larvae. This leads to the synchronized development and excystation of cyathostomins' larvae (Corning, 2009; Bowman, 2014).

Because of these two possible routes of development, the pathogenicity of cyathostomins can be observed in two forms. The first one occurs when larvae are continuously penetrating and rupturing the mucosa with no hypobiosis, leading to malabsorption of nutrients, chronic diarrhoea and, consequently, weight loss (Love et al, 1999). This protein losing enteropathy is recognized as cyathostominosis type I and has a high morbidity (Melo-Franco, 2014). However, when hypobiosis is present, the *en masse* emergence of the larvae can lead to a second clinical manifestation with emaciation and acute diarrhoea that becomes chronic. This is commonly known as larval cyathostominosis or cyathostominosis type II and is related to the occurrence of colics and infarctions that are sometimes fatal. This type of clinical manifestation is mostly seasonal but can also be triggered by recent deworming as the presence of luminal larvae and adults provides an important negative feedback to the encysted larvae (Love et al, 1999; Corning, 2009; Bowman, 2014; Reinemeyer and Nielsen 2018).

The treatment of this parasitic disease must be carefully considered because of the mentioned feedback and has been recently studied as more reports of anthelmintic resistance appear. Usual equine anthelmintics (benzimidazoles, pyrantel and macrocyclic lactones) are effective in removing luminal cyathostomins. The encysted larvae are more difficult to treat, since pyrantel and ivermectin have no effect on them (Taylor et al, 2016). Also, extra care should be taken because the massive die-off of these inhibited stages has been associated with higher gut dysbiosis and further inflammatory responses (Corning, 2009; Walshe et al, 2019). Moreover, with the emergence of anthelmintic resistance of cyathostomins, the other used drugs, like fenbendazole, have now very low efficacy in treating encysted larval stages and moxidectin is currently the only AH with some effect on these stages, even if very variable (Corning, 2009; Bowman, 2014, Bellaw et al, 2018). The equine nematode's anthelmintic resistance problem will be further developed.

3.4.3.2. Subfamily Strongylinae

This subfamily comprises of five genera of which only two will be addressed as they are the only two of interest to this thesis.

Figure 15 – A *Strongyus* spp. Egg with its characteristic morula inside. Adapted from Taylor, Coop and Wall, 2016.

3.4.3.2.1. Strongylus spp.

Genus *Strongylus* has long been considered one of the most important groups of parasites in equids because of their characteristic highly pathogenic larval migrations (Bowman, 2014; Reinemeyer and Nielsen 2018). Four species are described in this genus, *S. vulgaris*, *S. equinus*, *S. edentatus* e *S. asini*, with only the first three infecting horses. All these

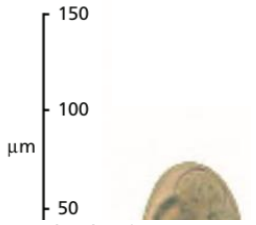
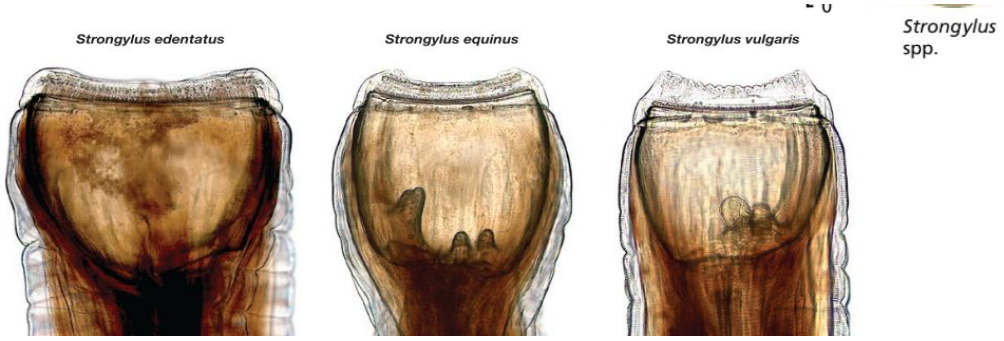


Figure 16 – Anterior end of *Strongylus* spp. evidencing each species buccal capsule, leaf crown and presence or absence (in the case of *S. edentatus*) of tooth-like structures at the base of the buccal capsule. Adapted from ESCCAP, 2019.



parasites are characterized by their deep buccal capsules anteriorly demarcated by two leaf crowns (Taylor et al, 2016). The three equine species are easily distinguishable by the teeth in their buccal capsules (fig. 16) and the parasitic stage of their direct life cycle is also very different. However,the free-living phase of these parasites is the same, starting with medium-sized eggs evidenced in fig. 15 (*S. vulgaris* e *S. equinus:* 61-75 x 37-46 µm; *S. edentatus*: 90-98 x 43-51µm). From the egg, a L1 hatches in two weeks under optimal conditions and later develops into L3, with its protective sheath, which is the infective stage (Lichtenfels et al, 2008; Taylor et al, 2016).

S. vulgaris is the smallest of the three species with females reaching 24mm, but its migration is considered the most pathogenic of them. After ingestion, the L3 loose their protective sheaths and penetrate in the mucosa of the cecum and ventral colon, moulting into L4 in the submucosa (fig. 17A). From here, the larvae enter the intima layer of small arterioles and migrate through the endothelium of the colic, cecal and finally cranial mesenteric artery, depicted in fig. 17B. This phase of the migration causes arteritis, thrombosis and embolism of these

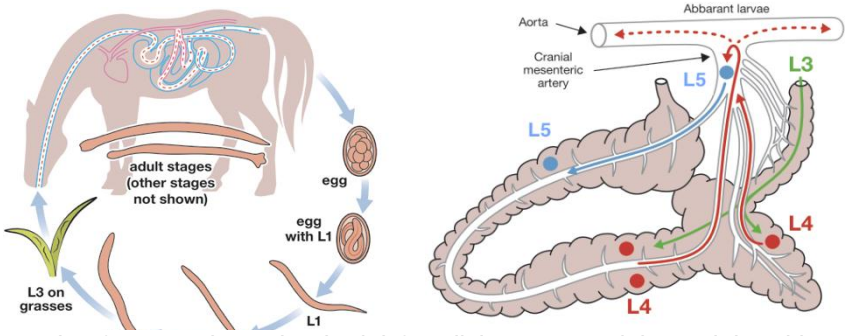


Figure 17 – Life cycle of *Strongylus vulgaris*: (A) free-living stages; (B) arterial and intestinal migration of larvae. Adapted from ESCCAP, 2019.

arteries and branches, sometimes leading to their complete occlusion. Then, when the growing phase has ended, the L4 penetrate again in the arterial lumen and reach the intestinal walls of the cecum and colon and become encapsulated. The moult to L5 takes place and the larvae rupture their capsule into the intestinal lumen, where they mature, with the prepatent period for this parasite being 6-7 months. For years, this species' migration was thought to be closely related to the occurrence of verminous colic in horses, but nowadays, with AH programs in place in the majority of equine farms, strongilosis by *S. vulgaris* and thromboembolic colic are less correlated (Owen and Slocombe, 1985; Kaplan, 2002; Bowman, 2014; Taylor, et al, 2016; Reinemeyer and Nielsen 2018).

A B

On the other hand, *S. edentatus* and *S. equinus* are the larger species of this genus with females reaching up to 44 mm and 47 mm, respectively. The prevalence of these strongylids is not as high as the one of *S. vulgaris*, and *S. equinus* is the least commonly found in domestic horses. The migrations of these parasites are also very different from *S. vulgaris*, as they don't move through the arterial intima layer. Instead, these species migrate through the hepatic parenchyma. Both species penetrate the mucosa of the cecum and colon, but *S. edentatus* reaches the liver via the portal vein whereas *S. equinus* via the peritoneal space. By the start of the migration, *S. equinus* larvae have already moulted into L4, but *S. edentatus* only moults in nodules in the liver. The returning route of these species also differs as *S. edentatus* returns to the large intestine's lumen through the retroperitoneal tissue, especially hepatic ligaments, *S. equinus* tends to pass through the pancreas. Both species' larvae use these tissues to undergo their final moult to L5 and only after do they migrate through the intestinal walls into the cecum and colon's lumen. The prepatent periods for *S. edentatus* and *S. equinus* are 10-12 months and 8-9 months, respectively (Owen and Slocombe, 1985; Bowman, 2014; Taylor et al, 2016; Reinemeyer and Nielsen 2018).

Although highly pathogenic, the damage of larval migrations of *Strongylus* spp. are healed overtime, if treatment for this parasitic disease is applied. Currently, equine AHs commonly used, such as benzimadoles, macrocyclic lactones and pyrantel pamoate, are

effective against this genus. Different dosages are described for adulticide or larvicidal purposes, with the exception of pyrantel pamoate, which doesn't have any effect in migrating larvae (Bowman, 2014). With passing years, some reports of anthelmintic resistance have been made and they will be addressed further below.

3.4.3.2.2. *Triodontophorus* spp.

The other large intestinal parasite genus of equids considred in this dissertation is *Triodontophorus*, comprising of five different species specific to these hosts. From these species, *T. brevicauda*, *T. serratus* and *T. tenuicollis* appear to be the most prevalent and are normally found attached to the mucosa of the ventral colon (Mfitilodze and Hutchinson, 1985; Anjos and Rodrigues, 2003; Chapman, Kearney and Klei, 2003; Reinemeyer and Nielsen, 2018). These parasites measure up to 25 mm, have three pairs of in-capsuled teeth and are usually found in mixed strongyles infections, contributing to the pathogenic effect of strongylosis with the formation of mucosal ulcerations (Taylor et al, 2016). Their eggs are very easily recognizable under the microscope as they are very large in comparison to other strongylid eggs (84-120 x 41-68 μm) (Lichtenfels et al, 2008, Reinemeyer and Nielsen, 2018).

Although currently considered part of the Strongylinae subfamily, Gao et al (2017) have found strong evidence analysing mitochondrial DNA that suggest that *Triondontophorus* spp. is more closely related to cyathostomins. Furthermore, despite its life cycle not being completely described still, it is thought to be similar to that of cyathostomins, already described (fig. 13), without any migratory stages (Taylor et al, 2016).

3.5. Anthelmintic resistance

The intestinal nematodes are genetically characterized by rapid rates of nucleotide sequence evolution because of their fast life cycles, which is exponentiated when considering their effective large population sizes, giving them a highly genetic diversity (Kaplan, 2004). Because of these features, it was only logical that strains of these parasites resistant to anthelmintic drugs would arise. These strains would be defined as populations where "the frequency of individuals able to tolerate doses of a compound is higher than in a normal population", with the capacity of transmitting this tolerance to newer generations, according to Prichard et al (1980). They also stated that this resistance could be directed to a particular drug compound with a similar mode of action (side-resistance) or other drugs of different AH groups (cross-resistance).

Nowadays, there are three broad-spectrum AH groups at the disposal of veterinarians to treat grazing animals: the benzimidazoles (BZD); imidazothiazoles (levamisole - LEV) and

hydropyrimidines (pyrantel, morantel - MOR); and macrocyclic lactones (ML) (ivermectin - IVM, moxidectin - MOX) (Kaplan, 2004; Coles et al, 2006; Bowman, 2014). However, the appearance of anthelmintic resistance (AHR) was surprisingly fast when considering that the first reports go back to the late 1950's, with lack of effectiveness of phenotiazine against *Haemonchus contortus* in sheep (Prichard et al, 1980; Drudge et al, 1957 cited by Kaplan, 2004). After the introduction of each new drug to the market, resistance has followed few years after (Table 1) and nowadays it is recognized as a major widespread problem in all livestock species (Prichard, 1994; Kaplan, 2004; Kaplan and Vidyashankar, 2012). A big part of the problem has been the lack of investment in new livestock AHs since the introduction of macrocyclic lactones in the 1980's, aside from monepantel use in sheep in few countries (Kaminsky et al, 2008; Kaplan and Vidyashankar, 2012).

Table 1 – Year of approval of broad-spectrum anthelmintic drugs in sheep and horses comparing to the first published report of its resistance. Adapted from Kaplan, 2004.

Drug	Host	Year of initial drug approval[a]	First published report of resistance[b]
Benzimidazoles			
Thiabendazole	Sheep	1961	1964
	Horse	1962	1965
Pyrimidines			
Levamisole	Sheep	1970	1979
Pyrantel	Horse	1974	1996
Macrocyclic lactones			
Ivermectin	Sheep	1981	1988
	Horse	1983	2002
Moxidectin	Sheep	1991	1995
	Horse	1995	2003

[a]Approval in the United States of America

[b]The first published report did not normally coincide with the first clinical reports of inefficacy

The intensive and regular deworming programs in place caused this alarming rate of AHR appearance in the great majority of farms, which impose a strong selection pressure for resistant strains of nematodes (Prichard, 1980). Related to this, another important factor is the deworming of animals that are not heavily infected, reducing the population of parasites that are not exposed to AHs. This population is called refugia and includes not only parasites in

non-dewormed hosts but also free-living stages of parasites (like the ones on the pasture) and parasitic stages in the host that are not affected by the AHs used (like encysted parasites in the large intestine wall) (Wyk, 2001; Kaplan, 2004; Kaplan and Nielsen, 2010). Currently, refugia is considered as important to tackle the advancements in the AHR problem as spared and alternate use of the drugs themselves (Wyk, 2001; Besier, 2012). Furthermore, other factors influence the appearance and advancement of resistance, like the fecundity of females, lifespan of mature worms, survival of free-living stages in the environment and the inheritance of resistance traits; levels of innate and acquired immunity and behavioral differences affecting exposure rates of the hosts (Churcher et al, 2010).

The impact of AHR is rapidly becoming visible as more farms shut down their production due to the presence of multiple drug resistance nematodes (Sargison, Jackson, Bartley and Moir, 2005; Blake and Coles, 2007). The diagnosis of AHR has been thoroughly reviewed over the years in order to find suitable ways to detect it in time to prevent these situations. Today, these diagnostic methods are divided in molecular techniques and more evidence-approached techniques, extensively reviewed by Coles et al (2006). This separation is extremely important as the former detect the presence of genetic resistance (alleles) in the population, which evolves slowly over time, whereas the latter detect the phenotypic manifestation of resistance in a host population that can appear suddenly (Kaplan and Vidyashankar, 2012). Despite molecular techniques like PCR being useful as sentinels for the detection of rising resistance, they still have limitations as they are allele-specific and drug-specific, with only benzimidazoles specific alleles established and don't yet quantify the influence of multiple alleles to the clinical manifestation of resistance (Taylor, Hunt and Goodyear, 2002; Kaplan and Vidyashankar, 2012).

Other than molecular techniques, multiple approaches have been developed either *in vitro* or *in vivo*. The first group includes methods like egg hatch assay for BZD, larval paralysis and motility test for BZD, PYR, LEV and MOR, larval development test for BZD, LEV and IVM, among other less common techniques. The *in vivo* techniques are mostly limited to the Fecal Egg Count Reduction Test (FECRT), with comparison of EPG in FEC prior and after treatment with AHs (Coles et al, 1992; Taylor, Hunt and Goodyear, 2002; Coles et al, 2006). Nonetheless, because all *in vitro* techniques imply a laboratorial assessment of AHR, FECRT has been assumed as the practical gold standard to determine resistance at the farm level in all livestock species and it can only be interpreted for the population and not individuals (Kaplan and Vidyashankar, 2012).

3.5.1. Resistance in equine nematodes

In the 1960's, the equine health management and welfare changed forever with the introduction of BZD as an AH for horses, generating a new epidemiological approach to parasite control in this domestic species. This new system was designed to control the infections by *Strongylus* spp., especially *Strongylus vulgaris*, based on an interval dose system of 6-8 weeks (Drudge and Lyons, 1966 cited by Kaplan, 2002), which prevented the maturation of any intra-luminal larvae development. The success of this deworming program was recognized worldwide in the equine community (Lyons et al, 1999) and by 1983, with the introduction of ivermectin as a larvicidal of *Strongylus* spp. migrating larvae, these parasites were already considered uncommon and dissociated as a cause of equine colic's development, maintaining this status until the beginning of 21st Century (Kaplan, 2002).

As a consequence, the main parasites of managed horses have changed, with cyathostomins becoming the most important pathogenic parasites since the 1980s (Love and Duncan, 1991; Lyons et al, 1999). However, the widespread interval dose programs directed for *Strongylus* spp, continued being applied, even with their stated decreasing prevalence. These dewormings used rotation of the equine AHs, which currently are the same three classes described before (Gokbulut and McKellar, 2018). As a result, for the past decades, there have been continuous reports of growing resistance to all classes of AHs worldwide in the other two major equine nematode groups: *Parascaris* spp. and cyathostomins (Table 2) (Kaplan and Nielsen, 2010; Reinemeyer, 2012; Peregrine et al, 2014).

Table 2 – Summary of reported resistance worldwide of the main equine nematodes to broad-spectrum anthelmintics. Adapted from Nielsen et al, 2019.

Drug Class	Cyathostomins	Large strongyles	*Parascaris* spp.
Benzimidazoles	Widespread	None	Early indications
Pyrimidines	Common	None	Early indications
Macrocyclic lactones	Early indications	None	Widespread

3.5.1.1. Diagnosing resistance in horses

In horse management, the practical gold standard at field level to analyse these resistances is FECRT, assessing the AH effectiveness in reducing the FECs (European Scientific Counsel Companion Animal Parasites [ESCCAP], 2019; Nielsen et al, 2019), as previously described. AAEP guidelines by Nielsen et al (2019) suggest the inclusion of at least 6 horses, preferably the ones with the highest FEC, and that these horses have not been previously dewormed for at least 8 weeks. With these requirements fulfilled it is possible to evaluate the efficacy of the AH drugs 14 days after they're used and FEC reduction thresholds

have been established (Table 3). Below these cut-off values, resistance of the parasite population in question can be inferred. If a continuous monitoring of FECs is maintained, another parameter that can indicate the rising of AHR in a population is the egg reappearance period (ERP), the time it takes for FECs to reassume significant values of egg shedding after deworming which was already established for cyathostomins (Table 4). A shorter ERP is suggestive of increasing AHR (Nielsen et al, 2019).

Even though FEC of mature horses are normally consistent overtime (Nielsen, Haaning and Olsen, 2006; Carstensen, Larsen, Ritz and Nielsen, 2013), many factors influence FEC and therefore FECRT. Among them, non-uniform distribution of the eggs in the feces, storage of the fecal samples and egg loss during technical processing are some of the most important factors (Vidyashankar et al, 2012). The method used to perform FECRT is extremely important in detecting AHR, as a more sensitive technique will provide more accurate FEC and, consequently, its reduction percentage.

Table 3 – Thresholds of fecal egg count reduction test results to determine the presence of anthelmintic resistance to equine broad-spectrum anthelmintics. Adapted from Nielsen et al, 2019.

Anthelmintic	Expected efficacy if no resistance	Susceptible (no evidence of resistance)	Suspetected resistance	Resistant
Febendazole/Oxibendazole	99%	>95%	90-95%	<90%
Pyrantel	94-99%	>90%	85-90%	<85%
Ivermectin/Moxidectin	99.9%	>98%	95-98%	<95%

Table 4 – Egg reappearance periods of equine broad-spectrum anthelmintics when the drug is fully effective on cyathostomins. Adapted from Nielsen et al, 2019;

Anthelmintic	Usual ERP when drug is effective	ERP when drug was first introduced	ERPs on farms with emerging resistance
Febendazole/Oxibendazole	4-5 weeks	6 weeks	-*
Pyrantel	4-5 weeks	5-6 weeks	-*
Ivermectin	6-8 weeks	9-13 weeks	3-5 weeks
Moxidectin	10-12 weeks	16-22 weeks	4-6 weeks

*Resistance so commonly reported that ERPs have not been measured

In Europe and in the United States of America (USA), AHR of *Parascaris* spp. to macrocyclic lactones has been recently documented (Schougaard and Nielsen, 2007) and extensively reviewed (Reinemeyer, 2009; Reinemeyer, 2012), pointing out the current

effectiveness of only benzimidazoles against these parasites. Something to consider when analysing the growing resistance of *Parascaris* spp., is that it is a dose-limiting parasite (DLP) for most equine AHs, which means that, in order to kill it, a higher dosage must be used. However, because most equine AHs today are considered broad-spectrum, using the recommended dosage will leave room for DLP's to grow resistant (Reinemeyer, 2009).

For cyathostomins, however, the problem is much bigger, as these parasites have been documented to be resistant to all classes of broad-spectrum drugs, except for macrocyclic lactones (Kaplan et al, 2004; Matthews, 2008; Corning, 2009; Bellaw et al, 2018). Their resistance against benzimidazoles has been constantly recorded and reviewed (Matthews, 2008; Corning, 2009) and Bellaw et al (2018) recently evidenced that these drugs can no longer be considered as effective against these parasites due to the widespread of resistant strains. Another important studied AH was PYR, which effectiveness was also evidenced to be decreasing (Kaplan et al, 2004). Nonetheless, PYR resistance is mainly recorded in the USA and Canada, the only two countries where a daily oral dosage of PYR in the diet is considered a normal approach to horse management. Some suggestions have been made that this might be the reason behind that increasing resistance and that this practice should be discontinued (Kaplan and Nielsen, 2010). Finally, the only still fully effective AH in horses against cyathostomins are macrocyclic lactones. However, even signs of rising resistance to this class have been pointed out, with commonly found shorter ERPs, from 8 weeks to 4 weeks (Lyons, Tolliver and Collins, 2009; Bellaw et al, 2018; Molena et al, 2018).

