**REVIEW**

# The equine ascarids: resuscitating historic model organisms for modern purposes

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## Abstract

The equine ascarids, *Parascaris* spp., are important nematode parasites of juvenile horses and were historically model organisms in the field of cell biology, leading to many important discoveries, and are used for the study of chromatin diminution. In veterinary parasitology, *Parascaris* spp. are important not only because they can cause clinical disease in young horses but also because they are the only ascarid parasites to have developed widespread anthelmintic resistance. Despite this, much of the general biology and mechanisms of anthelmintic resistance are poorly understood. This review condenses known basic biological information and knowledge on the mechanisms of anthelmintic resistance in *Parascaris* spp., highlighting the importance of foundational research programs. Although two variants of this parasite were recognized based on the number of chromosomes in the 1870s and suggested to be two species in 1890, one of these, *P. univalens*, appears to have been largely forgotten in the veterinary scientific literature over the past 100 years. We describe how this omission has had a century-long effect on nomenclature and data analysis in the field, highlighting the importance of proper specimen identification in public repositories. A summary of important basic biology, including life cycle, in vitro maintenance, and immunology, is given, and areas of future research for the improvement of knowledge and development of new systems are given. Finally, the limited knowledge regarding anthelmintic resistance in *Parascaris* spp. is summarized, along with caution regarding assumptions that resistance mechanisms can be applied across clades.

**Keywords** Equine · Ascarid · *Parascaris* · Parasite · Nematode · Anthelmintic resistance

## Introduction

Helminth parasites have been known to humans for thousands of years. The Egyptian Papyrus Ebers, circa 1550 BCE, refers to intestinal worms; the Greeks knew about helminths infecting other species; and the Romans also clearly described *Ascaris* parasites, including symptoms of clinical disease (Cox 2002). Carl Linnaeus described and named six helminths in 1758, including *Ascaris lumbricoides*, which ultimately led to an increasing number of helminths being described and formally named (Linnaeus 1758). Ascarids, also known as parasitic roundworms, encompass a large number of important helminth parasites that have three anterior lips in the superfamily Ascaridoidea, including ascarids of poultry, *Ascaridia* spp. (Dujardin 1845) and *Heterakis gallinarum* (Schrank 1788); fish, *Anisakis* spp. (Rudolphi 1809); canids, *Toxocara canis* (Werner 1782); felids, *Toxocara cati* (Schrank 1788); felids and canids, *Toxascaris leonina* (Linstow 1909); cattle, *Toxocara vitulorum* (Goeze 1782); mustelids and bears, *Baylisascaris* spp. (Sprent 1968); pigs, *Ascaris suum* (Goeze 1782); humans, *Ascaris lumbricoides* (Linnaeus 1758); and equids, *Parascaris equorum* (Goeze 1782) and *Parascaris univalens* (Boveri 1887). Many of these parasites can cause severe clinical diseases, including high mortality in cattle (Borgsteede et al. 1992; Gundran and More 1999; Chelladurai et al. 2015); loss of appetite and weight, anorexia, depression, and increased mortality in chickens (Kaufmann et al. 2011; Thapa et al. 2015; Sharma et al. 2019); and pneumonitis, dyspnea, and coughing in pigs (Yoshihara et al. 1983; Curtis et al. 1987; Stewart and Hale 1988; Holland 2013; Mateus et al. 2015), all of which can lead to millions of dollars in agricultural

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economic losses. Globally, an estimated 807 million to 1.2 billion humans are infected with *A. lumbricoides*, many of them children in impoverished countries, causing retardation of physical and mental growth, pneumonia, asthma, abdominal distension, intestinal obstruction, pancreatitis, and death (Bethony et al. 2006). Zoonotic infections can also occur with many ascarid parasites. *A. suum* can complete its life cycle in humans and cause ascariasis (Nejsum et al. 2005; Volk and Tormey 2017; Avery et al. 2018); however, others cannot, leading to a condition known as visceral larva migrans caused by aberrant larval migration. Cases have been reported with *T. cati* (Eberhard and Alfano 1998; Zibaei et al. 2014), *T. canis* (Hill et al. 1985; Xinou et al. 2003; Gakosso et al. 2020), *Baylisascaris* spp. (Saffra et al. 2010; Kelly et al. 2012), and *Anisakis* spp. (Kojima et al. 2013; Sohn et al. 2015), resulting in various clinical manifestations including coughing, rash, myalgia, liver lesions, myocarditis, visual impairment, neurological symptoms, and death in rare cases.

The equine ascarids, *Parascaris* spp., are considered the most pathogenic parasites infecting juvenile horses (*Equus caballus*, Linnaeus, 1758) globally and can cause coughing, nasal discharge, lethargy, poor appetite, diarrhea, and colic (Reinemeyer 2009; Nielsen 2016). Fibrotic liver lesions (Brown and Clayton 1979), lung lesions, hyperpnea, bronchiolitis, and lobular pneumonia (Clayton and Duncan 1978; Nicholls et al. 1978) have been reported in experimentally infected foals. Poor body condition has been associated with *Parascaris* spp. infection in working equids (Getachew et al. 2008, 2010); however, foals under parasite management programs have not exhibited these signs in recent studies (Bellaw et al. 2016; Nielsen et al. 2021). Small intestine impaction is one of the largest concerns with this parasite, often requiring hospitalization and surgery, and can ultimately lead to death (Nielsen 2016; Southwood et al. 1996; Cribb et al. 2006; Tatz et al. 2012). In 37 published cases where surgical intervention was necessary for impaction colic due to *Parascaris* spp., 31 horses survived until discharge, but only 11 survived more than 1 year (Nielsen 2016). While the cause of death was not confirmed in these cases, long-term complications resulting from surgery may have contributed to mortality (Santschi et al. 2000; van Loon et al. 2020). Losing young horses results in a direct financial loss from veterinary care and breeding fees; future losses in sales prices, which can be tens of thousands to millions of dollars; competitive winnings; and stud fees; as well as an emotional loss for the owners and caretakers associated with that horse.

