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Long live the worms: Methods for maintaining and assessing the viability of intestinal stages of
Parascaris spp. *in vitro*

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Longevity and viability of *Parascaris* spp. *in vitro*

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27

28 **Abstract**

29 *In vitro* maintenance of helminth parasites enables a variety of molecular, pharmaceutical, and
30 immunological analyses. Currently, the nutritional and environmental *in vitro* requirements of the
31 equine ascarid parasite, *Parascaris* spp., have not been determined. Additionally, an objective method
32 for assessing viability of *Parascaris* spp. intestinal stages does not exist. The purpose of this study was to
33 ascertain the *in vitro* requirements of intestinal stages of *Parascaris* spp., and to develop a viability
34 assessment method. A total 1045 worms were maintained in a total of 212 cultures. Worms obtained
35 from naturally infected foals at necropsy were immediately placed in culture flasks containing 200 mL of
36 culture media. A variety of media types, nutrient supplementation, and environmental conditions were
37 examined. A motility-based scoring system was used to assess worm viability. Worms maintained in
38 Roswell Park Memorial Institute- 1640 had significantly better viability than any other media ($p<0.0001$)
39 and all media types supplemented with any of the nutrients examined ($p<0.0001$). The use of a platform
40 rocker also significantly improved viability ($p=0.0305$). This is the first study to examine the
41 requirements for maintaining *Parascaris* spp. intestinal stages *in vitro* and to evaluate their viability
42 based on movement using an objective scoring system.

43 **Keywords:** *Parascaris*, *in vitro*, *helminth*, *maintenance*, *ascarid*, *viability*

44 **Key Findings**

- 45 • Identified *in vitro* maintenance requirements for *Parascaris* spp. intestinal stages
- 46 • Novel approach to assess viability of intestinal stages of *Parascaris* spp.
- 47 • *Parascaris* spp. had significantly better viability when maintained in RPMI media
- 48 • Glucose and CO₂ did not significantly affect worm viability
- 49 • Female worms had significantly better viability than males and L₄s

1. Introduction

Parascaris spp. is a clinically important helminth parasite infecting foals (Clayton and Duncan, 1978; Cribb *et al.* 2006; Tatz *et al.* 2012) with anthelmintic resistance reported world-wide (Peregrine *et al.* 2014). Anthelmintic resistance has not been described for other mammalian ascarid species. The ability to maintain gastro-intestinal helminths *in vitro* would enhance the experimental tractability of nematode parasites by facilitating the application of a range of molecular and biochemical tools and analyses in clinically relevant species. Such an advance would prompt a paradigm shift in parasitology research permitting progress in key areas including evaluation of anthelmintics and natural products with anthelmintic properties, (Rapson *et al.* 1985; Brownlee *et al.* 1997; O'Grady and Kotze, 2004; Jansen *et al.* 2013), application of transcriptomics to investigate the genetic mechanisms driving anthelmintic resistance (Jansen *et al.* 2013), induction of RNAi interference for the identification of novel drug targets (McCoy *et al.* 2015), analysis of excretory and secretory products (Young *et al.* 1995; Geldhof *et al.* 2000, Islam *et al.* 2004; Cribb *et al.* 2006; Burk *et al.* 2014; Thomas *et al.* 2016), and interrogation of host-parasite interactions (Kotze and McClure, 2001).

Most of the literature on *in vitro* culture and maintenance of ascarid parasites has focused on the pig nematode, *Ascaris suum*, where a variety of culture conditions have been employed. Some reports describe *in vitro* maintenance of larval stages (Douvres and Urban, 1983, 1986), however the size and nutrient requirements of the intestinal stages introduce new challenges to *in vitro* maintenance. Chehayeb *et al.* (2014) maintained adult *A. suum* collected from the small intestine of pigs for 24 hours in Locke's solution where glucose was provided as the main nutrient. Weisblat and Russel (1976) described culturing *A. suum* in artificial perienteric fluid (APF), and Brownlee *et al.* (1997) maintained worms in APF for five days. Islam *et al.* (2004) maintained adult *A. suum* under both aerobic and anaerobic conditions to observe changes in proteome expression patterns. Worms were maintained in Roswell Park Memorial Institute- 1640 (RPMI-1640) medium, and viability was maintained in both