3.5.2. Delaying or preventing advancements in resistance

As seen, the extensive use of intensive chemical deworming techniques has been very short-sighted (Kaplan, 2004). The approach to equine nematodes' control has to be reviewed and it needs to be integrated with non-chemical methods. Such methods can include, selective treatments, correct management of the pasture and its hygiene, biological control with nematophagous fungi and quarantine of new animals.

3.5.2.1. Selective anthelmintic treatment programs

The use of anthelmintics in equine management should not be abolished but reduced and consciously used instead. With the confirmation of AHR around the world, a new approach to AHs emerged based on two principles: egg shedding of mature horses is consistent (Nielsen et al, 2006; Scheuerle et al, 2016); and 80% of the shed eggs in pasture are resulting of contamination from just 20% of the population (Kaplan and Nielsen, 2010), or even a lower percentage of hosts (Lester et al, 2013; Relf et al, 2014). These two statements are the justification for the new selective anthelmintic treatment (SAT) programs and pretend to use

refugia as the buffer for the advancements of AHR (Kaplan, 2004; Kaplan and Nielsen, 2010; Besier, 2012; Pfister and van Doorn, 2018). Once in place, SAT programs attempt to: 1) understand the epidemiology of the present nematodes; 2) determine which drugs are effective in the farm; 3) use the right AH for the correct parasite developmental stage at the appropriate time of the year; 4) determine which horses require less or more frequent treatment; and 5) evaluate the overall success of parasite control (Kaplan and Nielsen, 2010).

In this type of deworming programs, no adult horses in the farm should be dewormed for 12 weeks and, after that, fecal samples of every horse should be analysed to obtain its FEC. Then, according to a certain cut-off value, only the horses exceeding it (medium and high shedders) should be treated with AH and the others (low shedders) should be left untreated to act as refugia for the population. This cut-off value as long been discussed in the equine community with some statements being made that it should be 200 EPG, as shown in Table 5 (Kaplan and Nielsen, 2010; Pfister and van Doorn, 2018; Rendle et al, 2019). However, a study reports that a range of up to 500 EPG should be used, as it holds a better relation between FEC and worm burden (Nielsen et al, 2010b). This range should also be considered according to the risk of infection in the farm in question (Table 7), with farms with lower risk being able to tolerate horses with 500 EPG as threshold (Rendle et al, 2019). Fecal egg counts should be performed in moderate climates during grazing season, from March to September (until October in mediterranean countries), preferably each 8-12 weeks (Rendle et al, 2019) and they should be performed in triplicates every time, as it reduces the variability inherent to this method (Nielsen et al, 2006; Vidyashankar et al, 2012).

Table 5 – Classification of horses according to their fecal egg count and their respective proportion in the population. Adapted from Nielsen et al, 2019;

Egg count level		**Adult population[a]**
Low shedders:	0-200 EPG	50-75%
Medium shedders:	200-500 EPG	5-15%
High shedders:	>500 EPG	10-30%

[a]These values are only estimates and the actual percentage of horses in each category will vary among farms depending on a multitude of factors.

In the first year of the implementation of SAT programs, the parasite monitoring may seem expensive and labouring, but, since adult horses maintain their egg shedding consistent, keeping up monitoring and control of high shedders is much easier afterwards (Pfister and von Doorn, 2018). The medium and high shedders should be dewormed with an effective AH and FECRT is recommended to be performed at least annually, after the ERP considered for the drug used in order to evaluate the appearance of AHR (Churcher et al, 2010; Nielsen et al, 2019, Rendle et al, 2019). Taking into consideration the current status of AHR and the

effectiveness of different AHs (Table 6), the most recommended drugs to use in these high shedders are IVM and PYR (Rendle et al, 2019) and rotation of AHs should not be encouraged as it has been proven not to delay AHR and there are few effective drugs to rotate (Kaplan and Nielsen, 2010; Leathwick, 2013; Shalaby, 2013). In order to prevent the further development of AHR in cyathostomins, treatments are most effective during winter (Sauermann, Nielsen, Luo and Leathwick, 2019). Guidelines for treating foals and other age-specific treatments were described by Pfister and von Doorn (2018), in AAEP (Nielsen et al, 2019) and ESCCAP (2019) guidelines.

SAT programs promote a logical, spared and justified application of AHs and provide a greater amount of refugia in the parasite population (Nielsen, 2012; Pfister and von Doorn, 2018). As a consequence of only treating high shedders, the contamination of the pasture will be significantly lower, as they are the ones who contribute the most for it and the surviving parasites with resistant genes will be diluted because of the greater refugia size (Besier, 2012). According to ESCCAP, the SAT approach should only be recommended for adult horses and exclusively designed for the control of small strongyles (ESCCAP, 2019). It is also important to state, however, that the true aim of SAT programs is to delay or even prevent the development of AHR and that clinical parasitic disease may still occur from pasture infection, namely by infection with *Strongylus* spp., particularly *Strongylus vulgaris*, which will increase the risk of horse colic. Thus, the integrated use of AHs and non-chemical control methods must be put in place after a good balance of the advantages and disadvantages of SAT for each horse farm and region, according to local and regional knowledge of horse parasite epidemiology, namely if no *S. vulgaris* L3 larval stages are found in monitoring faecal cultures (Besier, 2012; ESCCAP, 2019).

Table 6 – Adulticidal and larvicidal action of broad-spectrum anthelmintics in main equine nematodes. Adapted from Decision Tree Horse, accessed in June 2019.

Decision tree	**Benzimidazoles**	**Pirymidines**	**Macrociclic lactones**
Ascarids	Adult and larval stages and worm eggs	Adult stages	Adult and larval stages
Cyathostomins	All stages and worm eggs*	Adult stages (efficacy often less than 90%)	Adult and immature stages** (not L3 or encapsulated larvae)
Strongylins	All stages and worm eggs*	Adult stages (efficacy often less than 90% and particularly S. edentatus not very sensitive)	Adult and all larval stages (except for S. equinus only adult stages)

*To obtain a high efficacy against larval stages (in the mucosa or migrating) it is often recommended to treat with fenbendazole for 5 consecutive days.
**MOX is partially effective against encapsulated larvae and has a residual effect for two weeks

3.5.2.2. Pasture management

Pasture hygiene is very effective in preventing re-infection and it's of great use when considering a selective grazing species like the horse. The removal of feces twice weekly was shown to be a valuable practice in significantly reducing the contamination of the pasture and to be even more effective than treatment with AH. Furthermore, while grazing, horses determine defecation areas (roughs) where grazing is avoided and herbage is normally dense and feeding areas (lawns), making it easier to select areas for those cleanings. Because of this, horses are a grazing species that can greatly benefit from fecal removal as a non-chemical worm control measure but also as a way of increasing grazing areas in paddocks (Herd, 1990; Reinemeyer and Nielsen, 2018).

Other pasture management methods to interrupt parasite transmission and reduce the need of using AHs, include harrowing, rotation or mixed grazing of pastures. Harrowing pastures to break up fecal pellets and expose free-living parasitic stages can also be of valuable use to control parasite transmission in sub-tropical climates, such as in southern Europe, due to the higher temperatures registered in the summer. Pasture rotation can also be applicable to decrease the contamination of grazing areas in temperate climates and a 6-week grazing period per pasture with 18 weeks of rest was shown to be effective. Mixed or alternate grazing of pastures with ruminants has also been described to reduce strongyles infection in horses but care must be taken as some intestinal nematodes share both hosts (Hernández et al, 2018; Reinemeyer and Nielsen, 2018).

Table 7 – Assessment risk of infection from pasture considering various factors and practices in horse stud farms. Adapted from Rendle et al, 2019.

Low risk	Moderate Risk	High risk
Repeated negative FEC	Low/moderate FEC	High FEC
5–15 years old	>15 years old	<5 years old
Faecal collection > twice per week	Sporadic faecal collection	No faecal collection
Good pasture management	Moderate pasture management	Poor pasture management
Stable population	Occasional movement of the animals	Transient population
Low stocking density	Medium stocking density	High stocking density
No youngstock		Grazing with youngstock
Effective quarantine		No quarantine

No history of parasitic disease	History of parasitic disease
No history of colic	History of colic
	AHR identified on property by FECRT

3.5.2.3. Nematophagous fungi

A new promising biological control of worms has been extensively studied recently, consisting of feeding spores of nematophagous fungi to horses, with the purpose of controlling the free-living parasitic stages. The fungal chlamydospores are able to survive the intestinal tract of the horse and develop in the fecal environment, developing hyphae that trap and, consequently, kill larvae (Larsen, Nansen and Henriksen, 1995). *Duddingtonia flagrans* (Buzatti et al, 2015) and *Monacrosporium thaumasium* (Tavela et al, 2011) or *Mucor circinelloides* (Envagelista, 2018) are some fungi species that have demonstrated effectiveness in reducing equine infective larvae on pasture, with their larvicidal and ovicidal effect. Reduction rates of 90% and higher have been reported in the hosts, and 50-70% in the contamination pasture level (Fernanndez and Larsen, 1997; Madeira de Carvalho et al, 2007; Buzatti et al, 2012; Envagelista, 2018) and longer periods of unneeded treatment compared to the use of AHs have been documented (Hernández et al, 2016; Envagelista, 2018), making this one of the most promising measures in preventing further development of AHR.

3.5.2.4. Quarantine

Basic and careful hygiene and quarantine measures, both for horses in stables and on pasture are important to reduce the infection risk and consequently the need for treatment. According to ESCCAP, to prevent introduction of new parasite species and/or resistant parasite populations, each horse recently introduced to a farm should be quarantined and treated after arrival. Therefore, the animal should only be moved to pasture after a FEC performed five days post treatment has confirmed that the horse is negative concerning worm eggs and that deworming was successful (ESCCAP, 2019).

3.5.3. Current situation – farm practices and legislation

Even with all this information available about AHR, the integrated approach to deworming horses has slightly changed. In several recent questionnaires directed to horse farms, recommendations to prevent it are still being poorly implemented. The chemical approach to deworming horses appears to have changed, with farms decreasing AH administrations from as high as 6 per year (Lloyd et al, 2000) to 2-3 doses a year (Hinney et al, 2011; Nielsen et al, 2018) as a growing percentage of them adheres to SAT programs (Stratford et al, 2014). In some regions, however, 4-5 doses of AHs are still being given

(Elghryani, Duggan, Relf and de Waal, 2019). The rotation of these drugs seems to be also disappearing, with more horse farms stopping the simultaneous use of three drug classes per year (Lloyd et al, 2000) and relying only in macrocyclic lactones to deworm their animals (Hinney et al, 2011; Stratford et al, 2014; Nielsen et al, 2018). However, there are still some farms deworming using benzimidazoles despite all the recommendations to avoid them (Fritzen, Rohn, Schnieder and von Samson-Himmelstjerna, 2010; Elghryani et al, 2019). Another concerning fact is that FEC are still not widely implemented as part of the parasitic control programs as expected (Relf, Morgan, Hodgkinson and Matthews, 2012; Nielsen et al, 2018; Scare et al, 2018; Elghryani et al, 2019), with some farms having never performed a FECRT (Fritzen et al, 2010).

Pasture management seems to be increasing in horse farms (Nielsen et al, 2018), with about 40% of them removed feces twice weekly (Hinney et al, 2011; Stratford et al, 2014; Elghryani et al, 2019), but some still don't apply this practice (Fritzen et al, 2010). Sub-dosing of AH drugs is also a point of interest, since some horse farms still use unprecise methods of weighting animals (Fritzen et al, 2010; Relf et al, 2012; Hinney et al, 2011; Elghryani et al, 2019) while other are more prone to determine more precise weights before deworming (Stratford et al, 2014).

Perhaps the most concerning is that the great majority of horse owners are unaware of AHR in their farms and they are not very concerned about it (Stratford et al, 2014). This may indicate a distancing between horse owners and veterinarians, since AHs are so easily bought and administered to horses nowadays, in contrast to the past (Kaplan and Nielsen, 2010). Nonetheless, legislation has already been introduced in Europe to turn equine anthelmintic administration prescription-only, to prevent the further development of AHR. Denmark was the first country to adopt this strategy in 1999 (Nielsen, Monrad and Olsen, 2006) with significantly good results, as the use of AHs has gotten lower along with EPG and increased strongyles and AHR surveillance (Nielsen et al, 2014b; Becher et al, 2018). After that, the European Union followed with directives to apply restrictions on anthelmintic administration in livestock (EU, E.P.a.o.t.C, 2001, 2006) and currently the Netherlands, Finland, Sweden, Austria and Germany all have this approach to equine AHs (Becher et al, 2018). As a consequence of these changes, *Strongylus* spp. appears to be reemerging as interval dosing treatments are discontinued (Nielsen et al, 2012; Tydén et al, 2019), evidencing the importance of non-chemical approaches to parasitic control and the regular parasitological monitoring of horse farms.

3.5.4. Portugal's panorama

In Portugal, few studies have been made to study the presence of AHR. To the author's knowledge only two were performed in the last two decades. Both Madeira de Carvalho (2001) and Melo-Franco (2014) suspected resistance of cyathostomins and ascarids to doramectin in 1-year old foals, due to lower efficacy and shorten ERP. Benzimidazoles were shown to have standard efficacy *in vivo* (Melo-Franco, 2014), but resistance was detected in *in vitro* studies (Madeira de Carvalho, 2001). PYR failed to reduce EPG in foals and demonstrated shorter ERP, suggesting the presence of some level of resistance (Madeira de Carvalho, 2001).

4. Objetives

This study aimed to evaluate the performance of four diagnostic flotation methods currently used in veterinary parasitology, with different flotation solutions, on diagnosing different parasitosis in ungulates. The first part of the study intended to study the repeatability and reliability of these methods, in wild ungulates in a zoological context (red river hogs, greater kudus, Grevy's zebra). The second part evaluated the use of the same methods, with only two flotation solutions, as diagnostic and decision-making tools, when considering a horse stud farm and the present problem of anthelmintic resistance in this species.

5. Materials and Methods

5.1. Repeatability Study of Coprological Methods in Zoo Animals

5.1.1. Weather characterization

The weather in Amsterdam is characterized by four seasons, typically from atlantic moderate climates, as seen in graph 1: winter from November to February, with cold temperatures below 5°C and low precipitation; spring from March to May, with rising temperatures and moderate precipitation; summer from June to August, with moderate temperatures and low precipitation; and autumn from September to November, with decreasing temperatures and moderate precipitation.

Graph 1 – Temperature (°C) and precipitation (mm) in Amsterdam during the year of 2018, including the internship period (Weather underground [WU], 2019).

5.1.2. Sample collection and preservation

During the internship period, fecal samples of red river hogs (*Potamochoerus porcus*), greater kudus (*Tragelaphus strepsiceros*) and Grevy's zebra (*Equus grevyi*) were collected, in October, November and December 2018, respectively. The samples were obtained by the zookeepers in the course of their daily cleanings of the interior enclosures of the animals. Individual collections from the rectum were not possible due to the wild nature of the animals and necessary anesthetic procedures required to do so. In order to maintain the health status required by the veterinary team of the institution, the animals were immediately dewormed after the samples collection. Thus, only one sample from each species was collected.

The sample from the hogs represented feces of 3 adult male specimens kept together in their respective enclosure, which was separate from any other one. The samples from the kudus represented feces from 3 adult male specimens and the ones from the zebra were from

Figure 18 – One of the studied greater kudus, a giraffe and the studied zebra in their mixed enclosure at ARTIS (Original).

1 young female specimen, which were all kept in the savannah enclosure (fig. 18) along with a group of giraffes (*Giraffa camelopardalis*), the rest of the Grevy's zebras (*Equus grevyi*) and a flock of helmeted guineafowl (*Numida meleagris*). Nevertheless, each species had its own interior enclosure where the animals were kept overnight, making the collection of species-specific samples easier.

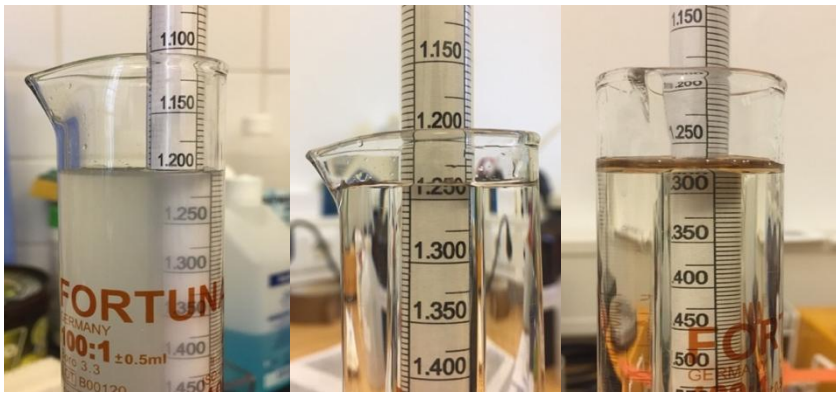
Once collected, all the samples were transported to the laboratory of the veterinary department at ARTIS and stored at 4ºC. Before storing, every sample was manually homogenized with the help of a wooden tongue depressor. When submitted to analysis, only a small volume of the total sample was kept on top of the lab bench at room temperature to prevent significant temperature variations and deterioration of the whole sample (Nielsen et al, 2010a). As soon as this amount was analyzed, another small volume was retrieved from the cooling storage.

5.1.3. Coprological methods

The samples were submitted to analysis by two qualitative methods and two quantitative methods. The qualitative methods applied in this study were the simple flotation (SF) and the modified centrifuge flotation (CF), while the quantitative methods were the McMaster (McM) and Mini-FLOTAC (MF) techniques. The technique's proceedings carried out by the author are described further below.

Every method was performed with 3 different flotation solutions: salt (NaCl), sugar and magnesium sulphate ($MgSO_4$). The salt and sugar solutions were made by the author in the laboratory of the Veterinary Department of ARTIS, according to Foreyt's formulation (2001), and the $MgSO_4$ solution was the Fecalizer Floating Solution from Henry Schein®. The specific gravities of these solutions were 1.200, 1.280 and 1.240, respectively, measured with a 1.000/1.600 specific gravity hydrometer from H-B Durac® (fig. 19). All methods were performed with 10 replicates for each species' sample, with the three solutions.

Figure 19 - Flotation solutions used in ARTIS with their specific gravity measured with a hydrometer. From left to right: salt, $MgSO_4$ and sugar solutions (Original).



5.1.3.1. Simple Flotation

For the simplest flotation method, 3g of feces were weighted and manually mixed in a plastic cup with 13mL of the flotation solution with a glass-stirring rod. The homogenization step took about 15 seconds for each sample. The mixed solution was then strained through a 500μm mesh sieve into a centrifugation tube, making sure to press out all the solution. After this, flotation solution was added until a positive meniscus was formed, the tube was covered with a 22x22mm glass cover and put in the tube rack to allow the eggs to float. In this method, the eggs were allowed to float for 15min, after which period the cover glass was pulled straight up and placed on top of a standard glass slide (75x25mm), ready for microscope viewing. The

simple flotation (SF) took about 18 minutes to complete, without the observation period. In order to give this method a semi-quantitative parameter, the number of eggs on the slide were counted.

5.1.3.2. Centrifugal Flotation

The centrifugal flotation (CF) method was performed as described by Taylor, Coop and Wall (2016) with slight modifications. This method also used 3g of feces which were put in a plastic container and manually homogenized with 13mL of water. The mixture was then poured through a fine mesh sieve (aperture 500μm) and collected into the centrifuge tube using a plastic funnel. The tube was centrifuged at 1500rpm for 2 minutes in a Hettich Universal 32 centrifuge, with a rotating motor of 7 cm of radii (fig. 20), after which the supernatant was discarded and the tube filled up to 1cm from the top with flotation solution. Before centrifugation, the sediment was manually homogenized with the solution. It was then centrifugated at 1500 rpm for 2 min and the tube was put on the tube rack. Finally, more flotation solution was added until a positive meniscus was formed at the top and the tube was covered with a 22x22mm glass cover. In this method, with two previous centrifugations followed by a flotation, the eggs were allowed 2min to adhere to the cover. Past this period, the cover glass was pulled straight up and placed on top of a standard glass slide (75x25mm), ready for microscope viewing. The whole proceeding took about 9 minutes to complete. With the objective of comparison between qualitative methods, the same semi-quantitative parameter described in the simple flotation method was applied in this method.

Figure 20 – Hettich Universal 32 centrifuge used in ARTIS' laboratory, with a rotating motor of 7 cm of radii (Original).

5.1.3.3. McMaster

This method was performed as proposed by Madeira de Carvalho (2001) using the different saturated solutions, like the sugar and salt ones. Initially, 2g of feces were weight and manually mixed with 28mL of flotation solution for 15 seconds in a plastic cup, using a stirring

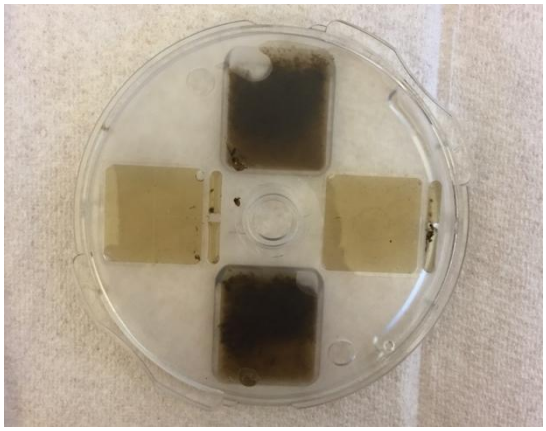
rod. The solution was filtered through a 500μm strainer into a plastic cup. Before filling each compartment of the McMaster (McM) chamber with a plastic Pasteur pipette, the mixture was stirred in the cup to assure the homogenized dispersion of the eggs. When the chambers were filled, the slide was left on the balcony for 4min to allow the eggs to float and adhere to the glass. After this period, the chamber was observed in the microscope with the 10x objective and the eggs were counted. A correction factor of 50 was used in this method to obtain the real EPG, also considered its detection limit. The whole proceeding usually took 6 min to complete, not including the counting period.