There are three available anthelmintic drug classes for the treatment of *Parascaris* spp. infection in horses: macrocyclic lactones, benzimidazoles, and tetrahydropyrimidines. Traditionally, foals were treated within their first 30 days of life, and then at either monthly or bimonthly intervals until their first birthday (Drudge and Lyons 1966; Ellingson and Coates-Markle 1996; Robert et al. 2015; Nielsen et al. 2018). Early reports questioning the efficacy of ivermectin against *Parascaris* spp. (Anderson 1984; Jones 1985) emerged in the mid-1980s, shortly after its introduction to the market, leading to a defense of the drug claiming that the parasite life cycle was misunderstood and errors were made during diagnostic fecal egg counts (Boraski 1987). Formal reports of ivermectin resistance started in the Netherlands in 2002, quickly followed by Canada in 2003 (Boersema et al. 2002; Hearn and Peregrine 2003). This was followed by reports of macrocyclic lactone resistance encompassing the global equine population, and more recently, reports of tetrahydropyrimidine and benzimidazole resistance (Table 1). Current recommendations reduce the overall number of anthelmintic treatments against *Parascaris* spp. in foals in an attempt to slow down the development of resistance (ESCCAP 2019; Nielsen et al. 2019; Rendle et al. 2019).

Reports of anthelmintic resistance in other ascarid species of veterinary and medical importance are few and far between. Only case reports of resistance are available for a few species, including *Ascaris lumbricoides* (Krücken et al. 2017), *Ascaridia dissimilis* (Yazwinksi et al. 2013; Collins et al. 2019), and *Heterakis gallinarum* (Yazwinksi et al. 2013; Collins et al. 2021), but it is clear from the widespread anthelmintic resistance of many important parasitic nematodes infecting livestock (Rose et al. 2015; Fleming et al. 2006; Sutherland and Leathwick 2011; Kaplan and Vidyashankar 2012; von Samson-Himmelstjerna 2012; von Samson-Himmelstjerna et al. 2021b), including *Parascaris* spp., that evolution of resistance is a concern. Anthelmintic resistance is an emerging concern in companion animals (Jimenez Castro et al. 2019; Jimenez Castro et al. 2021), and while there have been no reports of resistance in any companion animal ascarid species, frequent monthly treatment intervals necessitate robust anthelmintic resistance monitoring programs (von Samson-Himmelstjerna et al. 2021b). Previous reviews have discussed the need for medical parasitology to learn from veterinary parasitology and identify causes of anthelmintic resistance, modify anthelmintic treatment regimes, and monitor for resistance, in order to, at minimum, slow down the development of resistance (Beech et al. 2010; Vercruysse et al. 2011; Tinkler 2020; von Samson-Himmelstjerna et al. 2021b). This One Health approach and warning to reduce treatment efficacy has been mentioned for nearly two decades (Geerts et al. 1997; Geerts and Gryseels 2000, 2002; Thompson and Roberts 2001), yet little has changed, particularly in human public health (Tinkler 2020).

Despite widespread anthelmintic resistance in *Parascaris* spp. and the looming specter of resistance in other important ascarid species, little research has been conducted attempting to understand mechanisms of resistance in this species.

**Table 1** Publications reporting anthelmintic resistant populations of *Parascaris* spp., the anthelmintic class investigated, and location by continent and country

| Continent | Country | Publication | Anthelmintic class | | |
|---|---|---|---|---|---|
| | | | Macrocyclic lactones | Tetrahydropyrimidines | Benzimidazoles |
| Asia | Saudi Arabia | Alanazi et al. (2017) | X | X | X |
| | Turkey | Cirak et al. (2010) | X | | |
| Europe | Denmark | Schougaard and Nielsen (2007) | X | | |
| | Estonia | Lassen and Peltola (2014) | X | | |
| | Finland | Näreaho et al. (2011) | X | | |
| | | Hautala et al. (2019) | | X | |
| | France | Laugier et al. (2012) | X | | |
| | | Geurden et al. (2013) | X | | |
| | Germany | von Samson-Himmelstjerna et al. (2007a, b) | X | | |
| | Iceland | Martin et al. (2021b) | X | | |
| | Italy | Veronesi et al. (2009) | X | | |
| | | Veronesi et al. (2010) | X | | |
| | Poland | Studzińska et al. (2020) | X | | |
| | Sweden | Lindgren et al. (2008) | X | | |
| | | Lind and Christensson (2009) | X | | |
| | | Martin et al. (2018) | | X | |
| | | Martin et al. (2021a) | | | X |
| | The Netherlands | Boersema et al. (2002) | X | | |
| | UK | Stoneham and Coles (2006) | X | | |
| | | Relf et al. (2014) | X | | |
| North America | Canada | Hearn and Peregrine (2003) | X | | |
| | | Slocombe et al. (2007) | X | | |
| | USA | Craig et al. (2007) | X | X | |
| | | Lyons et al. (2008) | X | X | |
| | | Lyons et al. (2011) | | X | |
| Oceania | Australia | Armstrong et al. (2014) | X | X | X |
| | | Beasley et al. (2015) | X | | |
| | | Wilkes et al. (2017) | X | | |
| | New Zealand | Bishop et al. (2014) | X | | |
| South America | Argentina | Cooper et al. (2020) | X | | |
| | Brazil | Molento et al. (2008) | X | | |

This review aims to consolidate basic biological information about *Parascaris* spp. and also highlight the importance of robust foundational research programs.

## A tale of two cryptic species

Equine ascarids have an important place in the history of scientific discovery. The German zoologist Johann August Ephraim Goeze was the first to name *Ascaris equorum* in 1782. In the 1880s, Belgian embryologist Édouard van Beneden used the same parasite—now renamed *Ascaris megalocephala*—as a model species and showed that fertilization consisted of the union of haploid gametes to form a diploid zygote, contributing greatly to knowledge of both meiosis and mitosis (van Beneden 1883; Hamoir 1992). Over the course of the following 5 years, at least 27 papers—four by van Beneden—were published featuring *A. megalocephala* studying phenomena such as cell division, chromosome organization, and chromatin diminution (Boveri 1887, 1888).