74 systems for over two weeks. Dmitryjuk *et al.* (2014) sustained adult *A. suum* in *Ascaris* ringer's solution
75 (ARS) for 20 hours without any nutrient, while McCoy *et al.* (2015) maintained *A. suum* for eight days in
76 ARS without any nutrient. In contrast, only two studies have reported the *in vitro* maintenance of
77 *Parascaris* spp. Burk *et al.* (2014) reported culturing of larval stages and maintenance of adult stages to
78 investigate the production of excretory-secretory products. In that study, two adult worms were
79 maintained in RPMI-1640 medium at 37°C for five days. Jansen *et al.* (2013) maintained an undisclosed
80 number of adult worms in APF for 30 hours at 37°C for *in vitro* ivermectin exposure. No attempts have
81 been made to evaluate the requirements for long term *in vitro* maintenance of *Parascaris* spp. intestinal
82 stages, nor to characterize their preferred environment and nutrient requirements.

83 In order to determine the optimum *in vitro* requirements and monitor the effects of *in vitro* drug
84 exposure, it is necessary to ascertain helminth longevity and viability. *In vitro* evaluation of anthelmintic
85 efficacy in adult worms has been done by determining worm longevity by classifying them on an alive or
86 dead basis (Egualé *et al.* 2007a,b; Hu *et al.* 2013). While Hu *et al.* (2013) implemented a scoring system
87 on a 0-3 scale to assess worm movement, it was still largely subjective and the results considered worms
88 only on an alive (score 1-3) or dead (score 0) basis. Similarly, Richards *et al.* (1995), described a simple
89 method to monitor drug sensitivity of *Necator americanus* and *Ancylostoma caninum* based on the
90 observation of worm motility of treated versus control worms. Worms were characterized as either
91 active or inactive after gentle prodding. Neither the method proposed by Hu *et al.* (2013) nor Richards *et*
92 *al.* (1995) allows for the objective evaluation of worm viability over a series of time points. A similar
93 subjective method was reported by Dmitryjuk *et al.* (2014) to monitor the effects of *in vitro* anthelmintic
94 exposure to adult *A. suum*. Later, a motility assay was developed by O'Grady and Kotze (2004) that
95 utilized a scoring system to monitor anthelmintic efficacy against *Haemonchus contortus*. While the
96 scoring system allows one to observe a decline in viability over time, the definition of each score is
97 subjective as scores are assigned based on the investigators definition of significant movement, and a

set amount of time for each observation was not described. Marcellino *et al.* (2012) developed the WormAssay, a high throughput screening method to assess the anthelmintic efficacy against macroparasites based on motility. The WormAssay uses an open source computer software program and a camera to automatically assess worm movement and provide a quantitative measurement. Worms must be placed in microtiter plates, and the system is compatible with plates of either 6, 12, 24, 48, or 96 wells. The *Parascaris* species, however, are still too large for the well plates used in this system. Even the largest wells (6-well plate) measuring approximately 3.48 cm in diameter are not large enough for a mature *Parascaris* spp., which are commonly over 10 cm long (Clayton and Duncan, 1978). The Worminator uses a similar method but is specifically designed for determining the motility of microscopic nematode stages (Storey *et al.* 2014).

The purpose of this study was to characterize appropriate *in vitro* conditions for maintaining intestinal *Parascaris* spp., and to establish a scoring system to monitor worm viability over several time points.

2. Materials and Methods

2.1 Parasite sources

The study took place over the course of eight foal necropsies from October 2016 to October 2017. The foals were born in a herd housed at the University of Kentucky that has not been treated with any anthelmintics since 1979 and has been documented to harbor a variety of equine parasites through natural infection (Lyons *et al.* 1990). The foals employed in the study consisted of five colts and three fillies. Foals were humanely euthanized when they reached 4.5-5 months old and subsequently necropsied. The research was conducted following approval from the University of Kentucky's Institutional Animal Care and Use Committee (IACUC) under protocol number 2012-1046.

121

122 2.2 Study Design

123 During the first phase of this study (necropsies 1-3) worms were monitored on an alive/dead
124 basis in order to make initial observations on the necessary conditions for *in vitro* maintenance and
125 nutrient requirements of *Parascaris* spp. specimens. The second phase (necropsies 4-8) commenced
126 following the development of a scoring system to objectively assess the viability of *Parascaris* spp.
127 specimens under various environmental and nutrient conditions.