5.1.3.4. Mini-FLOTAC

As the other quantitative technique, the author chose the Mini-FLOTAC (MF) and Fill-FLOTAC (figs. 21 and 22) recently developed by the Department of Veterinary Parasitology of

Figure 221 – Fill-FLOTAC with its container, sampling kit and built in filter and the reading disk, Mini-FLOTAC, on the right (Original).

Figure 212 – Mini-FLOTAC full and locked ready to be read under the microscope (Original).



the University of Naples. According to Cringoli et al (2017), 5g of feces were put inside the Fill-FLOTAC along with 45mL of flotation solution. The feces were homogenized using the apparatus' conical collector for 15 seconds. Before using the plastic point to fill each chamber of the reading disc, the solution was further mixed by inverting the Fill-FLOTAC 5-6 times. The filled reading disc was left on the balcony for 10min and then rotated to be seen under the microscope for counting. Unlike the McMaster technique, the detection limit and correction factor applied with this method was just 5. The whole proceeding took about 12 min to finish, without the counting period.

5.1.4. Microscopic reading

After completion of the methods, every slide, chamber and reading disk was observed microscopically for egg counting. All the slides obtained with SF, CF and McM were inspected under a Leica DM750 with an ICC50 HD camera integrated. Due to the stage and stage clips'

structural layout, it was not possible to view MF's reading disks in this microscope. For those disks, a Leitz Laborlux 12 microscope was used, along with MF's microscope adaptor.

5.1.5. Data analysis and statistics

Data was organized and statistically analysed in Microsoft® Office Excel for Mac version 16.16.9.

Since all the data obtained for each species was obtained from one sample only, the assumption of independence required for statistical tests to assess statistical difference (Wilcoxon signed rank test, as the most basic test) was not fulfilled. Because of this, statistically-based inferences could not be drawn.

Nevertheless, one inherent characteristic of every quantitative diagnostic method is its precision, its ability to give consistent results, when applied to the same sample. Statistically, it can be inferred through the calculation of the coefficient of variation (CV) and the subtraction of this from 100, making a technique with a lower CV more precise. This approach was used for the analysis of the precision of the tested techniques in this part of the study. The standard error of the mean (SEM) is another measurement of variability of results and quantifies it. Both CV and SEM were possible to calculate in this part of the study and were used to delineate comparisons between the performed techniques.

5.1.6. Fecal Egg Count Reduction Test

FECRT were also calculated in this part of the study to infer about the presence of AHR in ARTIS. After 15 days, feces of the studied species were collected again and FEC were performed with the same methods and solutions in four replicates for each species' sample. The value of the test was obtained following the formula pointed by Nielsen et al (2019): $FECRT = \frac{\text{FEC pre-treatment} - \text{FEC post-treatment}}{\text{FEC pre-treatment}} \times 100$. The detection of AHR was determined according to cut-off values proposed by Coles et al (1992, 2006) and Nielsen et al (2019).

5.2. Performance Comparison of Coprological Methods – CIISA-FMV-ULisboa

5.2.1. Horse farm Alter do Chão

The samples analysed in this part of the study were collected from horses kept in the horse farm Alter do Chão, located in Portalegre, in Portugal (39°13'19,70'' N 7°41'16,30' W**)**. The farm was founded in 1748 by the king João V and became considered as the birthplace of the Lusitano breed horse, the most recognized Portuguese breed. After years of being at risk of closing its doors, this horse farm was saved by the intervention of Dr. Ruy d'Andrade

(Coudelaria de Alter, 2019), the same person who began restoring the Sorraia breed in 1937 from a small abandoned group (Associação de Criadores do Cavalo Sorraia, 2019). Nowadays, Alter Real is still one of the most prestigious horse farms for the Lusitano breed and, thanks to the intervention of Dr. Ruy d'Andrade, it possesses a small population of Sorraia horses. This population is currently composed of 5 stabled males and 12 females on rotating pastures.

5.2.1.1. Weather characterization

Portalegre is considered as having a temperate climate with hot and dry summers, according to Köppen-Geiger climatic classification, with mean temperatures of 25°C in summer and 17,5°C year-round (Instituto Português do Mar e da Atmosfera and Agencia Estatal Metereología, 2011). As seen in graph 2, that region is characterized by two marked seasons: a cold season from October to March, with increased precipitation during spring; and a hot season from May to September, with little to no precipitation.

Graph 2 - Temperature (ºC) and precipitation (mm) in Portalegre, as recorded in the Badajoz airport weather station (WU, 2019).

5.2.2. Sample collection and preservation

Seventeen fecal samples were collected in April 2019 from Sorraia horses. From these samples, 12 were from adult females on pasture (samples A-L) and 5 were from adult stabled males (samples 1-5). After collection, the samples were transported back in a cooled container to the Laboratory of Parasitology and Parasitic Diseases of the Centre for Interdisciplinary Research in Animal Health (CIISA-FMV-ULisboa), where they were preserved at 4ºC and later analyzed. During the processing of each sample, the same care already described to prevent sample deterioration in ARTIS was also taken with these samples.

5.2.3. Coprological methods

Before the samples were analysed, each of them was manually homogenized inside their plastic bags. The methods performed in this part of the study were the same as the ones performed at ARTIS, following the same proceedings. The only difference worthy of mention is the use of a centrifuge Centromix II-BL (fig. 23) instead of the one used at ARTIS, but also with a rotating motor of 7 cm of radii.

Figure 23 – Centromix II-BL centrifuge used in CIISA's laboratory, with a rotating motor of 7 cm of radii (Original).

5.2.4. Coprocultures

In this part of the study, coprocultures of all the samples were carried out, as described by Madeira de Carvalho (2001). A plastic cup was filled with about 50-60 g of fecal sample and a hole was drilled in the middle. The cup was covered with aluminium sheet pierced multiple times to provide air exchange and put in the incubator at 37°C with relative humidity of 70-80%. After 14 days, the culture was removed from the incubator and filled with water until the top. The cup was inverted into a petri dish that was then filled with water until a height of about 1 cm. After 24 hours, the liquid around the cup was collected to a 10 mL tube which was left on the tube rack to await a 24 hours sedimentation. Finally, 100 μL of the resulting sediment were collected with a pipette and put on a microscope slide for observation under a cover slip and identitification of L3 infective larval stages according to Madeira de Carvalho et al. (2008) and Santos et al. (2018).

5.2.5. Microscope reading

Equipment used for the counting of eggs in the slides were also different from the ones used in ARTIS. The microscope used in the Laboratory of Parasitology and Parasitic Diseases was an Olympus CX31. Another microscope, Olympus BX50, was used for taking pictures as it had a built-in micrometer.

5.2.6. Data analysis and statistics

Data was organized in Microsoft® Office Excel for Mac version 16.16.9 and analysed using IBM® SPSS® Statistics version 23.0.0.0. To calculate the confidence intervals for Cohen's Unweighted Kappa, Richard Lowry's VassarStats online calculators (http://vassarstats.net/) were used.

To study the difference in performance of the tested qualitative methods and evaluate the rate of agreement between them, a value of kappa (*k*) was calculated, as well as its prevalence index, bias index and confidence interval, according to Sim and Wright (2005). A minimum *k* value of 0.70 was needed to assume statistical meaning[2]. The strength of agreement between techniques were assessed according the classification proposed by Landis an Koch (1977), as follows: poor ≦ 0; slight = 0.01 – 0.20; fair = 0.21 – 0.40; moderate =0.41 – 0.60; substantial = 0.61 – 0.80; almost perfect = 0.81 – 1. In the cases where disagreement between the techniques performed was observed, a McNemar test was used to assess the statistical evidence of the differences.

The Shapiro-Wilk test was used to assess the normality of the data distribution for each method and solution. The strength of association between the different techniques was investigated through the calculation of Spearman's correlation coefficients. Statistical evidence of differences in-between techniques was sought using paired sample t-tests (or Wilcoxon signed rank test, when the former was not applicable). Also, to evaluate the relation between the presence of *Triondotophorus* spp. eggs and the EPG's obtained, the Fisher exact test was used. To compare the performance of the different techniques between the two sexes, a Friedman test was used.

A 5% significance level was considered for all statistical tests performed.

The CV and SEM were also calculated for the performed techniques in this part of the study and were used to compare their variability.

[2] In a two-tailed test, with the null hypothesis of value of *k* equal to 0.

6. Results

6.1. Repeatability Study of Coprological Methods – ARTIS

In the first part of the study, 3 samples of 3 different zoological species were analyzed with the four coprologic methods described previously, each of them repeated for three flotation solutions (salt, sugar and $MgSO_4$). From each sample, 10 replicates were analyzed for every method and solution. The 360 results are summarized in Table 9, where only the arithmetic means of the 10 replicates are presented.

For the analysis and comparison of the methods and solutions used, every association method-solution was perceived as an individual technique, for easier understanding of the results.

6.1.1. Red river hogs

In the hog sample, the only evidenced eggs in all the techniques performed were from *Ascaris suum* (fig. 24a). Concerning the qualitative techniques, CF_{MgSO4} performed with substantially higher median values of total egg counts (21.6 eggs) but its CV was very high (93.42%) and its SEM was the highest of all the qualitative methods (6,38 eggs). On the other hand, CF_{sugar} achieved high egg counts (9.2) with substantially lower SEM (1.75) and the lowest CV of this type of methods (60.15%). SF_{salt} and CF_{salt} performed with much lower means of total egg counts and higher values of SEM and CV. As for the quantitative techniques, McM obtained substantially higher median values of EPGs, with McM_{sugar} (207.50 EPG) being even five times higher than MF_{sugar} (41.50 EPG). The salt solution was the exception to this, with McM_{salt} having lower EPG than MF_{salt} (70 EPG *vs.* 79 EPG). MF_{salt} also obtained the highest EPG and the lowest CV for this method in this species. The qualitative techniques performed with the salt solution also had the highest CVs and SEMs.

6.1.2. Greater kudu

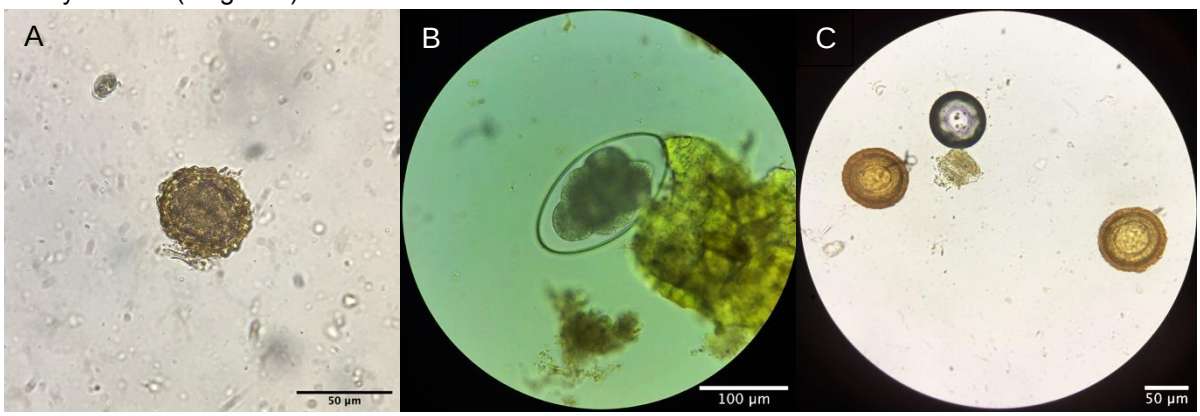
In the kudu sample, the only evidenced eggs were from *Nematodirus* spp. (fig. 24b). The qualitative techniques that performed with the highest total egg counts were CF_{sugar} (44.10 eggs) and CF_{MgSO4} (31.30 eggs), which were substantially different from the rest. In spite of this, it should be noted that all the qualitative techniques in this species performed, unexpectedly, with a CV higher than 100%, with the exception of CF_{MgSO4} (86.44%), which was the lowest of all. SF techniques constantly yielded lower SEMs. Between quantitative techniques, there were no substantial differences evidenced in the obtained EPG's, except for MF_{MgSO4} (177.50 EPG) which surpassed McM_{MgSO4} (120 EPG). MF techniques performed with

lower CV's, with the lowest being MF_{MgSO4} (18.60%), which also presented the lowest SEM (37.95 EPG).

6.1.3. Grevy's Zebra

In the zebra sample, the only evidenced eggs were from *Parascaris* spp. (fig. 24c). As for the qualitative techniques, the greatest difference was found between SF_{sugar} and CF_{sugar}, with the latter performing with the highest mean of total egg count (44.10 eggs) substantially different from the rest. CF_{sugar} also presented the lowest CV (37.50%). For the quantitative techniques, McM_{sugar} and McM_{MgSO4} obtained the highest EPGs (205 eggs) and SEMs (64.83 EPG) and McM_{salt} performed with the highest CV in this type of methods (44.51%). The differences in-between the other techniques' CVs were not substantial, but the one obtained with MF_{MgSO4} should be highlighted as the lowest of all (14%), in all three species.

Figure 24 – Helminth eggs found in zoo animals at ARTIS; A – *Ascaris suum* egg from feces of red river hogs; B – *Nematodirus* spp. from feces of greater kudus; C – *Parascaris* spp. from feces of Grevy's zebra (Originals).



6.1.4. Fecal Egg Count Reduction Test

After 15 days, FECs of the studied species were performed with every technique for later calculation of FECRT. The results are presented in Table 8.

Table 8 – Pre-treatment (D0) and post-treamtent (D15) FECs of the studied species at ARTIS. H – hogs; K – kudus; Z - zebra;

	D0						D15					
	McM			MF			McM			MF		
	Salt	Sugar	MgSO4	Salt	Sugar	MgSO4	Salt	Sugar	MgSO4	Salt	Sugar	MgSO4
H	70.0	207.5	147.5	79.0	41.5	56.0	31.3	37.5	37.5	101.3	65.0	88.8
K	125.0	175.0	120.0	153.0	161.0	177.5	0	0	0	3.8	6.3	1.3
Z	172.5	205.0	205.0	134.0	164.5	175.0	0	0	0	0	0	0

Table 9 - Descriptive statistics for the values obtained with the flotation techniques – ARTIS; SEM – standard error of the mean; CV – coefficient of variation.

			Salt				Sugar				$MgSO_4$			
			SF (total eggs)	CF (total eggs)	McM (EPG)	MF (EPG)	SF (total eggs)	CF (total eggs)	McM (EPG)	MF (EPG)	SF (total eggs)	CF (total eggs)	McM (EPG)	MF (EPG)
Sample	Hog	**Mean**	1.10	2.70	70.00	79.00	1.90	9.20	207.50	41.50	0.30	21.60	147.50	56.00
		Median	0.5	2.5	25.0	95.0	1.5	9.0	200.0	25.0	0	13.0	175.0	42.5
		Std. Deviation	1.60	1.64	119.49	53.58	1.52	5.53	151.41	52.97	0.48	20.18	94.61	44.46
		SEM	0.50	0.52	37.79	16.94	0.48	1.75	47.88	16.75	0.15	6.38	29.92	14.06
		CV (%)	145.01%	60.61%	170.70%	67.83%	80.20%	60.15%	72.97%	127.64%	161.02%	93.42%	64.14%	79.39%
	Kudu	**Mean**	0.50	3.40	125.00	153.00	2.40	44.10	175.00	161.00	6.90	31.10	120.00	177.50
		Median	0	1.5	125.0	155.0	1.0	15.5	187.5	170.0	2.5	25.5	112.5	180.0
		Std. Deviation	0.85	5.60	52.70	41.18	3.66	55.06	64.55	48.75	10.32	26.88	75.28	33.02
		SEM	0.16	1.08	39.53	48.38	0.76	13.95	55.34	50.91	2.18	9.83	37.95	56.13
		CV (%)	169.97%	164.75%	42.16%	26.91%	152.40%	124.85%	36.89%	30.28%	149.59%	86.44%	62.73%	18.60%
	Zebra	**Mean**	3.10	11.10	172.50	134.00	5.00	44.10	205.00	164.50	3.10	13.40	205.00	175.00
		Median	3.5	11.5	175.0	140.0	5.0	39.5	200.0	157.5	2.5	12.0	200.0	172.5
		Std. Deviation	1.45	7.78	76.78	39.43	2.16	16.54	46.84	30.32	1.97	6.59	68.52	24.49
		SEM	0.98	3.51	54.55	42.37	1.58	13.95	64.83	52.02	0.98	4.24	64.83	55.34
		CV (%)	46.75%	70.10%	44.51%	29.42%	43.20%	37.50%	22.85%	18.43%	63.52%	49.15%	33.42%	14.00%

6.2. Performance Comparison of Coprological Methods – CIISA-FMV-ULisboa

In the second part of the study, 17 samples of Sorraia horse feces were analyzed with the four coprologic methods described previously. Each of them repeated for two flotation solutions, salt and sugar. For SF and CF, samples were analyzed in triplicates and for McM and MF only duplicates were performed. The 340 results obtained are summarized in Table 10 and only the means of the replicates are presented. Samples identified by letters were females and the ones identified by numbers were stabled males.

As explained in the previous part of the study, each association method-solution was also perceived as a technique, for easier understanding of the results and comprehension of their significance.

Table 10 – Mean values obtained with the flotation techniques performed with faecal samples from Sorraia Horses– CIISA-FMV.

	Salt				Sugar			
Sample ID	SF (eggs/field)	CF (eggs/field)	McM (EPG)	MF (EPG)	SF (eggs/field)	CF (eggs/field)	McM (EPG)	MF (EPG)
A	8	46	3800	1630	4	17	5150	1525
B	8	28	1950	880	4	14	2650	770
C	7	40	1125	553	4	13	2875	725
D	7	18	2725	1063	1	23	3175	1105
E	12	47	5675	2773	7	27	5250	2410
F	15	23	3850	1570	7	10	4675	1615
G	7	19	1175	810	3	4	2350	958
H	9	28	3950	1265	3	5	4650	1438
I	8	9	1500	310	1	1	1025	393
J	4	29	7525	2553	6	5	6550	2130
K	4	27	2750	815	3	2	2200	1163
L	7	19	750	143	2	2	2200	298
1	3	2	175	98	6	7	1750	1045
2	3	7	200	90	6	5	2475	1273
3	5	6	625	183	6	21	1325	683
4	11	6	100	23	4	3	725	1283
5	9	3	150	60	10	3	2850	570

6.2.1. Qualitative techniques' assessment and sensitivity

In the two qualitative methods, SF and CF, the types of eggs recovered were registered for each of the flotation solutions. Only two categories of eggs were recovered in these samples: typical strongylid eggs (fig. 25) and large strongylid eggs, morphologically consistent with *Triodontophorus* spp. eggs (fig. 26).

Figure 26 – Typical strongylid eggs found (Original).

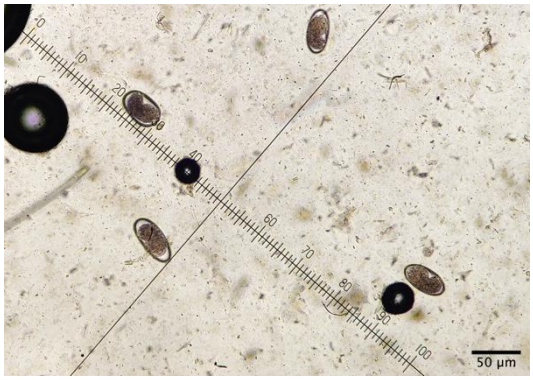


Figure 27 – *Triodontophorus* spp. egg (Original).

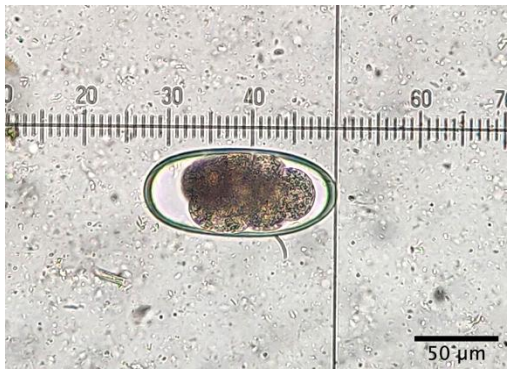


Table 11 – Prevalence of *Triodontophorus* spp., according to the qualitative technique and solution used.

Method	Prevalence
Salt	59%
SF_{salt}	53%
Sugar	41%
CF_{salt}	41%
SF_{sugar}	35%
CF_{sugar}	35%

All samples were positive for typical strongylid eggs with every technique, revealing a prevalence of infected animals of 100% (17/17). However, the detection of *Triodontophorus* spp. eggs was different according to different techniques, as seen by Tables 11 and 12. SF_{salt} and CF_{salt} were able to detect the presence of *Triodontophorus* spp. eggs in 53% (9/17) and 41% (7/17) of the samples, respectively. The sugar solution recovered this type of eggs in only 35% (6/17) of the samples, regardless of the method used. When comparing both solutions, 59% (10/17) of the samples were determined as positive with salt for the presence of *Triodontophorus* spp. eggs and 41% (7/17) with sugar.

Table 12 – Presence of *Triodontophorus* spp. eggs in qualitative methods with salt and sugar solutions (+ = positive).