Two variants of *Parascaris* were described and distinguished from one another in the late 1800s by counting the number of chromosomes present prior to the first cellular division, a process known as karyotyping (van Beneden 1883; Carnoy 1886/1887; Boveri 1887). *Ascaris megalocephala univalens* was initially described by van Beneden

(van Beneden 1883), and *A. meg. bivalens* by Jean Baptiste Carnoy (1886/1887) during cytological studies, but it was not until a few years later that Oskar Hertwig recognized them as different species (Hertwig 1890). These species received their currently accepted names in 1978: *Parascaris univalens*, which has one chromosome pair, and *P. equorum* which has two pairs of chromosomes (Bullini et al. 1978; Goday and Pimpinelli 1986). Hybrids between these two species have been described (Bullini et al. 1978; Goday and Pimpinelli 1986), although they are sterile (Goday and Pimpinelli 1986). Another species with three pairs of chromosomes, *P. trivalens*, was described in the 1930s but has not been described in the literature since (Li 1937; Tchou 1937).

The *Parascaris* species only have a slight morphological difference in their spicula, with *Parascaris univalens* having a distally truncated spicula and *P. equorum* having a distally rounded spicula (Biocca et al. 1978). Two methods have been used in the past to distinguish the two species of *Parascaris* from one another. The first is via karyotyping of primordial germ cells prior to the first cell division, which is an arduous process that requires collecting either parasites with germ cells in the proper stage (Goday and Pimpinelli 1986) or eggs from feces at the first mitotic division (Nielsen et al. 2014; Martin et al. 2018), and the second utilizes electrophoresis of twenty-seven enzyme loci, although this method has only been employed for a single study (Bullini et al. 1978). Karyotyping is a challenging process because it requires either live parasites or eggs that have yet to start developing, and due to this, it is rarely performed for parasitological studies utilizing equine ascarids.

In the nearly 100 years between Hertwig naming the two species and their modern name assignment in 1978, *Parascaris univalens* and *P. equorum* were not recognized as separate species and were instead recognized as different variants of *P. equorum* (Lin, 1954), which may explain why *P. equorum* was thought to be the only species. This ultimately led to *P. equorum* being the only species mentioned in veterinary textbooks and research for decades, with the only recognition of two species occurring in cell biology and cytogenetics, highlighting a lack of communication between disciplines. The last positive identification of *P. equorum* via karyotyping was in 1986 (Goday and Pimpinelli 1986), despite contemporary recognition of two species and an increased effort to karyotype specimens. Ultimately, lack of consensus on species versus variants and naming them led to a decades-long misclassification of specimens that had and continues to have far-reaching consequences.

## Phylogenetics

The phylum Nematoda (Cobb, 1932) consists of over 22,000 named species separated into five distinct clades (Blaxter

et al. 1998; Blaxter and Koutsovoulos 2015). The ascarid parasites fall under Clade III, which also includes pinworms, filarial nematodes, and parasites of millipedes (Blaxter et al. 1998). Within the Ascaridoidea superfamily, *Parascaris* spp. belongs to the monophyletic clade of Ascarididae along with *Baylisascaris* spp., *Toxoascaris leonina*, and *Ascaris* spp. (Nadler 1987; Nadler and Hudspeth 2000; Liu et al. 2016; Li et al. 2018). Parasitism in the Ascaridoidea includes prehistoric host-type switches correlated with global changes in sea level (Li et al. 2018), and tissue parasitism within Clade III evolved separately at least three different times (Nadler et al. 2007). Understanding these evolutionary relationships within Clade III and the Ascarididae clade provides important context when comparing these parasites to other groups within the Nematoda.

Some other well-known and heavily studied species, such as the model organism *Caenorhabditis elegans* (Maupas, 1900), a free-living nematode, and *Haemonchus contortus* (Rudolphi, 1803), the most pathogenic and economically significant nematode parasite of small ruminants and model organism for parasitic nematodes, fall under Clade V (Blaxter et al. 1998). Anthelmintic resistance is rampant in *H. contortus*, and it has therefore been heavily studied (Kotze et al. 2014; Kotze and Prichard 2016), along with substantial research on the same topic in *C. elegans* (Geary and Thompson 2001; Kotze et al. 2014). This means that much of the research conducted regarding anthelmintic resistance that will be described in subsequent sections has been broadly applied to ascarids despite the work being completed in organisms belonging to a completely different clade. The evolutionary distance between *Parascaris* spp., *C. elegans*, and *H. contortus* combined with distinct differences in the life cycle and parasitism suggests that direct comparisons and broadly applying information from one clade to another must be done with caution.

## Genetics

Genetic studies opened the door for contemporary recognition of two distinct *Parascaris* species, although earlier classification as variants named *Parascaris equorum* and *Parascaris equorum univalens* led to the majority of specimens in veterinary parasitology being called *Parascaris equorum*. One study utilizing electrophoresis of enzyme loci showed the opposite, with 93.5% of 2238 specimens collected in an abattoir identified as *P. univalens* (Bullini et al. 1978). This study, however, was published in Italian and appears to have gone unnoticed for several decades. A more recent population genetics study compared equine ascarid specimens from Sweden, Norway, Germany, Iceland, Brazil, and the USA and found that all of the parasites were genetically homogenous (Tydén et al. 2013b). One of the study populations was later karyotyped and found to consist only of *P.*

*univalens* (Nielsen et al. 2014). Additional populations in the USA (Nielsen et al. 2014), Sweden (Martin et al. 2018), Iceland (Martin et al. 2021c), and China (Han et al. 2022) were karyotyped and identified as *P. univalens*, which taken together suggests that the main species present in domestic horses globally is *P. univalens* and not *P. equorum*.

A phylogenetic analysis of *Parascaris* spp. parasites from the mountain zebra (*Equus zebra*, Linnaeus, 1758), domestic horse, and wild ass (*Equus asinus*, Gray, 1824) using the mitochondrial genes *cox1* and *nadh1*, demonstrated that the worms from *E. asinus* formed a distinct clade compared to specimens collected from the other two *Equus* species (Peng et al. 2019). A recent whole-genome study of specimens from horses, donkeys (*Equus africanus asinus*, Linnaeus, 1758), and zebras also indicated distinct clades for *P. univalens* specimens—some of which were confirmed via karyotyping—found within horses and those found in zebras and donkeys (Han et al. 2022). Another recent study completed a phylogenetic analysis for a select group of nuclear and mitochondrial genes using almost all *Parascaris* spp. Using DNA sequences from GenBank along with karyotyped specimens confirmed to be *P. univalens*, they found a small group of sequences, all from parasites collected from donkeys on a single farm in China, that formed a cluster (von Samson-Himmelstjerna et al. 2021a). Due to the genetic distance from *P. univalens* specimens from North America and Europe, the specimens in this cluster may represent another genotype or species of *Parascaris* (von Samson-Himmelstjerna et al. 2021a). This cluster could represent *P. equorum*, or even another species such as *P. trivalens*, which has only been described in a couple of studies using parasites from Chinese horses (Li 1937; Tchou 1937).

The first *Parascaris* spp. draft genome was published in 2017 for *P. univalens*, followed by a draft genome for *P. equorum* in 2019, both indicating over 14,000 coding genes present in the parasites (Wang et al. 2017; International Helminth Genomes Consortium 2019). When considering these genomes, however, the *Parascaris* species conundrum must be taken into consideration. The specimen reported to be *P. equorum* and used for the 50 Helminth Genomes Project was collected at necropsy from an abattoir, and there is no indication that karyotyping was performed to positively identify the species (International Helminth Genomes Consortium 2019). The previously described phylogenetic study using *Parascaris* sequences from GenBank along with karyotyped specimens indicated that nearly every sequence for the internal transcribed spacer-1 and spacer-2 and cytochrome oxidase I labeled as *P. equorum* was clustered within confirmed *P. univalens* specimens, indicating that they are likely all from *P. univalens* (von Samson-Himmelstjerna et al. 2021a). This information, combined with the lack of karyotyping, previous research suggesting that *P. univalens* is the predominant species in domestic horses, and the fact that *P.*

*equorum* has not been identified via karyotyping since 1986 (Goday and Pimpinelli 1986), suggests that the specimen in WormBase ParaSite, as well as many other data deposits in GenBank labeled as *P. equorum*, may be *P. univalens* (Nielsen et al. 2014; International Helminth Genomes Consortium 2019; von Samson-Himmelstjerna et al. 2021a). Currently, there are no GenBank deposits verified as *P. equorum* via karyotyping.

Incorrectly identified information in public repositories is detrimental to the field and can lead to misinterpretation of results. For example, one study comparing *Parascaris* mitochondrial genomes utilized fresh specimens that were not karyotyped but assumed to be *P. equorum* (Gao et al. 2019). These specimens were then compared to mitochondrial genomes from two karyotyped *P. univalens* isolates and one non-karyotyped isolate assumed to be *P. equorum*. The subsequent phylogenetic analysis clustered these four specimens into a single clade, and the authors concluded that *P. equorum* and *P. univalens* may represent the same species (Gao et al. 2019). This, however, is inaccurate given that no attempt was made to identify the collected specimen. Instead, the clustering of the two identified specimens strongly suggests that the collected specimen was *P. univalens*. Correctly identifying species is important not only for ensuring accurate research and performing future genome-wide research studies in a variety of disciplines, including the study of anthelmintic resistance, but also for developing molecular techniques, such as PCR, to identify *Parascaris* specimens to species (Doyle and Cotton 2019; von Samson-Himmelstjerna et al. 2021a).

## Chromatin diminution

After the first cell division, *Parascaris* spp. presomitic cells go through a process called chromatin diminution where chromosomes are fragmented and approximately 85% of the germline genome is eliminated, resulting in the creation of about 35 smaller chromosomes (Boveri 1887; Goday and Pimpinelli 1986; Muller and Tobler 2000; Niedermaier and Moritz 2000). The initial discovery of chromatin diminution was made with *Parascaris* spp. in 1887 and was later found to occur in other nematodes, including *Ascaris suum*, *A. lumbricoides*, and *Toxocara* spp., copepods, ciliates, hagfish, lamprey, and rat fish (Boveri 1887; Wang and Davis 2014). The *P. univalens* germline genome has an estimated 2500 megabases (Mb), whereas the somatic genome has an estimated 250 Mb, indicating a large loss of genetic information in an organism with only a single chromosome (Wang et al. 2017). Comparisons between *Parascaris* spp. and *Ascaris* spp. indicate that the mechanism for chromatin diminution is evolutionarily conserved between the two species and likely present in a common ancestor (Bachmann-Waldmann et al. 2004). Comparative analysis of *Parascaris*, *Ascaris*, and

*Toxocara* genomes shows that somewhere between 1000 and 2000 genes are eliminated, with 35% of those being expressed during spermatogenesis, and it is hypothesized that this allows for rapid adaptation and evolutionary change in the testes without causing deleterious effects because those genes are silenced and eliminated (Bachmann-Waldmann et al. 2004; Wang et al. 2017; Wang 2021).

Ultimately, the process of chromatic diminution, even though it causes a large loss of genetic information, is likely evolutionarily advantageous for *Parascaris* spp in some way. It is possible that chromatin diminution helps prevent events such as population bottlenecks due to the ability of the parasites to undergo rapid evolutionary changes in the germ line and may even have played a role in the evolution of parasitism (Bachmann-Waldmann et al. 2004). Evidence from cytogenetic studies suggests that there are differences in chromosome and heterochromatin organization between the two species (Goday and Pimpinelli 1984, 1986; Goday et al. 1985). It remains to be seen how this process differs molecularly and on a whole-genome level between the two species, particularly since the last karyotyped *P. equorum* specimen was identified in 1986, 2 years before the method for PCR was first published and 6 years before the first-ever whole genome sequence was completed (Goday and Pimpinelli 1986; Mullis and Faloona 1987; Fleischmann et al. 1995). If *P. equorum* was out-competed by *P. univalens* due to a fitness disadvantage as anthelmintic use became more prevalent, understanding chromatin diminution and comparing the two species could be an important key to understanding anthelmintic resistance development in ascarid parasites (von Samson-Himmelstjerna et al. 2021a).