	Method	A	B	C	D	E	F	G	H	I	J	K	L	1	2	3	4	5
Salt	SF	+		+	+	+	+			+	+		+				+	
	CF	+		+		+	+			+	+	+	+					
Sugar	SF	+		+		+	+				+	+						
	CF	+		+	+	+					+	+						

6.2.2. Qualitative techniques' agreement

Based on Table 12, the strength of agreement, prevalence index and bias index were calculated and are presented in Table 13.

Table 13 – Agreement statistics for associations between all methods and solutions – CIISA-FMV;

Associations	*k*	Strenght of agreement*	Prevalence index	Bias index	Confidence interval
SF_{salt}-CF_{salt}	0.648	substantial	0	0.06	0.287 – 1.00
SF_{sugar}-CF_{sugar}	0.742	substantial	0.294	0	0.407 – 1.00
SF_{salt}-CF_{sugar}	0.422	moderate	0.118	0.176	0 – 0.848
CF_{salt}-SF_{sugar}	0.761	substantial	0.176	0.118	0.449 – 1.00
SF_{salt}-SF_{sugar}	0.422	moderate	0.118	0.176	0 – 0.848
CF_{salt}-CF_{sugar}	0.742	substantial	0.294	0	0.407 – 1.00
Salt-Sugar	0.658	substantial	0	0.176	0.306 – 1.00

The strength of agreement between the qualitative techniques was classified as substantial for all associations, except for the associations SF_{salt}-SF_{sugar} and SF_{salt}-CF_{sugar} (k=0.422), which were considered moderate. However, only the associations CF_{salt}-SF_{sugar} (k=0.761), SF_{sugar}-CF_{sugar} and CF_{salt}-CF_{sugar} (k=0.742) showed evidence of significant agreement.

The other four associations, however, did not have a statistically significant agreement and the statistical difference in-between these techniques must be evaluated. Among the techniques in the non-agreeing associations, SF_{salt}, CF_{salt}, CF_{sugar} and SF_{sugar}, there was no evidence of statistical disagreement (McNemar's test, p>0.05), as well as between the salt and sugar solution.

The prevalence index evidenced the influence of the *Triodontophorus* spp. eggs prevalence in the determination of the *k* coefficient. On the other hand, the bias index represented the disagreement of the techniques on the proportion of positive cases for presence of this type of eggs. The contingency tables used for the calculation of these values are presented in Appendix C (Tables 22-27).

6.2.3. Data distribution

The distribution of the values obtained for each technique was examined. The qualitative techniques, all appear to have a distribution next to normal, except CF_{sugar}, as shown in fig. 27. On the other hand, McM and MF methods appear to have distributions next to normal only with the sugar solution, since, when performed with salt, their distributions are clearly

asymmetrically dispersed, as confirmed by the Shapiro-Wilk's Test of normality (Table 14) for McM_{salt} (p<0.05), MF_{salt} and CF_{sugar} (p<0.02).

Furthermore, the Shapiro-Wilk test was performed separately to males and female (Table 14). Only SF_{salt} (p<0.05), in females, and McM_{salt} and CF_{sugar} (p<0.02), in males, were found to have a non-normal distribution.

Table 14 – Shapiro-Wilk test of normality results for all techniques – CIISA-FMV. Significance values lower than 0.05 (in bold) evidence non-normal distribution.

	Global		Females		Males	
Method	Statistic	*p*	Statistic	*p*	Statistic	*p*
SF_{salt}	0.941	0.330	0.739	**0.023**	0.867	0.254
CF_{salt}	0.926	0.185	0.893	0.372	0.871	0.272
McM_{salt}	0.879	**0.031**	0.966	0.846	0.719	**0.015**
MF_{salt}	0.866	**0.019**	0.903	0.429	0.946	0.712
SF_{sugar}	0.945	0.387	0.883	0.325	0.828	0.135
CF_{sugar}	0.865	**0.018**	0.910	0.466	0.731	**0.019**
McM_{sugar}	0.933	0.243	0.799	0.079	0.972	0.887
MF_{sugar}	0.959	0.613	0.878	0.299	0.868	0.257

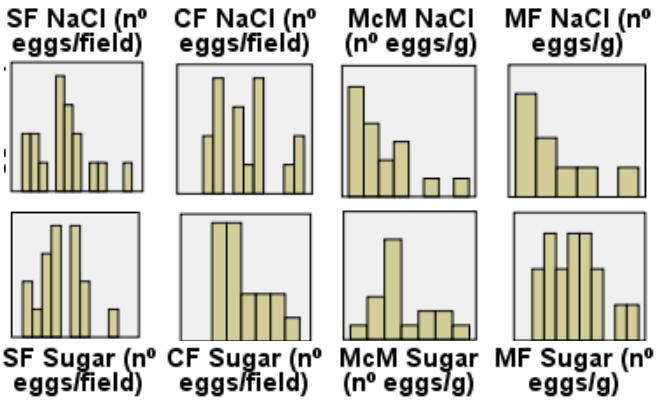


Figure 28 – Histograms of every technique's distribution, not considering sexes, evidencing the non-normal distribution of CF_{sugar}, McM_{salt} and MF_{salt}.

6.2.4. Descriptive statistics

The statistics of the techniques evaluated are presented in Table 16 and further evidence can be found of the non-normal distribution of the values for CF_{sugar}, McM_{salt} and MF_{salt}. These three techniques have a skewness higher or very close to 1 and the highest ratios skewness/standard error of skewness, even if only McM_{salt} is bigger than 2.

The significance values of all the techniques associations are summarized in Appendix D (Table 29).

Comparing the qualitative techniques, almost all of them were statistically different in-between them: SF_{salt}-CF_{salt} (paired samples t-test, p=0.001), SF_{sugar}-CF_{sugar} (Wilcoxon signed rank test, p=0.034), SF_{salt}-SF_{sugar} (paired samples t-test, p=0.005), CF_{salt}-CF_{sugar} (Wilcoxon signed rank test, p=0.007) and CF_{salt}-SF_{sugar} (paired samples t-test, p<0,001). The only exception was SF_{salt}-CF_{sugar} (Wilcoxon signed rank test, p=0.492). CF_{salt} and CF_{sugar} were the techniques with the highest counts of eggs/field (CF_{salt} $\bar{x}$=21.0; CF_{sugar} μ=9.5) solutions but higher CVs (CF_{salt}=68,97%; CF_{sugar}=86,07%), as well.

When it comes to the quantitative techniques, the influence of the methods and solutions used was also demonstrated as all the differences observed were also statistically different: McM_{salt}-MF_{salt} (Wilcoxon signed rank test, p<0.001), McM_{sugar}-MF_{sugar} (paired samples t-test, p<0.001), McM_{salt}-McM_{sugar} (Wilcoxon signed rank test, p=0.006) and MF_{salt}-MF_{sugar} (Wilcoxon signed rank test, p=0.035), McM_{salt}-MF_{sugar} (Wilcoxon signed rank test, p=0.022), MF_{salt}-McM_{sugar} (Wilcoxon signed rank test, p<0.001). In both solutions, the McM methods got the highest EPG (McM_{salt} μ=1500; McM_{sugar} $\bar{x}_r$=2650), but also standard deviations (SD) of more than half the mean and higher CVs (McM_{salt}=96%; McM_{sugar}=54%) and SEMs (McM_{salt}=519.43 EPG; McM_{sugar}=207.52 EPG).

The techniques performances render comparison between the two sexes, not because of the differences in management, but because of their impact in parasite burden classification. Statistically significant differences were found for the same technique between the two sex groups for: CF_{salt} (paired samples t-test, p=0.008); SF_{sugar} (paired samples t-test, p=0.001); McM_{salt} (Wilcoxon signed rank test, p=0.043); MF_{salt} (paired samples t-test, p=0.033) and McM_{sugar} (paired samples t-test, p=0.021). MF_{sugar} was the only technique that did not show significant differences between sexes.

Regarding each sex separately, the only techniques that were not statistically different in females were: SF_{salt}-CF_{sugar} (Wilcoxon signed rank t-test, p=0.432); McM_{salt}-McM_{sugar} (paired samples t-test, p=0.082) and MF_{salt}-MF_{sugar} (paired samples t-test, p=0.838). On the other side, the techniques that were statistically different in males were; McM_{salt}-MF_{salt} (Wilcoxon signed rank t-test, p=0.042); McM_{salt}-McM_{sugar} (Wilcoxon signed rank t-test, p=0.043); MF_{salt}-MF_{sugar} (paired samples t-test, p=0.006); and McM_{salt}-MF_{sugar} (Wilcoxon signed rank t-test, p=0.043 MF_{salt}-McM_{sugar} (paired samples t-test, p=0.011).

In the females, there was a significant difference between the results of the qualitative techniques, but, in males, no substantial differences were found. In this type of techniques, the

sugar solution performed with consistently higher CVs than the salt solution, in both sexes. As for the quantitative techniques, the McM method obtained statistically significant higher EPGs, except when using the sugar solution in males. Furthermore, in males, the EPGs values with this method seemed to be more variable than the ones obtained with MF, because of the higher CVs and SEMs. The same cannot be said for the females, where the latter method performed with slightly higher CVs but lower SEMs than McM, in both solutions.

The differences in EPGs of quantitative techniques in males should be highlighted. When performed with the sugar solution, McM and MF methods obtained results ten times greater than the ones obtained with the salt solution.

6.2.5. Correlations

It was also important to determine if the techniques' performances were correlated and so Spearman correlations were carried out, due to the non-normally distribution of some data. As described in Table 15, most of the techniques were indeed correlated, especially the quantitative ones. The strongest correlations observed were McM_{salt}-MF_{salt} (r_s=0.968, p<0.01), CF_{salt}-MF_{salt} (r_s=0.831, p<0.01), CF_{salt}-McM_{salt} (r_s=0.807, p<0.01) and MF_{salt}-McM_{sugar} (r_s=0.801, p<0.01). CF_{salt} was found to be correlated with every quantitative technique, whereas CF_{sugar} only correlated with MF_{salt} and McM_{sugar}. The fact that these qualitative techniques correlated in a highly significant way with quantitative ones should be highlighted.

Table 15 – Spearman correlations of the techniques performed at CIISA-FMV (n=17); ** – significant correlation.

		1	2	3	4	5	6	7
1 – SF_{salt} (eggs/field)								
2 – CF_{salt} (eggs/field)	r_s =	0.25						
	p =	0.34						
3 – McM_{salt} (EPG)	r_s =	0.22	**0.81****					
	p =	0.39	0					
4 – MF_{salt} (EPG)	r_s =	0.25	**0.83****	**0.97****				
	p =	0.34	0	0				
5 – SF_{sugar} (eggs/field)	r_s =	0.15	-0.07	-0.07	0			
	p =	0.57	0.80	0.78	0.99			
6 – CF_{sugar} (eggs/field)	r_s =	0.10	0.36	0.32	**0.50***	0.33		
	p =	0.71	0.16	0.21	0.04	0.20		
7 – McM_{sugar} (EPG)	r_s =	0.26	**0.72****	**0.75****	**0.80****	0.32	**0.52***	
	p =	0.31	0	0	0	0.21	0.03	
8 – MF_{sugar} (EPG)	r_s =	0.23	**0.49***	**0.62****	**0.64****	0.35	0.38	**0.64****

	p =	0.38	0.05	0.01	0.01	0.17	0.13	0.01

Table 16 – Descriptive statistics of all techniques performed at CIISA-FMV. For the separate sexes' statistics, the median was presented as it is a more conservative central tendency statistic for normal and non-normal distributed variables.

		Salt				Sugar			
		SF (eggs/field)	CF (eggs/field)	McM (EPG)	MF (EPG)	SF (eggs/field)	CF (eggs/field)	McM (EPG)	MF (EPG)
Global	*Minimum*	3	2	100	23	1	1	725	298
	Maximum	15	47	7525	2773	10	27	6550	2410
	Median (μ)	7	19	1500	810	4	5	2650	1105
	Mean ($\bar{x}$)	7.5	21.0	2236.8	871.7	4.5	9.5	3051.5	1140.2
	Std. Deviation (δ)	3.22	14.48	2141.65	855.64	2.37	8.20	1646.67	572.69
	SEM	0.78	3.51	519.43	207.52	0.58	1.99	399.38	138.90
	CV (%)	43.15	68.97	95.75	98.16	52.43	86.07	53.96	50.23
	Skewness	0.621	0.409	1.120	1.070	0.482	0.942	0.649	0.674
	Skewness ratio	1.13	0.74	2.04	1.95	0.88	1.71	1.18	1.23
Females	*Median*	7.5	27.5	2737.5	971.5	3.5	7.5	3025.0	1134.0
	SEM	0.25	0.97	169.00	68.26	0.17	0.72	137.14	53.93
	CV (%)	38.06	41.78	66.18	68.43	54.67	84.56	46.19	53.44
Males	*Median*	5	6	175	90	6	5	1750	1045
	SEM	0.73	0.43	42.57	11.87	0.44	1.51	171.43	66.16
	CV (%)	58.60	45.17	85.15	65.38	34.23	96.96	46.97	34.07

6.2.6. Association *Triodontophorus*-EPG

The association between the presence of *Triodontophorus* spp. eggs in the qualitative techniques and the EPG obtained with McM or MF methods was also investigated. The Fisher exact test did not show any significant difference for any of the quantitative techniques performed (p>0.07).

6.2.7. Coprocultures

All the cultures evidenced the presence of cyathostomins, concluding the prevalence of 100% (17/17) of these parasites in this horse population. However, the majority of larvae found were significantly degraded and lacking visible individual limits in-between intestinal cells, which made most of them impossible to identify. The identifiable cyathostomins were classified as being of type D (fig. 28), as they had 8 trapezoid or triangle shaped intestinal cells disposed in a single line. In addition, in one culture a larva of *Stongylus vulgaris* was identified (fig. 29). This was the only identifiable specimen in all the cultures, confirming the presence of this parasite in this population, with 5.9% prevalence.

Figure 308 – Cyathostomin type D from larval cultures of Sorraia horses (Original).

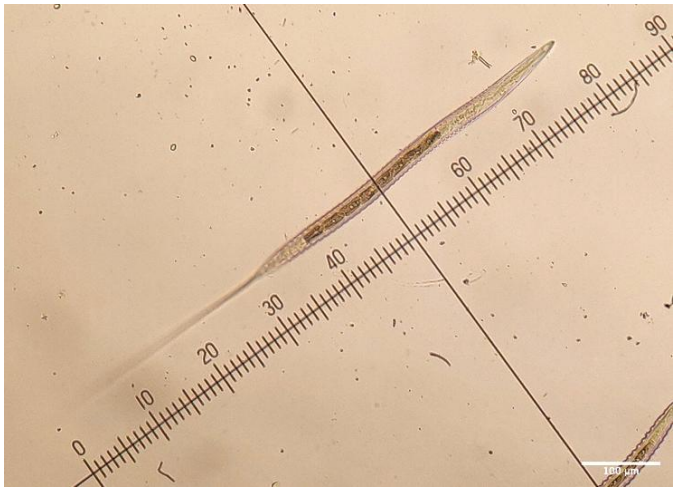


Figure 299 – *Strongylus vulgaris* larva from coprocultures of Sorraia horses (Original).

7. Discussion

The two most important characteristics to analyse when comparing two techniques are their accuracy and their precision (Went, Scare, Steuer and Nielsen, 2018). Accuracy represents the ability of a technique to measure the true value, whereas its precision reveals the consistency in results obtained with that technique, being also referred to as repeatability. The former parameter was impossible to determine in this study for any method or solution, since the true value of EPGs of all animals was unknown. However, through the determination of variance statistics, such as CVs and SEMs, precision could still be assessed (Noel et al, 2017; Scare et al, 2017) and was paramount to the comparison of the performed techniques.

When it came to the qualitative techniques, the amount of different parasite eggs they exposed and its consistency were more important to evaluate, as the purpose of these techniques is to evidence the biggest variety of eggs and allow the most accurate characterization possible of the parasite population in the feces (Hendrix and Robinson, 2012). Since only one species of parasites was detected in the flotation techniques performed in the first part of the study, the power to expose various types of eggs was perceived as the technique's ability to evidence higher total egg counts. This was based on the probability principle that, given a bigger quantity of eggs, the probability that different evidenced eggs will also be higher. The consistency in exposing these same eggs, the precision, was evaluated through the techniques' CV. In the second part of the study a better evaluation of the techniques ability to expose different eggs was possible.

When considering the quantitative techniques, besides their capacity to obtain higher EPG, the level of variability, evaluated with CVs and SEMs, was deemed as the other most important characteristic. Determination of the precision of egg-counting methods is essential nowadays, as they are more commonly used as decision-making tools in parasitic control programs (Duncan et al, 2002; Coles et al,2006; Pfister and van Doorn, 2018).

7.1. Repeatability of Coprological Methods – ARTIS

For a better organization, comparison and interpretation of the results, a summary table for the techniques performed at ARTIS can be found in Appendix E (Table 29), with all the results in decrescent order.

7.1.1. Red river hogs

In the hogs, CF_{MgSO4} performed with the highest median of total egg counts obtained, substantially different from the other qualitative techniques, evidencing its superiority as a

floating technique for *Ascaris* spp. eggs. However, it had a CV close to 100%, indicating that it is not very precise and lowering its reliability as a candidate for a better-suited qualitative technique for hogs. Considering this, CF_{sugar} seems to be the best compromise between higher values of eggs obtained and lower variation in results, since it had the second highest total egg count mean and the lowest CV of all the techniques of these type.

These findings seem to be in accordance with recent peer-reviewed protocols for diagnosing intestinal parasites in swine that support the centrifuge flotation method with sugar as the most sensitive and accurate for this type of animals (Pittman, Shepherd, Thacker and Myers, 2010). A study comparison on fecal analysis techniques in children's stools also confirmed this method as the best coprological method for the detection of *Ascaris* spp. eggs (Goodman et al, 2007).

The techniques performed with salt obtained substantially lower medians of total egg counts, suggesting that this solution might not be appropriate for the floatation of *A. suum* eggs. In 2014, an epidemiological study on intestinal parasitic infections in humans, dogs and pigs using a simple flotation method with salt did not find *Ascaris* spp. eggs, even though their presence was detected through PCR (Schär et al, 2014).

As for the quantitative techniques, McM_{sugar} performed with the highest EPG, but it also had the highest SEM, which represented a forth of its median EPG, evidencing a low precision in the results obtained. McM_{salt} obtained a lower EPG mean than McM_{sugar}, as opposed to what had been described before in the literature for counting *A. suum* eggs (Pereckienė, et al, 2007). These techniques also performed with very low levels of precision, as it can be seen by the high values of SEM and CV, in comparison to those previous reports. The reason behind this is probably the difference in the number of samples analysed, since, in 2007, Pereckienė et al used 30 samples for their study, whereas in this study only one was used.

This study seems to be the first report of the use of the McMaster method with a $MgSO_4$ solution and showed good results. McM_{MgSO4} had the second highest EPG, its SEM was in the middle of the SEM range for this technique, suggesting that it might be a better-suited egg-counting technique for *Ascaris* spp. in hogs, which use has never been described in the literature. In addition, it performed with the lowest CV of the quantitative techniques in this species, further evidencing its reliability.

When it comes to the other quantitative method tested, the Mini-FLOTAC had much lower EPG counts, comparing to the McMaster method. Even though the median EPG for MF_{salt} was the highest of the ones obtained with this method, it was only about half of the EPG of McM_{MgSO4}. The author found no previous studies on the use of Mini-FLOTAC in swine

species, rendering its accuracy comparison with McMaster difficult. Nonetheless, this technique performed with a lower SEM than McM_{MgSO4} and very similar CV, suggesting it to be more precise and reliable in red river hogs.

7.1.2. Greater kudus

In this species of ruminants, similarly to what was observed in the hogs, CF_{sugar} and CF_{MgSO4} achieved the highest medians of total egg counts, being substantially different from the rest of the qualitative techniques. The fact that the SF techniques performed poorly in comparison, supports the recent findings that they should not be used as qualitative techniques in ruminants (Rinaldi et al, 2011).

This superiority of the centrifugation method with sugar in detecting *Nematodirus* spp. infections is in line with previous studies reporting this finding for wild ruminants as well (Cebra and Stang, 2008). However, all qualitative techniques performed with a CV higher than 100%, evidenceding a low precision and repeatability of this type of techniques in greater kudus. This high level of variation was probably due to the compact nature of these ruminants' feces, as they are comprised of small acorn-sized compressed pellets (fig. 30). As reported by Cebra and Stang (2008), this is a major influential factor when detecting parasitic infections, since the liberation and homogenization of the eggs with the floating solution is compromised. Nonetheless, CF_{MgSO4} should be highlighted for having the lowest CV of all. Despite being very close to 100% as well, from all the tested techniques, this seems to be the best-suited to evidence *Nematodirus* spp. eggs in this species, taking in consideration the limitations related to the poor homogenization of this type of feces.

Figure 31 – Feces' morphology of different ungulate species from ARTIS. From left to right: red river hogs' fecal sample, much similar to dirt and with very loose matter; greater kudus' feces, as individualized acorn-sized pellets, coated with mucous; and Grevy's zebra's fecal bolus with obvious fiber content and easily broken apart (Originals).

When considering the quantitative methods, MF_{MgSO4} and McM_{sugar} performed with very similar and the highest EPGs. In addition, despite having the highest SEM, MF_{MgSO4} had the lowest CV, suggesting it to be the most precise of the tested techniques. Having the highest

SEM associated with the lowest CV of all techniques only means that this technique is very reliable, but, when it varies, it varies greatly. Repeated FECs are a viable solution to this.