# Life cycle and immunology

## Life cycle

*Parascaris* spp. are robust, cream-colored nematode parasites with a direct life cycle whose adult stages are found primarily within the small intestine of equids. Females are typically 10–20 cm in length with a diameter of 5 mm, whereas their smaller male counterparts are only 10–15 cm in length with a 3-mm diameter (Wells 1924; Clayton and Duncan 1979a). Males can be distinguished from females of a similar size by a curl in the posterior end and a lack of visible ovaries through the cuticle (Wells 1924).

Adult *Parascaris* spp. reproduce sexually in the small intestine via genital pores, and females lay their 40–70 μm eggs in the small intestinal content, from where they are excreted into the environment (Wells 1924). In the pasture, the fertilized parasite eggs embryonate and larvae develop within the eggs; it is this egg containing a second-stage larva that is infective (Wells 1924; Clayton and Duncan 1979a).

Once ingested by a foal, the eggs hatch in the small intestine, and the larvae penetrate the intestinal wall, where they subsequently migrate to the liver within a week of initial infection (Clayton and Duncan 1979a). Within 2 weeks after the initial infection, the larvae enter the lungs via the pulmonary circulation, where they emerge from arterioles and capillaries. The larvae are coughed up and then swallowed by the foal, making their way back to the small intestine 2 to 3 weeks after initial infection (Clayton and Duncan 1979a). At this stage, *Parascaris* spp. larvae are approximately 2–4 mm in length and, over the next 4.5 months, will grow 70–80× in size as they feed on intestinal content and mature into adults (Clayton and Duncan 1979a). While the general life cycle of *Parascaris* spp. has been described, the biological reasoning for larval migration is poorly understood. It has been suggested that tissue migration may be linked to increased body size and faster growth (Read and Skorping 1995) and may also play a role in immune evasion (Mulcahy et al. 2005; Deslyper et al. 2016, 2019). This, however, has not been directly studied in *Parascaris* spp., and no biological signals that may be required for parasite maturation have been identified. Understanding these signals would provide valuable insight into parasite biology, possible control mechanisms, and conditions necessary for in vitro culture from egg to adult.

## Culturing in vitro

Parasites can be difficult to maintain and grow in vitro because of their sometimes-complicated life cycles that are reliant on the correct host. This makes research complicated, particularly in the case of species whose hosts are either unethical to use as research subjects, such as humans, or too difficult—whether because of size, expense, or husbandry needs—to maintain as a research population. In the case of *Parascaris* spp., equine research herds are expensive to maintain, require a large amount of land, and obtaining adult parasites requires euthanasia of healthy foals that require eleven months gestation and another approximate five months before adult parasites can be harvested. There is one research herd known globally that is regularly used for this purpose (Lyons et al. 1990), and many specimens obtained elsewhere are from abattoirs (Janssen et al. 2013; Martin et al. 2020, 2021a; Trailovic et al. 2021) or collected opportunistically at diagnostic necropsies (Burk et al. 2014, 2016; Rakhshandehroo et al. 2016; Malekpour et al. 2019). Adult *Parascaris* spp. can be maintained in vitro for up to a week (Janssen et al. 2013; Scare et al. 2019; Martin et al. 2021a) but show transcriptional stress responses to culture conditions within the first 24 h compared to non-cultured worms via an increase in differentially expressed genes (Martin et al. 2020). Adult *Parascaris* in general do not maintain fitness well in culture, as evidenced by their

short survival time and stress responses, compared to their *Ascaris suum* counterparts that are able to be kept alive for at least 2 weeks (Islam et al. 2004). Despite these challenges, meaningful gene expression data have been obtained from current in vitro systems (Janssen et al. 2013; Scare et al. 2020; Martin et al. 2021a), as well as the development of a *Parascaris* spp. fitness scoring system (Scare et al. 2019).

L2/L3 larvae can be hatched from eggs (Burk et al. 2014; Rakhshandehroo et al. 2017; Martin et al. 2021a), which does not require sacrificing a horse, although they cannot be grown into L4 and L5 larvae or adults. The longevity of these larvae in culture is unknown, but they have been kept alive for at least 48 h (Martin et al. 2021a) and have been used in drug exposure (Rakhshandehroo et al. 2017; Martin et al. 2021a) and immunology (Burk et al. 2014, 2016) studies. Differences in protein transport gene expression between adults and larvae hatched from eggs in vitro (Martin et al. 2021a) must be considered when interpreting data and comparing results between life stages, but the larval culturing system is a promising path forward in *Parascaris* spp. research because it allows studies to take place without sacrificing young horses.

## Immunology

With a few exceptions, such as adult horses in tropical regions and donkeys (Vercruysse et al. 1986; Getachew et al. 2008, 2010; Lem et al. 2012), *Parascaris* spp. are generally found in juvenile horses up until the age of 6 to 8 months when an age-dependent immunity develops (Clayton and Duncan 1979b; Fabiani et al. 2016). Fecal egg shedding and worm counts in juvenile horses occur in an age-dependent manner, and in older foals, fewer larvae reach the small intestine, patent infections are less likely to develop, and fecal egg counts are lower (Clayton and Duncan 1979b; Donoghue et al. 2015; Fabiani et al. 2016). In a study where eight worm-free foals and two yearlings were experimentally infected with *Parascaris* spp., yearlings had a more severe respiratory response but maintained their body condition, whereas foals had a mild respiratory response and lost body condition, suggesting an age-dependent immune response, although the sample size was small (Clayton and Duncan 1978). Increased titers of antibodies to whole worm antigen have been shown to correlate with foal age and subsequent reduction in parasite prevalence (Bello 1985), and immune responses to migrating larvae in the lungs (Nicholls et al. 1978) and liver (Brown and Clayton 1979) have also been illustrated. There are no studies showing direct parasite death or fitness loss as a result of equine immune responses, and molecular evidence of immune response has yet to be demonstrated in horses, despite evidence of an age-dependent response. Understanding the equine immune response to *Parascaris* spp. at an in-depth molecular level would provide invaluable information regarding host-parasite dynamics and open the door for possible vaccine development.

Helminth excretory–secretory products, including micro-ribonucleic acids (RNAs) (Sotillo et al. 2020) and extracellular vesicles (Zakeri et al. 2021), are thought to play a role in immune evasion, elicit host immune response, and may allow for the development of vaccines and/or diagnostic tests (Lightowlers and Rickard 1988). In *Ascaris suum*, extracellular vesicles contain immunomodulatory proteins (Hansen et al. 2019), and microRNAs may be important for parasite development (Xu et al. 2013). An in vitro analysis of larval *Parascaris* spp. excretory–secretory products identified 19 kDa, 22 kDa, 26 kDa, and 34 kDa products that elicited an antibody response in sera of previously infected foals (Burk et al. 2014). Mares were shown to have antibodies against these products and passed them to foals via colostrum during the first suckling (Burk et al. 2016). These antibodies are not useful for diagnosis because the foals acquire them shortly after birth, and they are likely not useful for vaccination because, despite their presence, foals still become infected with *Parascaris* spp. There have been no studies to date examining *Parascaris* spp. extracellular vesicles or microRNAs, and ultimately, more research is necessary to determine the nature of equine immunity against *Parascaris* spp.

## The biology of resistance

In the early twentieth century, John D. Rockefeller committed over US$1 million to hookworm control and research, and Epsom salts, thymol, carbon tetrachloride, oil of chenopodium, tetrachlorethylene, and hexylresorcinol were all either used or investigated for use as anthelmintics (Horton 2003). Continuing into the twentieth century, various dyes and synthetic compounds were used to treat helminth infections, but many were ineffective, difficult to use, and/or toxic, causing a plethora of issues such as deafness, blindness, skin irritation, diarrhea, vomiting, organ damage, and death (Faust 1937; Horton 2003). The first safe, modern anthelmintics were phenothiazine, introduced in the 1940s, and piperazine, introduced in the 1950s. This was followed by the major anthelmintics currently used in horses, starting in 1961 with benzimidazoles and ending with the introduction of the macrocyclic lactones in 1981 (Brown et al. 1961; Campbell et al. 1983; Laing et al. 2016).

Anthelmintic resistance is rampant in veterinary parasitology (Rose et al. 2015; Fleming et al. 2006; Sutherland and Leathwick 2011; Kaplan and Vidyashankar 2012), and understanding how it developed in order to slow down the progression and preserve current anthelmintics for as long as possible, as well as preserve any new anthelmintic for as long as possible, is important. Husbandry practices and

anthelmintic treatment strategies as methods to slow down the development of resistance have been discussed in detail elsewhere (Reinemeyer 2009; von Samson-Himmelstjerna 2012; Matthews 2014; Nielsen 2016; Reinemeyer and Nielsen 2017; von Samson-Himmelstjerna et al. 2021b), although the effect of these practices is not always apparent. Anthelmintic mechanisms of action as well as mechanisms of resistance in parasitic nematodes have also been thoroughly reviewed in the past (Prichard 1994; Kotze et al. 2014; Whittaker et al. 2017; Kaplan 2020). This section will briefly describe drug mechanisms of action and resistance in general, with a focus on relevant research conducted using *Parascaris* spp.

## Benzimidazoles

Benzimidazole is a heterocyclic aromatic organic compound, and various modifications to this structure have resulted in the development of anthelmintic drugs (Townsend and Wise 1990). The first was introduced in 1961, followed by numerous formulations in the 1960s and 1970s (Brown et al. 1961; Harder 2002). Benzimidazole is a microtubule inhibitor that interacts with the colchicine-binding domain of β-tubulin and interrupts polymerization, disrupting vital cellular processes and causing parasite death (Friedman and Platzer 1978; Lacey 1988, 1990). Microtubules are polymers made of tubulin dimers consisting of α- and β-tubulin and are essential for cellular structure and processes such as intracellular transport and cell division (Lacey 1988, 1990). Benzimidazoles developed as anthelmintics have a higher binding affinity for nematode β-tubulin than mammalian β-tubulin, making them safe for use in horses and other mammalian species (Lacey 1988).

Benzimidazole resistance mechanisms are the most well-studied of the anthelmintic classes because of their rapid development in Clade V nematodes, in particular *Haemonchus contortus*, just 3 years after its introduction to the market (Drudge et al. 1964; Kotze and Prichard 2016). Benzimidazole resistance is associated with mutations in isotype-1 and isotype-2 β-tubulin genes that decrease the binding affinity of the drug for its target (Lubega and Prichard 1990; Lacey and Gill 1994; von Samson-Himmelstjerna et al. 2007a). There are a few single nucleotide polymorphisms (SNPs) that are associated with benzimidazole resistance in *H. contortus*, with phenylalanine to tyrosine substitution at codon 200 (F200Y) in the isotype-1 β-tubulin gene being the most common in wild type parasite populations (Kotze and Prichard 2016). Other mutations in isotype-1 β-tubulin linked to benzimidazole resistance include F167Y and E198A, with the former being quite rare and the latter conferring the highest level of resistance of the three (Ghisi et al. 2007; Kotze et al. 2012). Limited research regarding isotype-2 β-tubulin genes has been performed, but some

resistant populations of *H. contortus* show loss or decreased levels of the gene (Beech et al. 1994; Lubega et al. 1994).