No previous studies on the use of Mini-FLOTAC with $MgSO_4$ were found by the author, making these findings difficult to support based on literature. However, the superior precision of the Mini-FLOTAC method in comparison to McMaster in ruminants has already been reported in the literature (Dias de Castro et al, 2017; Bosco et al, 2018; Paras, Geroge, Vidyashankar and Kaplan, 2018), as well as other commonly used coprologic methods (Godber et al, 2015). Silva et al (2013) and Paras et al (2018) both showed that MF_{salt} could be used to obtain higher EPGs and lower CVs than McM_{salt} in ruminants, which was also observed in the present study. Increases in FEC of 116.5% for sheep and 27.2-53.6% for cattle were found when using MF_{salt} over McM_{salt}, whereas in this study the increase was only 18.3% in these wild ruminants. All these findings seem to support the claim that the Mini-FLOTAC might be a better-suited quantitative method than McM in ruminant species.

The differences between the three solutions used, in combination with the McMaster technique, have already been studied as well by Vadlejch et al (2011). In their study, McM_{sugar} obtained a higher mean EPG, followed by McM_{salt} and McM_{MgSO4}, with no statistical difference in-between them. This pattern was exactly the same as the one observed in this study, when taking into consideration the values for the McM techniques alone. However, the variability observed by Vadlejch indicated that McM_{salt} would be the most unconsistent of the three techniques, followed by McM_{MgSO4} and McM_{sugar}. This was not true in the present study, as McM_{MgSO4} had a substantially higher CV than McM_{salt}. Nonetheless, McM_{sugar} had the lowest CV of the three techniques in this study, supporting the findings of Vadlejch that it might be the most precise of the McM techniques.

The fact that the EPG values obtained with McM and MF techniques came from pooled samples of three specimens should not be seen as limitation of the study. Recent studies showed a positive correlation and little differences between individual FEC and FEC from pooled samples in sheep naturally infected with gastrointestinal strongyles and *Nematodirus* spp. (Rinaldi et al, 2014; Kenyon et al, 2016), rendering their close agreement when using MF_{salt}. In similarity to what was observed in the present study, Rinaldi et al (2014) also obtained higher EPGs with MF_{salt} in comparison to McM_{salt} in sheep, suggesting a greater accuracy of the former technique in ruminants.

7.1.3. Grevy's zebra

The results obtained for the qualitative methods of these wild equids were in line with what was found with the other wild species tested in the study. In this species, however, the

ability of CF_{sugar} in evidencing parasite eggs was proved by its superior median total egg counts, which were substantially higher than the other techniques. Furthermore, this technique also had the lowest CV of them all, underlining its precision and evidencing it as a good qualitative technique. A difference in the results achieved with techniques with centrifugation and without it is also noticeable, since the latter did not obtain total egg counts higher than 5 eggs per slide and the former all scored above 10 eggs per slide. Published information on qualitative methods in equine species is lacking, but these findings might suggest the centrifugal methods as the best-suited qualitative methods.

When it comes to the quantitative techniques, McM_{sugar} and McM_{MgSO4} had the highest median EPGs. Their SEMs and CVs were only surpassed by the salt techniques (McM_{salt} and MF_{salt}), evidencing their great inconsistency in results of the latter techniques. These findings have already been described before in the literature, where the McM method was found to perform with statistically higher mean EPGs and CV in horses, underlining its low precision (Dias de Castro et al, 2017). In this mentioned study, the variability of McM_{salt} was greater than MF_{salt} as observed in the present study. However, they were substantially different from the ones achieved here with feces from Grevy's zebras. Dias de Castro et al (2017) reported a variability, measured by CV (%), of 14.3% for MF_{salt} and 31.1% for McM_{salt}, in contrast to 29.42% and 44.51% obtained in the present study. This is probably due to the difference in equine species used as experimental models in both studies, since Dias de Castro et al (2017) used the domestic horse (*Equus caballus*).

Another recent study compared the reliability of McM_{sugar} and MF_{sugar} techniques in horses, when recovering strongylid and ascarid eggs (Nápravníková, Petrtýl, Stupka and Vadlejch, 2019). The ascarid recovered in this study was *Parascaris equorum* and the precision observed, measured by CV (%), were very similar to the ones obtained for the zebras in the present research. McM_{sugar} performed with a mean CV of 62.95% and MF_{sugar} with a mean CV of 18.95%, whereas, in the present study, the same techniques performed with 22.85% and 18.43%, respectively. The obvious difference in the CVs of both McM_{sugar} techniques might be because the value of the mentioned study resulted from the mean of CVs of different egg concentrations. Nevertheless, MF_{sugar} performed consistently better in both studies, suggesting it to be a very precise technique for evaluating FEC in equid species.

On the other hand, in the present study, MF_{MgSO4} performed with the second highest median EPG and a low SEM, both very close to the values obtained with McM_{salt}. It should be noted that the CV for MF_{MgSO4} was the lowest of all techniques in every species, indicating its superior precision as a quantitative technique. Published literature on the simultaneous use of

MF_{sugar} and MF_{MgSO4} is lacking, making a comparison between the two difficult to support, since they performed with very close values of mean EPG, SEM and CV.

7.1.4. General considerations

7.1.4.1. Diagnostic techniques

Based on the results obtained in this part of the study, it is possible to affirm that the chosen SGs of the floating solutions (salt – 1.20; sugar – 1.28; $MgSO_4$ – 1.24) were adequate for evidencing the parasitic eggs in question in these species. According to previous studies, the SGs of the parasites used as experimental modes are 1.13 and 1.09-1.10 for *Ascaris suum* and *Parascaris equorum*, respectively (David and Lindquist, 1982; Norris et al, 2018). The specific gravity of *Nematodirus* spp. eggs has not been fully studied yet and it is only known that they are able to float in solutions with a SG greater than 1.22 (O'Grady and Slocombe, 1980). However, in this research, this type of eggs was easily evidenced with techniques using the salt solution with a SG of 1.20, proving that their SG must be even lower than what was previously expected by O'Grady and Slocombe.

Taking into consideration the findings observed in this part of the study, centrifuge flotation seems to be a very good qualitative method for exposing a greater amount of parasite eggs in zoo ungulates, when compared to simple flotation. This should be highlighted since most of the parasitological surveys performed in zoological institutions do not include this method, using the simple flotation instead (Fagiolini et al, 2010; Dărăbuş, Afrenie, Hotea, Imre and Morariu 2014; Mir et al, 2016) or no flotation method at all (Lim, Ngui, Shukri, Rohela and Mat Naim, 2008; Mirzapour et al, 2018). Very few studies on this subject included this type of method (Gurler, Beyhan, Acici, Bolukbas and Umur, 2010; Thawait, Maiti and Dixti, 2014).

When it comes to the quantitative techniques, McMaster seems to be the most widespread method in zoological institutions for FEC assessment, based on previous studies (Goossens, Dorny, Boomker, Vercammen and Vercruysse, 2005; Melfi and Poyser, 2007). However, as seen with the present results, this method might not be the best to evaluate EPGs in some species, like greater kudus or Grevy's zebras. The Mini-FLOTAC performance appeared to be more precise and, in the case of the ruminant species, more accurate, suggesting it to be a better suited for this captive species. Even though this method was more time consuming than the McMaster, taking about 35 minutes to complete, in line with what is seen in the literature (Noel et al, 2017), it still has more advantages than the latter. One of those is the homogenization of the fecal sample it assures. The importance of homogenization in FEC has been constantly pinpointed as one of the most important factors affecting results (Vidyashankar et al, 2012) and is of great importance when considering all the different types

of feces of wild animals. In recent studies, the straining step in the McM method has been described as an important source of error (Paras et al, 2018; Went et al, 2018), making MF a more accurate coprological method because of its big pore-sized incorporated filter.

As for the flotation solutions used, sugar and $MgSO_4$ seem to be the best to evidence a greater amount of parasite eggs, as suggested by the observations made. Their superior SG, when compared to the salt solution, easily explains this. However, the relation between higher SG and higher total egg counts or EPG is not that straight forward, when comparing the two denser solutions. As seen from the results, the sugar solution was not able to obtain higher counts of eggs in all performed methods, suggesting that the combination method-solution may have an impact on the results obtained and that other characteristics of the solution might be of important evaluation. One example might be the solution's viscosity, since the sugar solution was clearly more viscous than the $MgSO_4$ one, rendering a slower flower rate of eggs floating to the surface, as established in Poiseuille's law[3] (Wilson, 2007).

7.1.4.2. Anthelmintic resistance at ARTIS

The deworming programs at ARTIS of up to 5 treatments a year for the zebra and the hogs in 2018 may be causing a great pressure on the selection of resistant strains of parasites in these species (Kaplan and Nielsen, 2010; Reinemeyer, 2012; Reinemeyer and Nielsen, 2018). Further evidence of the presence of resistant gastrointestinal parasites in these animals can be inferred from the constant presence of *A. suum* and *Parascaris* spp. in the feces of the hogs and the studied zebra, across the whole internship.

The AAEP guidelines (Nielsen et al, 2019) suggest an inclusion of at least 6 individuals in the valid calculation of FECRT or 10 in the case of pigs (Coles et al, 2006). It was not possible in this study, due to the lower number of specimens in this zoo. This constitutes a great limitation to the application of this tool for diagnosing AHR in zoological institutions, places where resistance is likely to appear. The main reasons for this are: the intensive deworming programs on behalf of the general animal health practiced in zoos, which, as explained before, increases the pressure on selection for resistant parasite strains; and the lack of accurate weight of wild species, which may lead to sub-dosing of AH (Fritzen et al, 2010; Relf et al, 2012, Hinney et al, 2011; Elghryani et al, 2019).

[3] This law states that the flow rate of a liquid is related to its viscosity, pressure gradient across the tubing, its length and radius.

Nevertheless, for the purposes of this dissertation, 15 days after deworming, FECRT were performed for these animals to assess the presence or absence of AHR in ARTIS. The results are presented in Table 17.

In the case of the hogs, the techniques performed with the McM method, recommended by Coles et al (2006), obtained much lower reductions than the stated threshold of 90% (Coles et al, 2006), confirming a possible presence of AHR in *A. suum* in these animals. Surprisingly, the MF techniques did not detect a reduction in FECs, instead higher EPGs were observed with this method after deworming. For the kudus, McM detected a reduction of 100% for all techniques, whereas the MF techniques registered more sensitive FECRT. Considering the results from MF, none scored lower than the 95% needed to confirm AHR (Coles et al, 1992), although this threshold should only be considered for oral formulations. The treatment in the Grevy's zebra appears to be 100% effective for all techniques, despite all the treatment failures witness during the internship. The fact that this animal was being dewormed with oral formulations can explain the continuous presence of *Parascaris* spp. throughout the internship without the existence of a resistant strain to IVM. This is because, if the zebra did not ingest enough AH for any reason, the treatment would not have the expected effect and the animal would not be properly dewormed. In turn, this would also increase the chances of appearance of a parasite strain resistant to IVM.

Table 17 – FECRT (%) values for the FECs performed at ARTIS, 15 days after treatment.

	Anthelmintic treatment	**FECRT (%)**					
		McM			MF		
		Salt	Sugar	$MgSO_4$	Salt	Sugar	$MgSO_4$
Red river hogs	IVM (Eraquell® tabs)	55.36	81.93	74.58	-28.16	-56.62	-58.48
Greater kudus	IVM (10mg/mL injection)	100	100	100	97.55	96.12	99.58
Grevy's zebra	IVM (Eraquell® tabs)	100	100	100	100	100	100

7.2. Performance Comparison of Coprological Methods – CIISA-FMV-ULisboa

7.2.1. Triodontophorus spp. prevalence

In this part of the study, a sensitivity comparison between all the qualitative techniques and solutions used for the detection of *Triodontophorus* spp. eggs was possible. There was a substantial difference in this parasite's prevalence according to techniques that used salt as a flotation solution, when compared to the sugar solution. Not only did the salt techniques

achieved higher prevalences (SF_{salt}=53%; CF_{salt}=41% *vs* SF_{sugar}/CF_{sugar}=35%), but also, when considering the salt solution as a variable, it obtained a considerable higher prevalence (59%) than the sugar solution (41%). This suggests that the former solution might be better suited for evidencing *Triodontophorus* spp. in qualitative methods, being more sensitive than sugar.

All the recent literature relative to coprological methods focus on quantitative techniques and the comparison between them, with very few studies about sensitivity of qualitative methods. One of these studies by Tomczuk et al (2014), concluded that a centrifugal flotation method would be more sensitive in detecting the presence of *Anoplocephala perfoliata*, an important cestode in equines. It also states that a higher SG solution considerably increases the sensitivity of the method. Although this parasite has very distinctive epidemiological characteristics from the strongylids studied here, its SG is very similar to the latter ($SG_{A.\ perfoliata}$=1.06; $SG_{strongylid}$=1.05) (Norris et al, 2018). Because of this, it is possible to infer that this centrifugal technique might also be the preferred qualitative method to evidence strongylid eggs. However, this was not observed in the present study. Not only the SF method detected higher prevalence of *Triodontophorus* spp., but the salt solution, with a lower SG than sugar, showed higher prevalence as well. These findings suggest that other variables must be influencing the detection of this parasite, such as the flotation solution's viscosity mentioned above or even the centrifugal impact on this type of eggs. No literature was found regarding these subjects and further studies must be developed.

7.2.2. Qualitative techniques' agreement

As previously said, only the associations SF_{sugar}-CF_{sugar}, CF_{salt}-SF_{sugar} and CF_{salt}-CF_{sugar} showed a significant agreement. In other words, in the practical use of these techniques, there is no apparent statistical difference in the outcome of detecting *Triodontophorus* spp*.* eggs, when using one instead of the other, in each association. The other three techniques associations, SF_{salt}-CF_{salt}, SF_{salt}-CF_{sugar} and SF_{salt}-SF_{sugar}, showed a low rate of agreement, in comparison to the ones mentioned before, evidencing that there was indeed a difference between the performance of both solutions with SF and between SF_{salt} and the three other techniques, as it is the common factor in all three associations. This confirms the differences in prevalence of *Triodontophorus* spp. eggs, when using SF_{salt}, already mentioned. However, this difference was not proved to be statistically different as well.

When considering both solutions, the Salt-Sugar association did not show a significant level of agreement. This is clearly evidenced in their performance in detecting *Triodontophorus* spp. eggs, with the prevalence obtained using the salt solution being higher than the sugar solution (53% *vs.* 41%). Nevertheless, the disagreement observed between both solutions was

not statistically different. In addition, it was also possible to assess the influence of the solution used in the detection of this parasite, via the bias index of the combination. This is particularly important in associations of the same method, for example, SF_{salt}-SF_{sugar} or CF_{salt}-CF_{sugar}. In the former association, the bias index was the highest (bias index=0.176), indicating that the positive results obtained with SF are influenced by the solution used in a greater way than the CF method, which does not seem to be influenced by the solution whatsoever (bias index=0).

Although none of the mentioned associations proved to be statistically different, the differences observed may be of clinical importance in the daily use of these techniques. When comparing the prevalence of each method, it is clear in Table 12 that false-negative results were obtained with CF_{salt}, SF_{sugar} and CF_{sugar}, having SF_{salt} as a gold-standard due to its higher detected prevalence. This finding is not very clinically relevant in this case, given that the AHs of choice to deworm against *Triodontophorus* spp. and cyathostomins are the same, since they belong to the same parasite family. Because of this, BZs and IVM are effective against adult stages of these parasites and MOX is effective against both adult and immature stages (University of Utrecht, 2019).

The same cannot be said for differences in detecting ascarids, like *P. equorum*, for which the recommended AH belongs to the pyrimidines drug family, for example PYR (Rendle et al, 2019). Unfortunately, ascarids were not found in this study so conclusions on their differential detection cannot be drawn. Studies on the detection of these and other types of equine parasites, using qualitative methods, are encouraged as different techniques' performances might give variable results. This, in turn, may lead to unwarrented treatment decisions, with consequent treatment failure and, probably, further development of anthelmintic resistance.

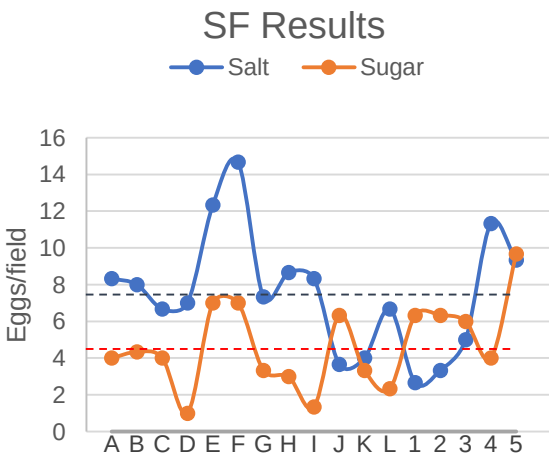
7.2.3. Techniques comparison

When observing the Table 16, differences among the qualitative techniques are clear. It should be also highlighted that all of these techniques were significantly different, with the exception of SF_{salt}-CF_{sugar}, as previously mentioned. Because of this, it is safe to say that CF_{salt} was the best technique in evidencing a greater amount of eggs for identification, like it is depicted in Graphs 3 and 4. Its capacity to present higher eggs/field counts greatly surpassed any other qualitative technique, in a statistically significant way, and can be seen as an advantage of this technique. These findings seem to be in line, method-wise, with what was observed with the results from the wild equid studied in ARTIS, which also determined CF_{sugar} as the most reliable and precise technique to detect *Parascaris* spp. in a captive Grevy's zebra.

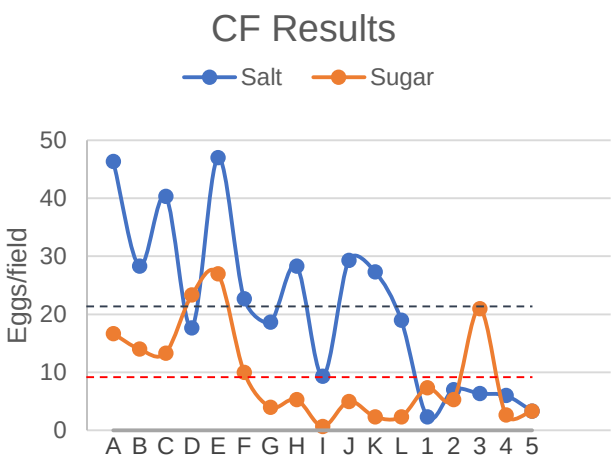
The literature on qualitative methods for equine parasites is lacking, as already said. However, previously mentioned studies with this type of methods and equine cestodes have already validated the use of CF as a more sensitive method for the detection of parasites such as *A. perfoliata* (Proudman and Edwards, 1992; Tomczuk et al, 2014). Therefore, further studies on qualitative methods are encouraged to determine if the same may be true for other equine gastrointestinal parasites.

Nonetheless, it should be noted that SF_{salt} performed with statistically significant lower variation than CF_{salt}, in the second part of the study. In addition, in previous sections, the former technique has been pointed as the one with better chances of evidencing other parasites' egg, such as *Triodontophorus* spp., supported by its substantially higher detected prevalence studied here. These findings seem to support SF_{salt} as the true qualitative method of choice, as the objective of this type of methods is to determine which type of eggs are present. One thing is clear, the salt solution performed with higher means of eggs/field with both methods, as depicted in Graphs 3 and 4, suggesting it to be best suited to qualitative methods. The fact that the salt solution also performed with significantly lower CVs, further evidences this solution as the best fit for qualitative methods. However, more studies must be developed, on the performance of simple and centrifugal flotation in equine feces, to further support the qualitative technique of choice.

The qualitative results on the two sex groups differ. As previously said, the only qualitative techniques that were statistically different were the ones performed with female fecal samples. This is easily recognizable in Graphs 3 and 4, as these samples scored higher values of eggs/field in almost every tested sample. However, this tendency is not present in the male samples, with only sporadic substantial differences, probably due to homogenization inconsistencies in the sample (Vidyashankar et al, 2012), as these feces were much more dried and fibrous than the ones from the females.

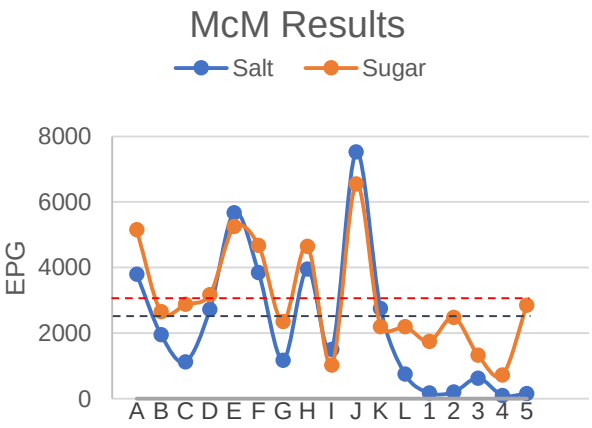


with both salt and sugar solutions for Sorraia horses at CIISA-FMV. The arithmetic means for both solutions are presented as a broken line.