Benzimidazoles are still an effective anthelmintic for the treatment of *Parascaris* spp. infections and resistance has only been reported in three studies beginning in 2014 (Table 1). Due to this limited emerging anthelmintic resistance, only a few studies have examined the resistance-related SNPs or transcriptional responses to benzimidazoles in *Parascaris* spp. Five studies have sequenced *Parascaris* spp. β-tubulin genes, including one using a known benzimidazole-resistant isolate, and none found any known resistance-related SNPs, suggesting a potentially different mechanism of resistance in ascarid parasites to this anthelmintic class (Tydén et al. 2013a, 2014; Malekpour et al. 2019; Martin et al. 2021b; Özben et al. 2022). Interestingly, it has been shown that isotype-1 and isotype-2 β-tubulin genes are expressed at higher levels in *Parascaris* spp. eggs, and while isotype-1 remains at similar levels of expression in larvae and adults, isotype-2 gene expression is very low in adults, suggesting differing functions throughout the life cycle (Tydén et al. 2016). Additionally, in vitro exposure to benzimidazoles significantly increased gene expression of isotype-1 β-tubulin genes in one study using eggs (Tydén et al. 2016), whereas in vitro studies using adult parasites showed either downregulation of isotype-2 β-tubulin (Martin et al. 2020) or no differential expression of β-tubulin genes (Scare et al. 2020). Previously discussed enzymes aiding in the removal of xenobiotic compounds, as well as genes related to detoxification, microtubule polymerization, regulation of membrane potential, and muscle contraction were also differentially expressed following in vitro exposure to benzimidazoles (Martin et al. 2020; Scare et al. 2020).

The β-tubulin genes targeted by benzimidazoles are different even within Clade V nematodes (Saunders et al. 2013), and resistance-related β-tubulin SNPs have a low frequency in benzimidazole-resistant equine cyathostomins, another Clade V parasite group, suggesting that they may not fully describe benzimidazole resistance even within the clade (Pape et al. 2003; von Samson-Himmelstjerna et al. 2003, 2007a; James et al. 2009). Considering these dissimilarities within Clade V and the lack of identification of known resistance-related SNPs in benzimidazole-resistant *Parascaris* spp., it is possible that the mechanism of resistance in Clade III ascarid-type nematodes is different and thus using these SNPs for anthelmintic resistance surveillance is inadvisable (Diawara et al. 2009, 2013; Rashwan et al. 2017; Zuccherato et al. 2018; Palma et al. 2020).

## Tetrahydropyrimidines

The tetrahydropyrimidines include two formulations of pyrantel using different salts: pyrantel pamoate and pyrantel tartrate. Drugs in this class act as agonists of acetylcholine

receptors (AChRs) and cause them to stay open, leading to prolonged muscle contraction and paralysis in the parasites (Harrow and Gration 1985; Robertson et al. 1994). Nicotinic acetylcholine receptors are ligand-gated ion channels activated by acetylcholine, a neurotransmitter, and are made up of five subunits surrounding a central pore (Beech and Neveu, 2015). The AChR repertoire of parasitic nematodes is not widely studied, with only a few subtypes having been described in nematodes in general, and even fewer when the scope is narrowed to *Parascaris*. Recently, a *Parascaris* ACR-16 receptor subunit was described, and it was found that parasitic nematodes have two AChR subunits, ACR-26 and ACR-27, that are not found in free-living nematodes (Courtot et al. 2015; Charvet et al. 2018). In *Parascaris* spp., these two subunits have a higher affinity for pyrantel than acetylcholine (Courtot et al. 2015).

*Parascaris* spp. resistance to pyrantel has only been reported in seven studies globally starting in 2007, and thus has limited research due to the lack of resistant parasite populations (Table 1). There has, however, been one in vitro study investigating transcriptional responses in *Parascaris* spp. when exposed to pyrantel, ivermectin, and thiabendazole (Martin et al. 2020). Expression of eight transcripts orthologous to AChR was differentially expressed, but with no clear pattern between drug classes (Martin et al. 2020). Differential expression was also found in genes coding for enzymes that aid in the removal of xenobiotic compounds including short-chain dehydrogenases/reductases and flavin-containing monooxygenases, but these enzymes have not been characterized in parasitic nematodes and thus more research must be completed to understand their possible involvement in anthelmintic resistance (Martin et al. 2020).

## Macrocyclic lactones

Macrocyclic lactones are a group of drugs derived from avermectin produced by *Streptomyces avermitilis* (Campbell et al. 1983; Kim and Goodfellow 2002) or milbemycins produced by *S. hygroscopicus* (Takiguchi et al. 1980) or *S. cyaneogriseus* (Carter et al. 1988) and consist of some of the most well-known and widely used anthelmintics in the world. The avermectin derivatives—particularly ivermectin—have had a large impact in both veterinary and human medicine and the 2015 Nobel Prize in Physiology or Medicine was awarded to William C. Campbell and Satoshi Ōmura for its discovery (Nobel Prize 2015). Ivermectin was first introduced in 1981, and by the end of the decade, it was the best-selling animal health product in the world (Laing et al. 2016). Moxidectin, a milbemycin derivative, was introduced in the mid-1990s and has a longer half-life and higher potency than ivermectin (Lyons et al. 1992; Afzal et al. 1997). Macrocyclic lactones irreversibly activate glutamate-gated chloride channels (GluCls) that are present in nematode neuron and muscle cells, inhibiting neuronal and muscle activity and ultimately causing paralysis and death (Wolstenholme 2012; Laing et al. 2016). Within nematodes, even those that are within the same clade such as *Caenorhabditis elegans* and *Haemonchus contortus*, GluCls are highly divergent, making comparisons between species, let alone clades, difficult when studying both the mechanism of action and development of resistance (Laing et al. 2016).

Macrocyclic lactone resistance was first reported in *Haemonchus contortus* in 1987—just 6 years after ivermectin hit the market—and continued to spread globally (Carmichael et al. 1987; Van Wyk et al. 1987; Prichard 1994). Despite widespread anthelmintic resistance to ivermectin in some species of nematode parasites, the mechanism for resistance remains poorly understood. Similar to benzimidazole resistance, ivermectin resistance has been studied in *H. contortus*, as well as in *Caenorhabditis elegans* (reviewed in Lespine et al. 2011; Doyle and Cotton 2019), but little research has been conducted in ascarid parasites. P-glycoproteins (Pgp) are cell membrane efflux proteins that pump foreign substances out of cells and were first associated with ivermectin resistance in parasitic nematodes in the late 1990s (Xu et al. 1998). Subsequently, they are one of the most widely studied putative mechanisms for macrocyclic lactone resistance and the only one that has been studied in *Parascaris* spp. Similar to benzimidazole resistance, the bigger picture is complex. Macrocyclic lactone resistance is likely multigenic (Choi et al. 2017; Khan et al. 2020) and the molecular mechanism is not fully understood (Laing et al. 2016; Rezansoff et al. 2016).

Ten *Parascaris* spp. Pgps have been identified to date, along with their tissue-specific expression levels and some evidence for interaction with ivermectin: *Pun*-Pgp-2, *Pun*-Pgp-3, *Pun*-Pgp-9, *Pun*-Pgp-10, *Pun*-Pgp-11.1, *Pun*-Pgp-11.2, *Pun*-Pgp-12, *Pun*-Pgp-16.1, *Pun*-Pgp-16.2, and *Pun*-Pgp-18 (Janssen et al. 2013; Chelladurai and Brewer 2019; Gerhard et al. 2020; Martin et al. 2021a). Their role in anthelmintic resistance, however, is unclear. Transgenic expression of *P. univalens Pun*-Pgp-9 and *Pun*-Pgp-11 in *Caenorhabditis elegans* decrease susceptibility to ivermectin (Janssen et al. 2015), and *Pun*-Pgp-9 does so in a tissue-specific manner, with intestinal expression conferring a protective effect, and depends on active ingestion via pharyngeal pumping (Gerhard et al. 2021). Comparisons between ivermectin resistant and susceptible *Parascaris* spp. populations revealed the presence of SNPs in *Pun*-Pgp-11 and increased *Pun*-Pgp-11 mRNA levels correlating to decreased macrocyclic lactone susceptibility (Janssen et al. 2013), but drug exposure assays showed no change in Pgp expression in response to ivermectin exposure (Gerhard et al. 2020; Scare 2020; Martin et al. 2021a). Differentially expressed genes for enzymes aiding in the removal of xenobiotic compounds and other cellular processes were similar to those

previously described for other drugs after ivermectin exposure, with the only exception being the upregulation of a gamma-aminobutyric acid subunit (Martin et al. 2020; Scare et al. 2020). While these studies suggest some potential candidates for ivermectin resistance mechanisms, it is similar to the other two previously discussed drug classes in that mechanisms for resistance are unclear.

## Novel anthelmintics

Several novel anthelmintic candidates have been tested for *Parascaris* spp., many of them involving plant extracts. Wild tarragon (*Artemisia dracunculus*, Linnaeus, 1753), pennyrile (*Mentha pulegium*, Linnaeus, 1753), *Zataria multiflora* (Boiss), cinnamon (*Cinnamomum zeylanicum*, Blume), pomegranate flower (*Punica granatum*, Linnaeus, 1753), and pepper (*Capsicum annuum*, Linnaeus, 1753) extracts were all lethal to L2/L3 larval *Parascaris* in vitro (Rakhshandehroo et al. 2016, 2017). Zinc oxide nanoparticles showed in vitro anthelmintic efficacy against *Parascaris* spp., including changes to morphological appearance (Morsy et al. 2019). The monoterpenic phenol isomer carvacrol, isolated from herbs, also showed in vitro anthelmintic activity against *Parascaris* spp. by inhibiting acetylcholine-induced currents and stopping muscle contractions, suggesting that it is an antagonist of AChRs similar to pyrantel (Trailovic et al. 2021). The *Bacillus thuringiensis* (Berliner, 1915) crystal protein Cry5B has shown efficacy against *Parascaris* spp. when administered to foals via nasogastric tube, dropping fecal egg counts to zero, and is the only in vivo experimental drug efficacy study that has been completed recently (Urban et al. 2021). While these treatments have shown some efficacy, there is little information regarding the mechanisms of action, which will be an essential piece of information if they make it to the commercial market in order to help prevent the development of resistance.

## Conclusions

Anthelmintic resistance mechanisms are poorly understood in helminths, particularly ascarid parasites due to *Parascaris* spp. being the only ascarid exhibiting widespread anthelmintic resistance. Limited research suggests that mechanisms of resistance in Clade III ascarids may be different from those in Clade V members such as strongylid parasites and *C. elegans*, and applying information learned from these Clade V nematodes to ascarids must be done cautiously. Continued research on resistance mechanisms in ascarid parasites using *Parascaris* spp. is important for the understanding of genetic and molecular mechanisms of resistance in order to preserve anthelmintics currently used in other ascarid species and develop new treatment options for the future.

The recent development of egg-hatching larval culture methods for *Parascaris* spp. makes it a prime candidate for research regarding anthelmintic resistance in ascarid-type parasites, although in vitro systems have limitations such as short *Parascaris* spp. lifespan, differences in gene expression between life stages due to culture conditions, and specimen acquisition. Continued improvement of in vitro systems for both adults and hatched L2/L3 larvae, particularly increasing lifespan in culture and decreasing stress by optimizing culture conditions, would not only increase the quality of data collected but also allow for a larger number of studies to be completed.

Open access data repositories such as GenBank and WormBase Parasite are used by scientists globally for various research applications, and it is important that the information is updated as necessary. *Parascaris* spp. is an example of the issues that incorrect submissions can cause due to mislabeled accessions. Samples must be properly identified prior to genetic analysis and submission, as illustrated by the confusion between *P. univalens* and *P. equorum*. This mislabeling and lack of identification of species is not only something that needs to be rectified in date repositories, but also serves as a warning regarding proper specimen identification and labeling. Additionally, the *Parascaris* species discrepancy highlights the importance of developing a molecular method for *Parascaris* species identification.

**Author contribution** Jennifer L. Cain conceptualized the work, performed the literature search and data analysis, and drafted and revised the work. Martin K. Nielsen revised the work and acted as supervisor.

## Declarations

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