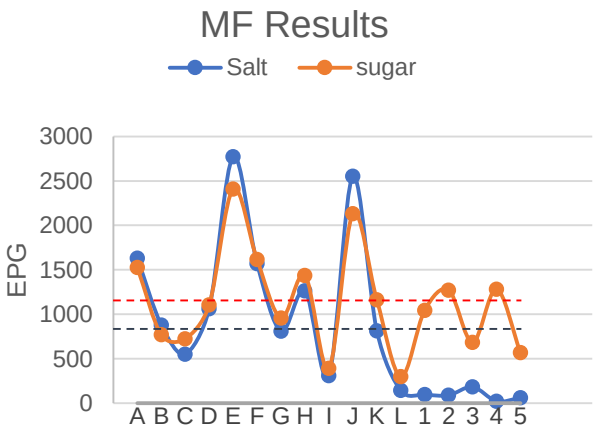


means for both solutions are presented as a broken line.

As for the quantitative techniques, every single technique was shown to be statistically different from the rest, making McM_{salt} and McM_{sugar} the techniques with significantly higher EPGs, as seen in Graphs 5 and 6. This justifies the comparison of the two sex groups, separately from each other, allowing the evaluation of the tested techniques in lower and higher parasite burdens. The McM method had SEMs for both solutions that were more than double the SEM for MF, showing that its precision is very low in comparison to MF. It should also be noted that McM_{salt} and MF_{salt} had lower medians and higher CVs than 95%, indicating that the salt solution should not be used as flotation medium with these techniques. On the other hand, McM_{sugar} and MF_{sugar} performed with much lower CVs and higher means, making it a better alternative to use with quantitative methods. This solution also had lower SEM, further evidencing its better precision. All these findings seem to suggest MF_{sugar} as the most precise technique for horses.



Graph 4 – McMaster (McM) results obtained with both salt and sugar solutions for Sorraia horses at CIISA-FMV. The means for both solutions is presented as a broken line.



Graph 5 – Mini-FOTAC (MF) results obtained with both salt and sugar solutions for Sorraia horses at CIISA-FMV. The means for both solutions is presented as a broken line.

The majority of the comparison studies found in the literature concerning McM and MF seem to follow the recommendations of Cringoli et al (2017) and used a salt solution as a floating medium for the eggs (Silva et al, 2013; Maurelli et al, 2014; Godber et al, 2015; Britt et al, 2017; Bosco et al, 2018; Nápravníková et al, 2019). Very few tested these methods using a sugar-only solution and, even then, the SG of that solution was the same as the 1.2 SG of the salt solution (Alvarado-Villalobos et al, 2017; Dias de Castro et al, 2017). However, some studies used a sugar-salt solution (Noel et al, 2017; Scare et al, 2017; Went et al, 2018) or a sodium nitrate solution (Bortoluzzi, Paras, Applegate and Verocai, 2018; Paras et al, 2018) denser than salt (SG=1.25) to evaluate the two methods.

Taking this into account, a more reliable comparison with those studies is made possible by considering the solutions used with a SG of 1.2 similar to the salt solution used here and the others similar to the sugar solution. Despite all the studies mentioned above recommending MF as an accurate and precise method, only the ones relative to horses are relevant to the present comparison with the results obtained in the present study.

From these studies, the majority of those which used a flotation solution with a SG of 1.2 registered higher results using McM than with MF (Dias de Castro et al, 2017; Bosco et al, 2018), as it was observed in the present study. These studies credited the different multiplication factors of the methods (McM = 50 *vs.* MF = 5) for the wrongful superiority of McM. In addition to this, all of them concluded that MF was a more precise method than McM, evidencing lower CVs that ranged from 16.8%-52% (Britt et al, 2017; Bosco et al, 2018; Dias de Castro et al, 2017; Nápravníková et al, 2019). This was not observed in the present study as well, where MF_{salt} performed with a CV slightly higher than McM_{salt} (98.16% *vs.* 95.75%), both very close to 100%, indicating a great level of variability.

In light of this, it must be stressed that, in this study, two groups of horses under different health managements were studied and this probably contributed to this high variability of results. If analyzed separately, as seen in Table 16, the females' samples, with significantly higher EPGs, had CVs around 70%, with McM_{salt} still performing more consistently than MF_{salt} (66.18% *vs.* 68.43%). However, the males' samples had much less variability as seen by the lower CVs, with MF_{salt} performing more consistently than McM_{salt} (65.38% *vs.* 85.15%). These findings are clearly visible in Graphs 5 and 6.

The results obtained in the present study are a bit higher than the ones registered on studies with other horses on pasture – CV_{MF}: 52%; CV_{McM}: 71% (Britt et al, 2017) – and in paddocks - CV_{MF}: 16.8%; CV_{McM}: 46.3% (Bosco et al, 2018). The explanation for this finding might reside in a recent study previously mentioned that concluded McM was better suited, in comparison to MF, for FECs of strongylid type eggs, as it was the case in the present study (Nápravníková et al, 2019). Despite its higher variability in results, this method accuracy in determining strongylids EPGs was always greater than MF (97.53% *vs.* 74.18%).

As for the mentioned studies that used a flotation solution with a higher SG, MF performed with higher EPGs than McM, either significantly (Scare et al, 2017) or not (Paras et al, 2018). Paras et al (2018) justified these discrepancies with different levels of egg loss during the processing of the feces, where the Fill-FLOTAC straining lid was proven to be more accurate than the cheesecloth straining method used commonly with the McM method. The superiority of MF was not observed in the present study, as McM_{sugar} still performed with

statistically significant higher EPGs than MF_{sugar}, in similarity to what was described by Noel et al (2017). Interestingly, a straining mesh was preferred to the cheesecloth straining method in the present study, one that was twice as wider than the one in Fill-FLOTAC (500μm-250μm). This might explain why the results obtained by Scare et al (2017) and Paras et al (2018) were not observed here, suggesting the exact opposite effect described by them.

In addition, the CVs obtained here for MF_{sugar} were also lower than for McM_{sugar}, as described in all three studies. However, the level of variability was much greater in the present study in comparison to the mentioned studies, where the CVs for MF ranged 14.3% to 35.66% and the McM ones from 31.1% to 50.88%. Although the McM's CV here was in close line to the one registered by Scare et al (2017) (53.96% *vs.* 50.88%), MF_{sugar} performed with much greater variability than previously observed with solutions with higher SG (50.23%). The reason behind this might be the mean of the analyzed samples, which was substantially higher than the ones reported in the mentioned studies, indicating a more extensive parasite infection in the horses studied here.

When analyzing the two sex groups, the mean EPGs obtained with McM_{sugar} continued to be significantly higher than the ones for MF_{sugar} in both groups. The CV of McM_{sugar} decreased to about 46% for both sexes, with MF_{sugar} being higher (53.44%). The greatest change is seen in the CV of MF_{sugar} of males that decreases to 34.07%, which is in the mentioned range of the literature.

7.2.4. Correlations

The assessment of possible correlations between the tested techniques was an important feature of the study in order to determine if any of the techniques performed alone could predict the outcome of any other technique. In fact, some of the quantitative techniques proved to be correlated in a highly significant way. McM_{salt}-MF_{salt} and MF_{salt}-McM_{sugar} correlated significantly in more than 80% of the results. This first association, in particularly, should be highlighted as it scored the strongest correlation, reaching almost 97%, much stronger than previously reported by Noel et al (2017) (r^2= 0.614, p<0.00001).

In addition, CF_{salt} positively correlated with every quantitative technique, especially McM_{salt} and MF_{salt}, whereas CF_{sugar} only correlated with MF_{salt} and McM_{sugar}. These findings indicate that when a high value of eggs/field is obtained with that qualitative technique, the probability of obtaining a high EPG value in any quantitative method performed is high as well. Another conclusion that can be taken from this is that CF_{salt}, as performed here, definitely has a semi-quantitative character, when performed alone. It would be interesting if, in future

studies, the extension of this quantitative quality of CF_{salt} could be further investigated and explore if any thresholds might influence it, if any exist.

7.2.5. Association *Triodontophorus*-EPG

The significant association between the presence of *Triodontophorus* spp. and higher EPGs obtained with quantitative techniques was not found. However, if such finding was observed, it would further support theories and evidences already described in the literature of interparasitic relations.

Although negative interactions, such as population reduction or interactive site segregation, are more commonly seen in parasite ecology (Poulin, 2001), synergetic interspecific relations amongst parasites has already been described in different species of trematodes and between trematodes and acanthocephalans (Cézilly, Perrot-Minnot and Rigaud, 2014). A recent study even suggested the existence of these same interactions between equine gastrointestinal nematodes, specifically *Parascaris* spp. and *Cyathostomum* type C or *Triodontophorus serratus* and *Posteriostomum* spp. (Jota Baptista, 2019).

7.2.6. Coprocultures

During the duration of the incubation period of the coprocultures, the hoven where they were kept suffered a malfunction, turning the incubation temperature from 27°C to 5°C. The cultures remained in these conditions for the remaining of the two weeks of incubation, until they were checked. Because the samples were being used to perform the various techniques studied and there was a chance that they would not be sufficient for another coprocultures, the decision was made to re-incubate these coprocultures in another hoven for another two weeks at 27°C.

The results of this part of the study revealed very degraded larvae with intestinal cells barely distinguishable, as they were used as a feeding source for the development period of these larvae. Many of them were unidentifiable but a few remained that allowed the detection of cyathostomins in every analysed sample, indicating a prevalence of 100% of these parasites in this population. This has already been described many times in the literature (Tolliver, Lyons and Drudge, 1987; Pereira and Vianna, 2006; Morariu et al, 2016). In Portugal, this high level of cyathostomins prevalence was also detected (Madeira de Carvalho, 2001; Reis, 2011; Melo-Franco, 2014). Furthermore, in a previous epidemiological study on the gastrointestinal parasites of the same Sorraia horses in April 2011, the prevalence of cyathostomins was found to be 100% as well, suggesting that these parasites are present virtually in every horse in the country (Osório, 2011).

The first larval type identified was *Cyathostomum sensum latum (s.l.)* type D, with 8 trapezoid intestinal cells in a single line and a length of about 739-782 μm (Santos et al, 2018). According to other parasitological studies conducted in Portugal, this morphotype of *Cyathostomum s.l.* is expected to be the third most common, after type A and C (Madeira de Carvalho, 2001; Reis, 2011). In addition, as the AH treatment of the horses from this population were kept at a minimum, with only 2-3 dewormings/year, a greater diversity in cyathostomin species was to be expected (Young et al, 1999). The reason behind this unexpected lack of variety in identified larvae was probably the poor conditions that they were kept in during the first incubation period, where some of those more common larvae may have hatched faster than the type D larvae and died.

The other identified larvae species was *Strongylus vulgaris*, with 29 intestinal cells displayed in two rows. As expected, according to Osório (2011), the number of this type of larvae found was inferior to the ones of cyathostomins. In this case, only one larva was found, possibly because of the conditions described before that lead to the deterioration of the majority of other larvae. Even though it was only possible to identify one specimen, it confirms the continued presence of *S. vulgaris* in this horse population, with a prevalence of 5.9% (1/17). Considering the pathogenic effect of this parasite's multi-organ migrations (Owen and Slocombe, 1985; Reinemeyer and Nielsen 2018), the AH treatment of these animals must be considered a priority in this farm management to reduce the probability of colic occurrence associated to strongilosis (Wright, 1972; Reinemeyer and Nielsen, 2009; Nielsen et al, 2016).

7.2.7. Parasitic burden

In the studied population of Sorraia breed horses, the lowest mean value of parasitic infection was 871.7 EPG (range: 23 – 2773) for MF_{salt}, being considered a medium level of parasitic infection (Madeira de Carvalho, 2008). All the other quantitative techniques obtained values higher than 1000 EPG, indicating a high level of parasitism. These latter values greatly surpasse the recommended clinical threshold to deworm horses of 200-250 EPG or 500 EPG, already established in the literature (Nielsen et al, 2010b; Rendle et al, 2019). Furthermore, none of the 17 horses was negative for any of the performed techniques. These findings are imperative to point out as they put this population at a high risk of gastrointestinal parasitic disease.

When analyzing each sex group separately, every quantitative technique performed differently between sexes in a statistically significant way (Friedman test, $p<0.05$), except for MF_{sugar} (Friedman test, $p=0.665$), which significances are presented in Table 28. As seen in Table 16, the female samples, from individuals kept on pasture, are significantly higher than

the stabled male ones, for the mentioned techniques, indicating that horse management is clearly an important variable in gastrointestinal parasitism. These findings are consistent with other results found in Poland, where stabled males, with no access to grass, had significantly lower EPGs than females kept on pasture (Kornaś, Cabaret, Skalska and Nowosad, 2010).

The results observed in the present study also confirm that the samples from females on pasture are the great contributors to the high mean EPGs observed with the different techniques. This is easily explained when considering that the detected parasites all have direct life cycles, making transmission from the contaminated pasture very easy. Marchand (2000), in a doctoral thesis, stated that a parasitized horse had a mortality risk of dying from colic that was twice higher than a non-parasitized horse. The risk was even greater for females with ages between 5-15 years. This is very alarming for the female population studied, when considering the extremely high EPGs detected in some samples. In addition, the detection in coprocultures of the presence of *S. vulgaris* in this population should be emphasized, as it is considered one of the three horse parasites more likely to be manifested as colic, alongside with *P. equorum* and *A. perfoliata* (Reinemeyer and Nielsen, 2009).

Given that the Sorraia breed is at great risk of extinction, derived from low population numbers and high consanguinity levels amongst individuals (Pinheiro, 2008; Carolino et al, 2013; Pinheiro et al, 2013), the threat to survival caused by parasitic disease is one that this breed can do without, since it is easily manageable through a sustainable and appropriate worm control program that should be seriously considered.

7.2.8. Influence of the used technique in the classification of shedders

The mean levels of parasitism changed according to the different techniques, due to different individual sample results obtained with each one of them, as evidenced in Table 18. On the observation of this table, it is visually possible to confirm that the great majority of the female samples maintain the same classification throughout the different techniques, whereas the male ones changed dramatically. This is especially visible in the male samples when comparing the salt and sugar solutions. All the low-level parasitized male samples changed classification to high, with great changes in the different classifications' proportions, as seen in Table 19. Based in these results, it would appear that the sugar solution performed more accurately than the salt one.

Surprisingly, in contrast to what was seen with the male samples, the low-level female samples maintain their classification throughout both solutions. These samples were extremely more liquid, almost like diarrhea, in comparison to the ones from the males, which were very dry and fibrous. This is a direct consequence of the management system in which the horses

were kept, since the females on pasture were able to graze all day and the stabled males were only fed hay. Thus, this macroscopic difference of the feces seems to be the real reason behind the differential classification of parasitism of both solutions. These findings evidence that the sugar solution obtained significantly higher and more accurate EPGs in dry fecal samples, explaining the great differences in classification seen in the males' section of Table 18. This would also explain the higher EPG mean of the sugar solution seen in Graphs 5 and 6 for the quantitative techniques.

The results registered on Table 19 show a great variation from the shedders classification's proportion proposed by Nielsen et al (2019), presented in Table 5. From all the tested techniques, MF_{salt}'s global proportion is the most in line with the mentioned above, suggesting it to be an accurate technique of the real classification of shedders. However, as discussed previously, its results were probably influenced by the drier macroscopic conditions of the fecal samples, undermining this claim.

Table 18 – Classification of shedders according to different techniques. Red table cells indicate a high level of parasitism; yellow table cells a medium level of parasitism; and green table cells indicate a low level of parasitism, according to Madeira de Carvalho (2008).

	Salt		Sugar	
Sample ID	McM	MF	McM	MF
A	3800	1630	5150	1525
B	1950	880	2650	770
C	1125	553	2875	725
D	2725	1063	3175	1105
E	5675	2773	5250	2410
F	3850	1570	4675	1615
G	1175	810	2350	958
H	3950	1265	4650	1438
I	1500	310	1025	393
J	7525	2553	6550	2130
K	2750	815	2200	1163
L	750	143	2200	298
1	175	98	1750	1045
2	200	90	2475	1273
3	625	183	1325	683
4	100	23	725	1283
5	150	60	2850	570

Table 19 – Percentage of high, medium and low level of parasitism of the tested samples, globally and sex-dependently, according to Madeira de Carvalho (2008) – CIISA-FMV.

	Level of parasitism	Global	Females	Males
McM_{salt}	High	64.7	91.7	0
	Medium	11.8	8.3	20
	Low	23.5	0	80
MF_{salt}	High	35.3	50	0
	Medium	23.5	33.3	0
	Low	41.2	16.7	100
McM_{sugar}	High	94.1	100	80
	Medium	5.9	0	20
	Low	0	0	0
MF_{sugar}	High	58.8	58.3	60
	Medium	29.4	25	40
	Low	11.8	16.7	0

When assessing the different values obtained with the performed techniques, it was possible to determine the mean differences for each association of techniques, which have been registered in Table 20. There, it is possible to confirm that, when using MF instead of McM, the global mean EPG was about 160% lower, despite the solution used. This rehiterates that McM obtained significantly higher EPGs than MF, as already mentioned when analyzing Table 16. However, this differs from two previously mentioned studies that obtained EPGs with MF that were higher 4.8% (Paras et al, 2018) and 8% (Noel et al, 2017) than McM. Nevertheless, superior McM values have already been described (Dias de Castro et al, 2017),

although to a lesser extent of only up to 15% in comparison to MF. The reason behind these great differences in comparisons between methods is probably due to the means of the analyzed samples in each study, as their means were substantially lower than the ones observed in the present study. This, in turn, in association with the different multiplication factors of the methods, might result in higher variability of the results. Dias de Castro et al (2017) observed a mean of about 600 EPG for both methods, Noel et al (2017) of 700 EPG and Paras et al (2018) a mean of about 200 EPG; all substantially lower to the ones obtained here.

No literature was found concerning the comparison between salt and sugar solutions as flotation mediums alonel, but the significantly higher ability of the sugar solution to obtain higher EPGs is clear from the observation of Table 20. Increases of about 25% were detected globally, suggesting the great influence that this solution has on the results obtained with these methods.

Table 20 – Mean differences (%) between the obtained results for quantitative techniques performed with Sorraia breed horse samples – CIISA-FMV.

	Global	Females	Males
McM_{salt} *vs.* MF_{salt}	-156.5	-181.8	-94.4
McM_{sugar} *vs.* MF_{sugar}	-167.7	-166.8	-67.5
McM_{salt} *vs.* McM_{sugar}	26.7	9.5	90
MF_{salt} *vs.* MF_{sugar}	23.5	14.3	91.4

8. Conclusions

8.1. Repeatability of Coprological Methods – ARTIS

1. For the red river hogs, the detected parasite was *Ascaris suum.* The best qualitative technique seems to be CF_{sugar} has it performed with the second best mean of total egg counts (9.20) and the lowest CV of the qualitative techniques (60.15%). As for the quantitative techniques, McM_{MgSO4} and MF_{salt} performed with the lowest CVs and low SEMs as well, suggesting a good reliability of these techniques. Lack of literature on the accuracy of these methods in pigs did not allow for a solid conclusion on which technique might be better suited for this species.
2. In the greater kudus, a *Nematodirus* spp. parasitic infection was detected. Even with the poor homogenization conditions of the fecal samples, CF_{MgSO4} had the second highest mean of total egg counts (33.10) and the only CV below 100% (86.44%). MF_{MgSO4} seems to be the best suited quantitative method for this species. Also, MF_{salt} was concluded to be a precise technique to analyze pooled samples of ruminants has previously mentioned in the literature.
3. The studied Grevy's zebra was infected with *Parascaris* spp.. The best qualitative technique performed here was, undoubtably, CF_{sugar}, with its highest mean of total egg counts (44.10) and lowest CV (37.50%). In the quantitative techniques, MF_{MgSO4} and MF_{sugar} performed with the lowest CVs and low SEMs, rendering them as the most reliable techniques. Without support from the literature, it is difficult to support one as the best suited based on the results obtained here.
4. The chosen solutions and their SGs were adequate to evidence the present parasites and evidence was found that the SG of *Nematodirus* spp. eggs is lower than what previously stated in published papers.
5. Centrifugal flotation, especially performed with a sugar solution, appear to be the best qualitative method in this part of the study and should be considered by zoological institutions as a valid and reliable technique for wild captive animals.
6. MF seems to be a very precise method and can be applied to wild species.
7. Higher density solutions seem to be better suited to evidence greater amounts of parasite eggs, but other less studied solution's parameters are suggested to be taken into consideration, for example, their viscosity.

8.2. Performance Comparison of Coprological Methods – CIISA-FMV

8. Concerning the detected *Triodontophorus* spp*.* infection detected, the salt solution was shown to be more sensitive in the detection of eggs and the SF techniques showed to be influenced by the flotation medium used, in contrast to the CF ones. None of the qualitative

techniques performed significantly better than the rest, although SF_{salt} obtained the best results, suggesting it to be a more reliable technique.

9. False-negatives for the detection of *Triodontophorus* spp. were observed with the majority of the qualitative techniques, rendering that these techniques cannot be used as stand-alone tools in the decision-making of treatments.
10. The salt solution performed with significantly higher means of global total egg counts and lower CVs for the qualitative methods, suggesting it to be a good fit with this type of fecal assessment.
11. The sugar solution performed with significantly higher means of global total eggs counts and lower CVs and SEM for the quantitative methods, suggesting it to be a very good fit with this type of fecal assessment.
12. CF_{salt} was concluded to have a semi-quantitative character, in correlation to all the quantitative techniques performed. Furthermore, the detection of *Triodontophorus* spp. eggs was shown to be correlated with higher mean EPGs, suggesting the existence of a synergetic interspecific relation of these parasites and other strongylids, like cyathostomins detected here.
13. Coprocultures confirmed the presence of *Cyathostomum s.l.* type D and *Strongylus vulgaris* in the studied horse sample.
14. The parasitic burdens observed in the studied horses were considered high, according to the literature, and, taking into consideration the presence of *S. vulgaris*, it evidences the great risk of these horses face on develop clinical gastrointestinal parasitosis. A sustainable and appropriate worm control program is recommended, given the fragile state of this horse breed.
15. MF_{sugar} appeared to be a more precise technique than the rest of the techniques performed, due to its low CV and substantially lower SEM, and more accurate than techniques performed with salt.
16. Evidence was found that dry and fibrous horse fecal samples might benefit from the use of techniques with sugar solution, as they performed with significantly higher EPG means.

9. Future Perspectives and Recommendations

The suspected presence of AHR in ARTIS animal collection is alarming and should be addressed. The constant treatment inefficacy in red river hogs and Grevy's zebra supports the existence of potential resistant strains of parasites in these animals and are probably a consequence of the high intensity-deworming program at place at the institution. This program exists in order to maintain the global animal health in the zoo, but other measures of worm control are recommended to delay the development of AHR. As discussed in the bibliography review of this thesis, rotation of AH should be avoided (Kaplan and Nielsen, 2010; Leathwick, 2013; Shalaby, 2013). To prevent reinfection, biological control measures like the administration of spores of nematophagous fungi (Larsen et al, 1995; Tavela et al, 2011; Buzatti et al, 2012; Buzatii et al, 2015; Hernández et al, 2016; Hérnandez et al, 2018) and more frequent cleanings of the enclosures (Herd, 1990), are recommended. A SAT program is not recommended to be applied in this zoological institution, as the animal health concept of ARTIS' veterinary team does not include the presence of any parasitical agents in the specimens.

Although it was not possible to determine the real EPGs of the analyzed samples in any of the parts of this study, there are many published articles in the literature comparing the accuracy between McM and MF. These studies used egg-spiking techniques to determine the real EPG in their samples and all of them seem to favor MF as more accurate than McM. Noel et al (2017), did this with equine strongyle eggs and presented an accuracy percentage that was almost double the one for McM (42.56% vs. 23.54%). Furthermore, they found MF to be more sensitive (83.33% vs. 54.17%) and precise (83.2% vs. 53.7%) than McM. The superior accuracy of MF, was also proved in comparison to McM (64.51% *vs*. 21.67%) (Scare et al, 2017). Maurelli et al (2014), found the same superior sensitivity of MF when analyzing fixed fecal samples of dogs, where MF performed with 100% sensitivity. Another study performed by Paras et al (2018) followed the same study design in cattle and concluded that MF was significantly more accurate than McM, being able to recover up to 70.9% of total eggs and McM only 55.0%.

Other factors to account for are the greater loss of eggs associated with straining procedures in McM, in comparison to MF (Scare et al, 2017; Paras et al, 2018; Went et al, 2018), and its higher multiplication factor, that has already been pointed as disadvantage to this method's accuracy (Dias de Castro et al, 2017; Bosco et al, 2018). With all this in mind, it is clear that the present guidelines recommending McM as a suitable method to perform FECRT, which require precision and accuracy, to determine AHR (Coles et al, 1992; Coles et

al, 2006; Nielsen et al, 2019) must be reviewed and include MF as more precise and accurate method, as ESCAAP already did (ESCAAP, 2019).

9.1. Limitations of the study

The major limitations and investigation directions that can be pointed based on this study are:

- It was only possible to have one sample per species for analysis at ARTIS, since the global animal health of the zoo had to be looked after and keeping animals parasitized was not in line with the health vision of ARTIS' veterinary team. Statistically this makes every conclusion drawn from the results obtained difficult to sustain, but they should be reported as well.
- The homogenization of fecal samples from species like greater kudu is a major influence in the coprological results as seen here and in published literature. This was not taken into consideration while processing the kudus' samples and a longer time for homogenization should be allowed for samples comprised of fecal pellets such as these, in order to make sure that they are properly mixed.
- As seen in the discussion of this thesis, literature on coprological techniques on wild species, pigs and horses is lacking, probably due to pre-conceived ideas that flotational methods and flotation solutions do not significantly differ in their results and that McM and the SF are already established as the "gold-standard" of the coprological assessment. However, this study proves these ideals wrong and unfounded and encourages future studies to develop more research on the following issues:
    a) this type of study with different flotation mediums other than the ones commonly used in parasitology;
    b) the accuracy comparison of McM and MF in various species, as well as with different flotation mediums with MF;
    c) on other solutions' parameters, such as viscosity, and its impact on flotation of eggs;
    d) particularly studies on qualitative methods on equine fecal samples, as these seem to be majorly lacking from the literature.

Without these studies, no parasitological decisions regarding coprological diagnostic procedures can be said to be based on solid and justified statements.

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APPENDIX A – Poster communication for the Proceedings of the 18th International Conference "Life Sciences for Sustainable Development", 26th to 28th of September, 2019, Cluj-Napoca, Romania

Comparison study between four coprological methods and two flotation solutions in Sorraia horse (*Equus ferus caballus*) – Preliminary results –

Introduction

Anthelmintic resistance in gastrointestinal parasites of horses has been continuously described all over the world in the last three decades. In order to delay, or even prevent, the further development of resistance, a new deworming approach must be taken, which include biological worm control measures, sustainable use and selective treatments with anthelmintics, considering the diagnostic tools available nowadays [1-3]. This study aims to pinpoint differences in performance of four commonly employed coprological methods, using two different flotation solutions, and make some practical recommendations on which method might be better-suited to face this emerging problem in horse management.

Material and Methods

In this trial, 17 fecal samples of Sorraia horses were collected in April 2019 (12 females on pasture – identified with letters – and five stabled males – identified with numbers) and submitted to coprological examination using simple flotation (SF), centrifuged flotation (CF), McMaster (McM) and Mini-FLOTAC (MF) methods. Each method was performed with a salt (specific-gravity - SPG=1.20) and a sugar solution (SPG=1.28). Each sample was analyzed in triplicates for SF and CF and in duplicates for McM and MF. As a semi-quantitative parameter in SF and CF, the number of eggs were counted in 10 fields of each slide and the arithmetic mean was obtained for each replicate.
Pairwise comparisons were performed using parametric (non-paired T test) and non-parametric tests (Wilcoxon and Spearman correlation tests), all with a significance level of 0.05.
Each method-solution association was perceived as an individual technique for better interpretation of the results.

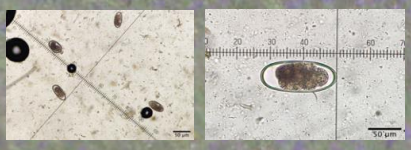


Figure 1 – Qualitative assessment of the parasitic infections detected in Sorraia horses. Left to right: typical strongylid eggs and *Triodontophorus* spp. egg (Originals).

Background photo credit: António Vicente | http://autoctones.ruralbit.com

Table 1 – Prevalence detection of *Triodontophorus* spp. eggs according to different techniques.

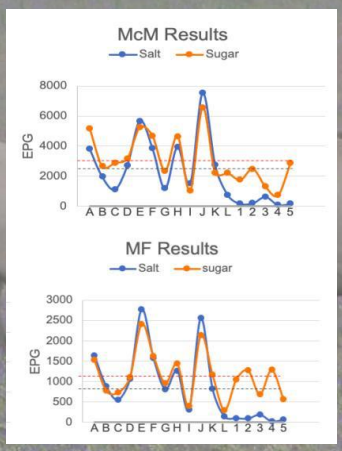
Technique / Solution	Prevalence
Salt	59%
SF_{salt}	53%
Sugar / CF_{salt}	41%
SF_{sugar} / CF_{sugar}	35%

Table 2 – Precision statistics for all tested techniques. SEM – standard error of the mean; CV – coefficient of variation.

	Mean	SEM	CV (%)
SF_{salt}	7	0.78	43.15
CF_{salt}	19	3.51	68.97
McM_{salt}	1500	519.43	95.75
MF_{salt}	810	207.52	98.16
SF_{sugar}	4	0.58	52.43
CF_{sugar}	5	1.99	86.07
McM_{sugar}	2650	399.38	53.96
MF_{sugar}	1105	138.90	50.23

Results and Discussion

The horse samples analyzed showed the presence of parasitic infection by *Triodontophorus* spp. and other strongylids (fig. 1).
Evidence of difference in eggs/field counts was found between qualitative techniques, with CF presenting the highest counts (tab. 2) and significantly higher coefficients of variation (CV). In addition, SF_{salt} was capable of detecting a higher prevalence of *Triodontophus* spp. eggs (53%) (tab.1), suggesting it to be the qualitative technique of choice.
The quantitative techniques with McM (sensitivity of 50 eggs per gram - EPG) and MF (sensitivity of 5 EPG) were all shown to be statistically different from each other, with McM method having the highest EPGs and standard error of the means (SEMs). However, in previous studies, this apparent superiority of the McM method was credited to its bigger multiplication factor and lower sensitivity [4,5].
Despite that, the CVs were not that different in-between techniques. This is indicative that both methods vary in a similar degree but differ in the quantification of that variation, as seen by the significantly higher SEMs of McM techniques. This has already been described before as well, when other studies concluded MF to be the most precise of the two methods, in part, thanks to its incorporated filter mesh, which prevented egg loss associated to straining [6,7].
The CVs of McM_{salt} and MF_{salt}, however, were very close to 100%, but it needs to be stressed that two sample groups, under different management conditions were studied here and this might have been the cause for this great variances. The graphs 3 and 4 make it possible to affirm that the differential performance of the quantitative techniques was only substantial for the male group kept stabled. This also confirms the influence of the equine management system on gastrointestinal parasites [8] and evidences that the sugar solution, especially MF_{sugar}, might be better-suited as a quantitative technique across different equine health managements.



Graph 1 and 2 – McM and MF results with both salt and sugar solutions. The means for both solutions is presented as a broken line.

Conclusion

Based on the results obtained in this trial, it is possible to infer that SF_{salt} was the best-suited qualitative method, due to its higher sensitivity in detecting different parasites, when comparing to CF techniques. It was also shown that MF_{sugar} performed more consistently than McM techniques, mainly because of its lower multiplication factor and, consequently, higher sensitivity. Surprisingly, a tendency was detected with the salt solution being better-suited for qualitative techniques and the sugar solution for the quantitative ones.

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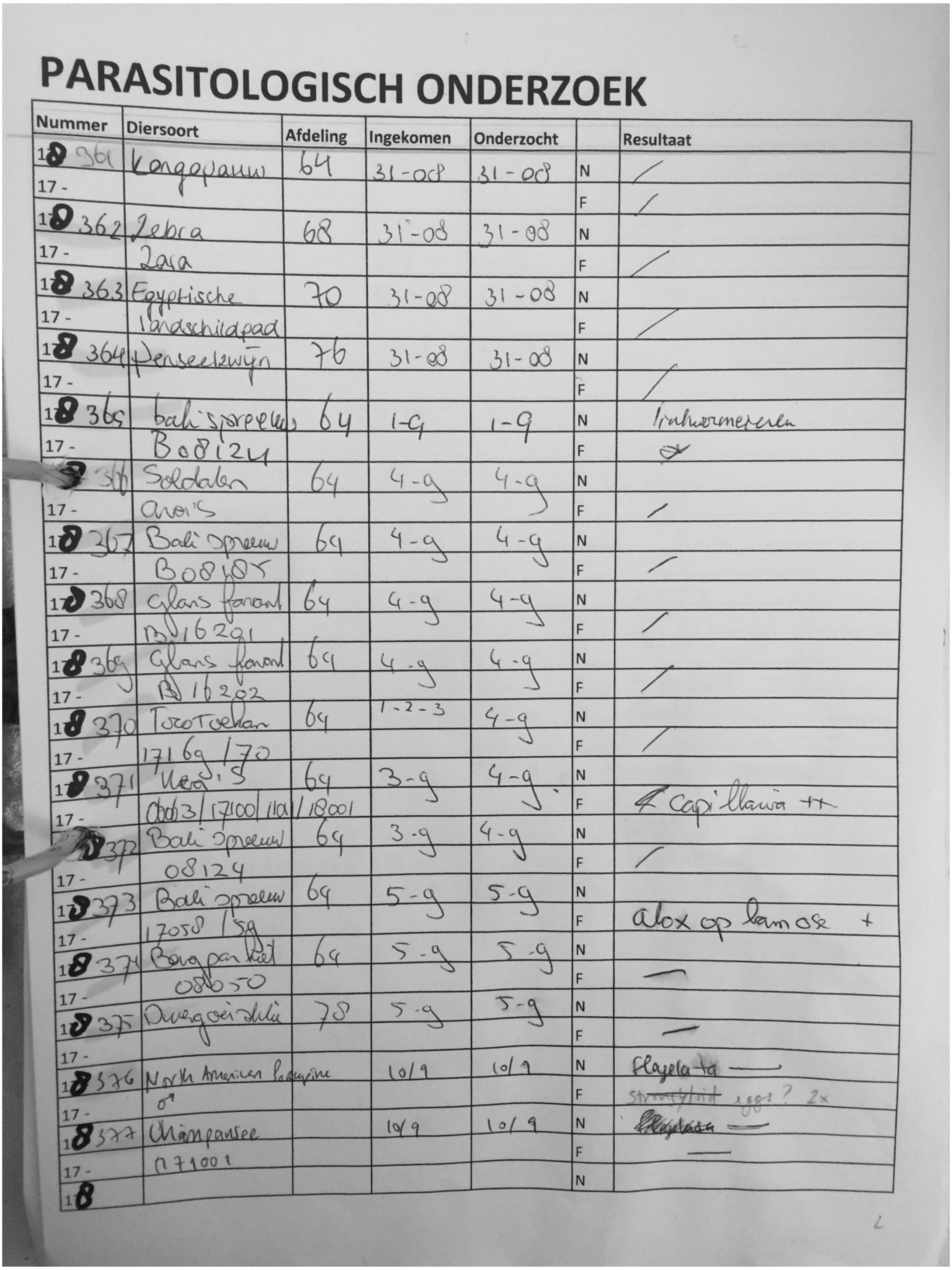
[6] Paras, K. L., George, M. M., Vidyashankar, A. N., & Kaplan, R. M. (2018). Comparison of fecal egg counting methods in four livestock species. *Veterinary*

APPENDIX B – Parasitology Logbook of ARTIS' Veterinary Department from September to December 2018



PARASITOLOGISCH ONDERZOEK

Nummer	Diersoort	Afdeling	Ingekomen	Onderzocht		Resultaat
18 361	Kongopauw	64	31-08	31-08	N	/
17 -					F	/
18 362	Zebra	68	31-08	31-08	N	
17 -	Zara				F	/
18 363	Egyptische	70	31-08	31-08	N	
17 -	landschildpad				F	/
18 364	Penseelzwijn	76	31-08	31-08	N	
17 -					F	/
18 365	bali spreeuw	64	1-9	1-9	N	lintwormeieren
17 -	B08124				F	⊘
18 366	Soldaten	64	4-9	4-9	N	
17 -	ara's				F	/
18 367	Bali spreeuw	64	4-9	4-9	N	
17 -	B08108				F	/
18 368	Glans fazant	64	4-9	4-9	N	
17 -	B16201				F	/
18 369	Glans fazant	64	4-9	4-9	N	
17 -	B16202				F	/
18 370	Toco toekan	64	1-2-3	4-9	N	
17 -	17169/170				F	/
18 371	Kea's	64	3-9	4-9	N	
17 -	06063/17100/11011/18001				F	capillaria ++
18 372	Bali spreeuw	64	3-9	4-9	N	
17 -	08124				F	/
18 373	Bali spreeuw	64	5-9	5-9	N	
17 -	17058/59				F	alox op lamose +
18 374	Bergparkiet	64	5-9	5-9	N	
17 -	08650				F	—
18 375	Dwergoeistiti	78	5-9	5-9	N	
17 -					F	—
18 376	North American Porcupine		10/9	10/9	N	flagellata —
17 -	♂				F	~~strongyloid~~ eggs? 2x
18 377	Chimpansee		10/9	10/9	N	~~flagellata~~ —
17 -	17001				F	—
18					N	

PARASITOLOGISCH ONDERZOEK

Nummer	Diersoort	Afdeling	Ingekomen	Onderzocht		Resultaat
18 378	Maleise jaarvogel		11-9	11-9	N	—
17 -	B08133				F	—
18 379	Kippen				N	—
17 -	B18033		11-9	11-9	F	—
18 380	Rendier 3♂		12-9	12-9	N	—
17 -					F	—
18 381	Kameel 3♂		12-9	12-9	N	—
17 -					F	—
18 382	Ezel 3♀		12-9	12-9	N	—
17 -					F	—
18 383	Steenbok 9♂,5♀		12-9	12-9	N	—
17 -					F	—
18 384	Watussi 1♂,1♀		12-9	12-9	N	—
17 -					F	—
18 385	Carlo 63		14-9	14-9	N	—
17 -					F	—
18 386	Bali Starling ♂		14-9	14-9	N	—
17 -	B0P124				F	Ascaridia + —
18 387	Zebra Zara		17-9	17-9	N	[illegible] —
17 -					F	Parascaris sp. ++ —
18 388	Toko Toekan		16-9	17-9	N	[illegible] —
17 -	B17169/B17170				F	—
18 389	Kea		16-9	17-9	N	[illegible] —
17 -	B1800, 06063, 171	00,11011	[illegible]		F	Capillaria sp. +++ —
18 390	wit gezicht saki		18-9	18-9	N	—
17 -	0.1. Quarantine				F	—
18 391	[illegible] Lioness		18-9	18-9	N	—
17 -					F	—
18 392	Maleise Horsers		17-9	18-9	N	—
17 -	crested				F	—
18 393	Macaque ♀ [illegible]		[illegible] 19-9	20-9	N	Flagellates ++ —
17 -					F	—
18 394	Bank monkey		20-9	20-9	N	—
17 -	M 11130				F	
18					N	

PARASITOLOGISCH ONDERZOEK

Nummer	Diersoort	Afdeling	Ingekomen	Onderzocht		Resultaat
18395	Swan		21-9	21-9	N	ciliates —
17 -					F	Ascaridia/Heterakis ? + —
18396	Boomstekel		24-9	24-9	N	—
17 -					F	—
18397	Paardje (Spotty)		24-9	24-9	N	—
17 -	Ajd 67				F	Strongylus type eggs —
18398	Paardjes		24-9	24-9	N	—
17 -	Ajd 67				F	Strongylus type eggs — ~~free roaming?~~
18399	Kea				N	
17 -	B11011 + B18001 + B17100 + B06063		24-9	24-9	F	Capillaria —
18400	Satyr Tragopan				N	—
17 -	B18120, 10148, 18117, 18119, 17086, 11817		21-9	25-9	F	Capillaria —
18401	groenvleugel ara				N	—
17 -	B09017		24-9	25-9	F	—
18402	Bartlett dolksteekduif		24-9	25-9	N	—
17 -	~~B11890~~				F	—
18403	Rood Arara Amazone		24-9	25-9	N	—
17 -	B15179; B15182				F	—
18404	Kongo Pau		24-9	25-9	N	—
17 -	B12174				F	—
18405	Rode ibis		24-9	25-9	N	—
17 -	Whole flock				F	—
18406	Saki o.l.		25-9	25-9	N	—
17 -	Quarantine				F	—
18407	Roodkraagmaki	60	26-9	26-9	N	—
17 -	G Q121				F	eggs with living larvae +
18408	Roodkraagmaki	60	26-9	26-9	N	amoebas, flagellates —
17 -	E Q121				F	eggs with living larvae +
18409	Roodkraagmaki	60	26-9	26-9	N	amoebas —
17 -	Q121 C				F	—
18410	Palawan Pau				N	—
17 -	B16043 + B13192		26/9	26/9	F	Capillaria —
18411	Purperkaplori		26/9	26/9	N	—
17 -	B08151 + B12176				F	—
18					N	

17

PARASITOLOGISCH ONDERZOEK

Nummer	Diersoort	Afdeling	Ingekomen	Onderzocht		Resultaat
18 412	Lion		27-9	27-9	N	—
17-	M18057				F	—
18 413	Snails	323	28-9	28-9	N	Amoebas +++, flagellates ++ —
17-	068 (ø)				F	living eggs —
18 414	Snails	"	28-9	28-9	N	ciliates +, flagellates ++, amoebas + —
17-	063 ø				F	eggs (larvae/mess)
18 415	Snails	"	28-9	28-9	N	ciliate +, flagellates, amoebas +++ —
17-	065 (ø)				F	eggs (blastocysts/mess)
18 416	Snail	"	28-9	28-9	N	flagellates, Shaky, 1.b.
17-	067 (ø)				F	~~nematode~~ eggs (~~larvae~~)
18 417	Snails	"	28-9	28-9	N	flagellate, 1-b —
17-	062 ø				F	eggs (larvae/mess)
18 418	Malaysian porter		28-9	28-9	N	—
17-	M11130				F	—
18 419	Rood cockatoo		~~27~~-9	28-9	N	—
17-					F	—
18 420	Pelawan lauf		28-9	28-9	N	X
17-	B13192+B16643				F	—
18 421	Black necked Swan		~~30~~-9	1-10	N	X
17-					F	—
18 422	Kea's		30-9	~~1~~-10	N	amoebas, ciliates —
17-					F	Capillaria —
18 423	Toko Toekans		30-9	1-10	N	—
17-					F	Capillaria —
18 424	Zwarthalszwaan		1-10	1-10	N	—
17-	B08040				F	—
18 425	Toko toekans		1-10	1-10	N	—
17-	B17169+B17270				F	capillaria —
18 426	Gorillas		1-10	1-10	N	—
17-					F	—
18 427	NA porcupine		1-10	2-10	N	—
17-	M17040				F	—
18 428	Saki duoroantine		2-10	2-10	N	—
17-	M18056				F	—
18					N	

17

PARASITOLOGISCH ONDERZOEK

Nummer	Diersoort	Afdeling	Ingekomen	Onderzocht		Resultaat
18 429	Seriema	231-A5	3-10	3-10	N	
17 -					F	
18 430	Hamerkop	231 E3	3-10	3-10	N	
17 -	B				F	
18 431	Dubbele D.1	231. E4.c	3-10	3-10	N	
17 -	B17002				F	
18 432	Dubbele 1.0		3-10	3-10	N	
17 -	14034				F	
18 433	hiyacinth ara		3-10	3-10	N	
17 -	B07014 + B07118				F	Heterakis sp. x1
18 434	Blauwkuif ara		3-10	3-10	N	
17 -					F	Heterakis sp. x1
18 435	Edel papegaai		3-10	3-10	N	
17 -					F	
18 436	North white-faced owl		3-10	3-10	N	
17 -					F	
18 437	roodoogmaki	60	5-10	5-10	N	flagellaten ++++
17 -	kikker				F	
18 438	1-0 Leeuw	71	7-10	7-10	N	
17 -					F	
18 439	Saki		9-10	9-10	N	
17 -	N18056				F	
18 440	Kea's		10-10	10-10	N	
17 -					F	
18 441	chimp.		12-10	12-10	N	ciliaten ++++
17 -	N71001				F	
18 442	Saki				N	
17 -	N18056		15-10	15-10	F	
18 443	Boomstekels		15-10	15-10	N	
17 -	N17040/N10100				F	
18 444	Ronkikker		15-10		N	larvae +++ ; amoeben
17 -	A18006				F	eggs with larvae (2)
18 445	Ronkikker		15-10		N	larvae ++++
17 -	A18007				F	eggs with larvae ++
18					N	

PARASITOLOGISCH ONDERZOEK

Nummer	Diersoort	Afdeling	Ingekomen	Onderzocht		Resultaat
18446	A18008	61	15-10	15-10	N	larvae++ —
17 -	Ronkilders				F	eggs with larvae++ —
18447	Rood Buulbuul	67	15-10	17-10	N	~~—~~
17 -	B18032				F	—
18450	Bonte Parus	78	17-10	18-10	N	—
17 -	N11180				F	—
18451	Sneeuwuil	77	22-10	23-10	N	—
17 -	B18139				F	coccidiosis —
18452	Sneeuwuil	77	26-10	26-10	N	—
17 -					F	coccidia —
18453	Red-eye tree frog ~~Sneeuwuil~~	61	29-10 ~~26-10~~	29-10 ~~26-10~~	N	eggs with living larvae —
17 -	Q121 E				F	" " ", fly debris++
18454	toko toekans	232	29/10	29/10	N	—
17 -	B17169, B17170				F	capillaria (12 eggs) —
18455	roodoogmaki	61	29/10	29/10	N	wormen +++ (darmen, sectie)
17 -	Q121 G				F	
18456	roodoogmaki	61	29/10	29/10	N	— (darmen sectie)
17 -	Q121 E				F	—
18457	Rode Vari	78	30-10	30-10	N	—
17 -	(all)				F	—
18458	Hyacintharas	74	30-10	30-10	N	—
17 -	B07118 + B07014				F	—
18459	Blauwkeel ara	74	30-10	30-10	N	—
17 -	(all)				F	=
18460	Keas	64			N	—
17 -	B17100 + B18001		31-10	31-10	F	Amidostomum sp. (1) —
18461	Zwarthals zwan	74	31-10	31-10	N	—
17 -	808040				F	
18462	Penseel	76A	31-10	31-10	N	—
17 -	(2 wijnen)				F	Ascaris suum (10) —
18463	Maki's kas	78	31-10	31-10	N	cilliates++ —
17 -					F	—
18464	Zeeleeuw Blok	68	2-11	2-11	N	/
17 -					F	/
18465	Edelpapegaai B17076	231	4-11	5-11	N	/

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PARASITOLOGISCH ONDERZOEK

Nummer	Diersoort	Afdeling	Ingekomen	Onderzocht		Resultaat
17-8466	gorilla's	76	5-11	5-11	N	
17-					F	ascaris lumbricoides 1ei
17-8467	Panel hoenders	6PC	6-11	6-11	N	—
17-					F	capillaria x5 —
17-8468	Wallaby	63	6-11	6-11	N	—
17-					F	—
17-8469	Alpaca/Lama	63	6-11	6-11	N	—
17-					F	—
17-8470	Vicogne	63	6-11	6-11	N	—
17-					F	—
17-8471	Spoonbills		5-11	6-11	N	—
17-					F	—
17-8472	Nylgaus		6-11	6-11	N	—
17-					F	—
17-8473	Tinamoe		6-11	6-11	N	— (capillaria (x) —
17-	B17085				F	—
17-8474	Stekel varkens		6-11	6-11	N	—
17-					F	—
17-8475	Maleise jaarvogel		6-11	7-11	N	flagellaten ++ —
17-	14101/ 09042				F	—
17-8476	Waver Vogel zaal 3		6-11	7-11	N	flagellaten+ —
17-					F	—
17-8477	intrasteen vogel		6-11	7-11	N	—
17-					F	—
17-8478	Tinamoe		6-11	7-11	N	flagellaten —
17-					F	—
17-8479	Vogels zaal 3		6-11	7-11	N	—
17-					F	—
17-8480	vogels zaal 2		6-11	7-11	N	—
17-					F	—
17-8481	Jaarvogel normaal		6-11	7-11	N	—
17-					F	—
17-8482	Kraan vogel	Quarant.	9/11	9/11	N	—
17-					F	—
17-8483	Tinamoe B17085		9/11	9/11	N	flagellaten —
					f	—

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PARASITOLOGISCH ONDERZOEK

Nummer	Diersoort	Afdeling	Ingekomen	Onderzocht		Resultaat
18 484	Kraais		9/11	12/11	N	X
17 -	B18001/817160				F	—
18 485	Toko toekans	232	9/11	12/11	N	X
17 -	B17169				F	—
18 486	Toko toekans	232	9/11	12/11	N	X
17 -	B17170				F	capillaria (2) —
18 487	Grave B18173		13/11	14/11	N	—
17 -	Quarantine				F	—
18 488	Pandas		14/11	14/11	N	—
17 -	♀				F	—
18 489	Chimps		14/11	14/11	N	cilliates —
17 -	(all)				F	—
18 490	Kibo mark varkens		14/11	14/11	N	—
17 -	M10148/M10147				F	—
18 491	Kibo mark kippen		14/11	14/11	N	—
17 -	B18033				F	—
18 492	Kibo mark schaapjes		14/11	14/11	N	—
17 -	M14058				F	—
18 493	Kibomark geiten		14/11	14/11	N	—
17 -	M15034/M15032				F	—
18 494	Anoas	63	14/11	14/11	N	—
17 -	(2)				F	
18 495	Springbokken		15/11	17/16 - 4	N	—
17 -					F	—
18 496	Kudu		15/11	15/16 - 11	N	—
17 -					F	Nematodirus (9 eggs) —
18 497	Thomson's gazelle		15/11	15/16 - 11	N	—
17 -					F	—
18 498	Kraan Vogel	495A	16/11	16/11	N	—
17 -					F	coccidia (17) —
18 499	Giraffen		16/11	16/11	N	—
17 -					F	Nematodirus (3 eggs) —
18 500	Gorillas	76A	19/11	20/11	N	living larvae and eggs +++ —
17 -	(all)				F	larvae + eggs —
18 501	Rood oog maki kikker		17/11	19/11	N	—
	Q-121-8				F	—

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PARASITOLOGISCH ONDERZOEK

Nummer	Diersoort	Afdeling	Ingekomen	Onderzocht		Resultaat
18 502	Chimp	76B	19/11	19/11	N	—
17 -	D71001 (Nargot)				F	— X
18 503	Red Eye Tree frogs		19/11	19/11	N	—
17 -	Q-121-C				F	X
18 504	Diana meerkatten	76A	20/11	21/11	N	flagellates —
17 -	(both)				F	—
18 505	Algenellen	68	20/11	21/11	N	—
17 -					F	—
18 506	Beer valley	63	23/11	23/11	N	
17 -					F	—
18 507	Keas		25/11	26/11	N	—
17 -	B17100; B18001				F	—
18 508	Boshuis		26/11	26/11	N	flagellates + —
17 -					F	unknown eggs (—
18 509	Stokstaan tea	76	26/11	26/11	N	—
17 -	(all)				F	—
18 510	Toko toekans		26/11	26/11	N	X
17 -	B17170				F	capillaria (4) —
18 511	Toko toekans		26/11	26/11	N	X
17 -	B17169				F	capillaria (1) —
18 512	Caria's		26/11	28/11	N	X
17 -					F	—
18 513	Chimp (Nargot)		27/11	27/11	N	ciliates ++ —
17 -					F	X
18 514	Pelikanen		28/11	28/11	N	—
17 -	(all)				F	—
18 515	wallabys		29/11	29/11	N	flagellates —
17 -	(all)				F	—
18 516	Knaanvogel		30/11	30/11	N	living larvae (1) —
17 -	B18173 (Quarantine)				F	—
18 517	Black necked swan		30/11	30/11	N	—
17 -	(bath)				F	—
18 518	Gorillas	76A	3/12	4/12	N	—
17 -	(whole group)				F	—
18 519	~~Satyr Tragopan~~		~~3/12~~		N	

~~B [illegible]~~

PARASITOLOGISCH ONDERZOEK

Nummer	Diersoort	Afdeling	Ingekomen	Onderzocht		Resultaat
18 520	Sakis	7B	3/12	3/12	N	—
17 -	M1203+/M18058				F	—
18 521	Kroonvogel		5/12	5/12	N	—
17 -	Q95A				F	— (3 larvae)
18 522	Bonte maki	7B	6/12	6/12	N	—
17 -	M11130				F	—
18 523	Balispreeuw		6/12	6/12	N	—
17 -	B09095				F	—
18 524	chimp.		7/12	7/12	N	Ciliates (26)
17 -	Mango				F	X
18 525	Satya Thagopan		7/12	7/12	N	—
17 -	B10148, 17086, 18117, 18118, 18119, 18120				F	Syngamus trachea ? (1)
18 526	Guinea Pig		7/12	7/12	N	—
17 -	(new male)				F	—
18 527	Muntjak	7B	10-12	10-12	N	Ciliaten + Blastocystis flagellaten
17 -					F	
18 528	Grondkoeskoes		10-12		N	—
17 -	(one of both)				F	—
18 529	Toekans	E3	7-12	10-12	N	—
17 -	day 1				F	—
18 530	Toekans	E3	8-12	10-12	N	—
17 -	day 2 B17170				F	capillaria (1)
18 531	Toekans B17169	E3	9-12	10-12	N	capillaria (1)
17 -	day 3				F	capillaria (4)
18 532	Satya Thagopan	A4	8/12	10-12	N	—
17 -					F	—
18 533	Satya Thagopan	A4	9/12	10-12	N	—
17 -					F	—
18 534	Ikea's			10-12	N	capillaria (1)
17 -	B06463, B17100, B18001				F	capillaria (11)
18 535	Muntjac ♂	63	10-12	11-12	N	—
17 -					F	—
18 536	Muntjac ♀	63	10-12	11-12	N	—
17 -					F	—
18 537	Flamingos (all)		11-12	11-12	N	—
					F	—

PARASITOLOGISCH ONDERZOEK

Nummer	Diersoort	Afdeling	Ingekomen	Onderzocht		Resultaat
18 538	Zebras	68	11-12	13-12	N	—
17 -	(all)				F	—
18 539	Wolven	62	12-12	12-12	N	—
17 -	(Soth)				F	—
18 540	Kaedoe	68	13-12		N	—
17 -	Punk				F	Positief Strongylidae
18 541	Guinea Pigs	Qg5	14-12	14-12	N	—
17 -	Q 95B 1.0				F	—
18 542	Chuckwalla	77	17-12	18-12	N	
17 -	R11026/12033				F	—
18 543	Jaguar	71	17-12	18-12	N	
17 -	(all)				F	—
18 544	Leeuwen	71	18-12	18-12	N	
17 -					F	✓
18 545					N	
17 -					F	
18 546					N	
17 -					F	
18 547					N	
17 -					F	
18 548					N	
17 -					F	
18 549					N	
17 -					F	
18 550					N	
17 -					F	
18 551					N	
17 -					F	
18 552					N	
17 -					F	
18 553					N	
17 -					F	
18 554					N	
17 -					F	
18 555					N	

APPENDIX C – Calculation of *k* value of agreement

Table 22 – Contingency table for the association SF_{salt}-SF_{sugar};

		SF sugar		
		Present	Not present	Total
SF salt	Present	5	4	9
	Not present	1	7	8
Total		6	11	17

Table 21 – Contingency table for the association CF_{salt}-CF_{sugar};

		CF sugar		
		Present	Not present	Total
CF salt	Present	5	1	8
	Not present	1	10	9
Total		6	11	17

Table 24 - Contingency table for the association SF_{salt}-CF_{salt};

		CF salt		
		Present	Not present	Total
SF salt	Present	7	2	9
	Not present	1	7	8
Total		8	9	17

Table 23 - Contingency table for the association SF_{sugar}-CF_{sugar};

		CF sugar		
		Present	Not present	Total
SF sugar	Present	5	1	6
	Not present	1	10	11
Total		6	11	17

Table 26 - Contingency table for the association CF_{salt}-SF_{sugar};

		SF sugar		
		Present	Not present	Total
CF salt	Present	6	2	8
	Not present	0	9	9
Total		6	11	17

Table 25 - Contingency table for the association SF_{salt}-CF_{sugar};

		CF sugar		
		Present	Not present	Total
SF salt	Present	5	4	9
	Not present	1	7	8
Total		6	11	17

		Sugar		
		Present	Not present	Total
Salt	Present	7	3	10
	Not present	0	7	7
Total		7	10	17

Table 27 - Contingency table for the association the salt and sugar solutions;

APPENDIX D – Significance values

Table 28 – Summary of the significance values (*p*) for the tested associations of techniques performed.

	Test	Statistic	*p*
Global			
SF_{salt}-CF_{salt}	Paired samples t-test	-3.760	0.001
SF_{sugar}-CF_{sugar}	Wilcoxon signed rank test	2.116	0.034
SF_{salt}-SF_{sugar}	Paired samples t-test	3.029	0.005
CF_{salt}-CF_{sugar}	Wilcoxon signed rank test	-2716	0.007
CF_{salt}-SF_{sugar}	Paired samples t-test	4.541	<0.001
SF_{salt}-CF_{sugar}	Wilcoxon signed rank test	0.687	0.492
McM_{salt}-MF_{salt}	Wilcoxon signed rank test	-3.622	<0.001
McM_{sugar}-MF_{sugar}	Paired samples t-test	4.520	<0.001
McM_{salt}-McM_{sugar}	Wilcoxon signed rank test	2.771	0.006
MF_{salt}-MF_{sugar}	Wilcoxon signed rank test	2.107	0.035
McM_{salt}-MF_{sugar}	Wilcoxon signed rank test	-2.296	0.022
MF_{salt}-McM_{sugar}	Wilcoxon signed rank test	3.621	<0.001
Between sexes			
SF_{salt}	Friedman test	1.800	0.18
CF_{salt}	Friedman test	5.000	0.025
SF_{sugar}	Friedman test	5.000	0.025
CF_{sugar}	Friedman test	5.000	0.025
McM_{salt}	Friedman test	5.000	0.025
MF_{salt}	Friedman test	1.800	0.18
McM_{sugar}	Friedman test	5.000	0.025
MF_{sugar}	Friedman test	0.200	0.665
Females			
SF_{salt}-CF_{salt}	Wilcoxon signed rank test	3.061	0.002
SF_{sugar}-CF_{sugar}	Paired samples t-test	-2.740	0.019
SF_{salt}-SF_{sugar}	Wilcoxon signed rank test	-2.909	0.004
CF_{salt}-CF_{sugar}	Paired samples t-test	6.380	<0.001
CF_{salt}-SF_{sugar}	Paired samples t-test	7.933	<0.001
SF_{salt}-CF_{sugar}	Wilcoxon signed rank test	0.785	0.432
McM_{salt}-MF_{salt}	Paired samples t-test	5.015	<0.001
McM_{sugar}-MF_{sugar}	Paired samples t-test	7.345	<0.001
McM_{salt}-McM_{sugar}	Paired samples t-test	-1.916	0.082
MF_{salt}-MF_{sugar}	Paired samples t-test	-0.209	0.838
McM_{salt}-MF_{sugar}	Paired samples t-test	4.423	0.001

MF_{salt}-McM_{sugar}	Paired samples t-test	-8.396	<0.001
Males			
SF_{salt}-CF_{salt}	Paired samples t-test	0.753	0.494
SF_{sugar}-CF_{sugar}	Wilcoxon signed rank test	-0.137	0.891
SF_{salt}-SF_{sugar}	Paired samples t-test	-0.108	0.919
CF_{salt}-CF_{sugar}	Wilcoxon signed rank test	0.730	0.465
CF_{salt}-SF_{sugar}	Paired samples t-test	-0.948	0.398
SF_{salt}-CF_{sugar}	Wilcoxon signed rank test	0.135	0.893
McM_{salt}-MF_{salt}	Wilcoxon signed rank test	-2.032	0.042
McM_{sugar}-MF_{sugar}	Paired samples t-test	1.861	0.136
McM_{salt}-McM_{sugar}	Wilcoxon signed rank test	2.023	0.043
MF_{salt}-MF_{sugar}	Paired samples t-test	-5.447	0.006
McM_{salt}-MF_{sugar}	Wilcoxon signed rank test	2.023	0.043
MF_{salt}-McM_{sugar}	Paired samples t-test	-4.507	0.011

APPENDIX E – Comparison of techniques performed at ARTIS

Table 29 – Summary of the comparison between all the techniques performed with the three species at ARTIS. Every parameter is organized in a decrescent order and techniques in bold represent the best compromises between parameters: high means and low SEM and CV.

Species	Qualitative		Quantitative		
	Mean (total eggs)	CV (%)	Mean (EPG)	SEM (EPG)	CV (%)
Hogs	21.60 – CF_{MgSO4}	161.02 - SF_{MgSO4}	207.50 – McM_{sugar}	47.88 - McM_{sugar}	170.70 - McM_{salt}
	9.20 – **CF_{sugar}**	145.01 - SF_{salt}	147.50 – **McM_{MgSO4}**	37.79 - McM_{salt}	127.64 - MF_{sugar}
	2.70 – CF_{salt}	93.42 - CF_{MgSO4}	79.00 – **MF_{salt}**	29.92 – **McM_{MgSO4}**	79.39 - MF_{MgSO4}
	1.90 – SF_{sugar}	80.20 - SF_{sugar}	70.00 - McM_{salt}	16.94 - **MF_{salt}**	72.97 - McM_{sugar}
	1.10 – SF_{salt}	60.61 - CF_{salt}	56.00 – MF_{MgSO4}	16.75 - MF_{sugar}	67.83 - **MF_{salt}**
	0.30 – SF_{MgSO4}	60.15 - **CF_{sugar}**	41.50 - MF_{sugar}	14.06 - MF_{MgSO4}	64.14 - **McM_{MgSO4}**
Kudus	44.10 - **CF_{sugar}**	169.97 - SF_{salt}	177.50 - **MF_{MgSO4}**	56.13 - **MF_{MgSO4}**	62.73 - McM_{MgSO4}
	31.10 - **CF_{MgSO4}**	164.75 - CF_{salt}	175.00 - McM_{sugar}	55.34 - McM_{sugar}	42.16 - McM_{salt}
	6.90 - SF_{MgSO4}	152.40 - SF_{sugar}	161.00 - MF_{sugar}	50.91 - MF_{sugar}	36.89 - McM_{sugar}
	3.40 - CF_{salt}	149.59 - SF_{MgSO4}	153.00 - MF_{salt}	48.38 - MF_{salt}	30.28 - MF_{sugar}
	2.40 - SF_{sugar}	124.85 - **CF_{sugar}**	125.00 - McM_{salt}	39.53 - McM_{salt}	26.91 - MF_{salt}
	0.50 - SF_{salt}	86.44 - **CF_{MgSO4}**	120.00 - McM_{MgSO4}	37.95 - **McM_{MgSO4}**	18.60 - **MF_{MgSO4}**
Zebra	44.10 - **CF_{sugar}**	70.10 - CF_{salt}	205.00 - McM_{sugar}	64.83 - McM_{sugar}	44.51 - McM_{salt}
	13.40 - CF_{MgSO4}	63.52 - SF_{MgSO4}	205.00 - McM_{MgSO4}	64.83 - McM_{MgSO4}	33.42 - McM_{MgSO4}
	11.10 - CF_{salt}	49.15 - CF_{MgSO4}	175.00 - **MF_{MgSO4}**	55.34 - **MF_{MgSO4}**	29.42 - MF_{salt}
	5.00 - SF_{sugar}	46.75 - SF_{salt}	172.50 - McM_{salt}	54.55 - McM_{salt}	22.85 - McM_{sugar}
	3.10 - SF_{MgSO4}	43.20 - SF_{sugar}	164.50 - **MF_{sugar}**	52.02 - **MF_{sugar}**	18.43 - **MF_{sugar}**

3.10 – SF_{salt}	37.50 - **CF_{sugar}**	134.00 - MF_{salt}	42.37 - MF_{salt}	14.00 - **MF_{MgSO4}**