128 A variety of different media types nutrient supplements and environmental conditions were
129 examined (see Section 2.4.1 and 2.4.2). The number of worms evaluated for each media, nutrient and
130 environmental condition (CO₂ and platform rocker) is described in Table 1.

131

132 2.3 Collection of *Parascaris* spp.

133 Following necropsy, the small intestine was detached from the stomach and cecum. The
134 intestinal contents were milked out onto a 425 μ mesh sieve. Room temperature (RT) tap water was
135 slowly added to the sieve to dilute the contents to better visualize the worms. Intestinal stages of
136 *Parascaris* spp. (adult and fourth larval stage, L₄) specimens were recovered using a spay hook and
137 placed in a container of RT media of either ARS (see Table 2 for composition) (necropsies 1-6) or RPMI-
138 1640 (R8758, Sigma-Aldrich, St. Louis, MO, US) (necropsies 7 and 8). The container was placed into a
139 water bath maintained at 37°C for transport to the laboratory. Worms were classified as adult or L₄, and
140 adult worms were further characterized by sex. Worms were considered adults when gonads were
141 visible as white material in the mid-section of the worm. Males were differentiated from females by
142 being smaller and having less gonad material than females, and occasionally presented with a curved
143 hook in the tail. Immature worms (L₄) did not have any visible gonad material.

144

145 2.4 *In vitro* maintenance of *Parascaris* spp.

146 Worms were maintained in vented TPP tissue culture flasks (300 cm², MidSci, St. Louis, MO)
147 containing 200 mL of the pre-assigned medium. Media were changed every 12 hours. This was done by
148 placing a cell strainer of 400 µm pore size (pluriSelect Life Science, Leipzig, Germany) over the mouth of
149 the flask and allowing the old media to flow through while keeping the worms in the flask to limit
150 handling and subsequent damage. New media, pre-warmed to 37°C, were then added to the flask. The
151 flasks were kept in the pre-determined incubator with or without CO₂ (5%) supplementation at 37°C.

152 In the first phase of the study (necropsies 1-3) worms were maintained in groups of four or five,
153 containing two males and at least one female and one L₄ worm. In the second phase of the study
154 (necropsies 4-8) a total of five worms were placed in each culture flask consisting of either two males,
155 one female, and two immatures, or three males, one female, and one L₄ worm. The variation in worm
156 stage/sex within each cohort was due to the number of worms per category collected at each necropsy.

157

158 2.4.1 *Preparation of culture media*

159 Media (ARS, APF, ARS 3x Tris, APF 2x NaCl, physiological saline (PS) (Hospira Inc, Lake Forest, IL,
160 US), homemade physiological saline (HMPS), and RPMI-1640; see Table 2) were freshly prepared, stored
161 at 4°C, and then warmed to 37°C prior to adding to the culture flasks. Streptomycin (1mg /1L), Penicillin
162 (1000 U/1L) and Amphotericin-B (10 µg/1L) were added to all media types, except when *Escherichia coli*
163 was added as a nutrient (see 2.4.2). All media types were employed within 24 hours of preparation.

164

165 2.4.2 *Nutrient supplementation*

A list of the nutrients and their respective concentrations can be found in Table 2. *Escherichia coli* OP50 (University of Kentucky) was prepared in the following manner. LB (lysogeny broth) (Miller formulation, ThermoFisher Scientific, Waltham, MA) and LB-agar (Fisher Scientific, Hampton, NH) were prepared according to the manufacturer's instructions. *Escherichia coli* OP50 (University of Kentucky) were cultured in 15 mL of LB broth overnight at 37°C in a shaking incubator at 225 rpm. Following incubation, cells were pelleted by centrifugation at 3220g for eight minutes. After centrifugation, the supernatant was decanted and pelleted. *E. coli* were re-suspended in 15 mL of filter-sterilized culture media. Colony forming units (CFUs) were determined for the *E. coli* suspension by plating ten-fold serial dilutions to determine the starting culture concentration (*i.e.* input). The remaining suspension was equally divided and added to the assigned flasks. One flask was kept without worms as a control. Prior to the media changes, an aliquot of the media from the culture flasks, including the flask without worms, was plated to determine the final concentration (*i.e.* output) of surviving *E. coli*.

2.4.3 Environmental conditions

The environmental conditions assessed were the use of a 5% CO₂ incubator and platform rocker. The number of flasks assigned to each condition can be found in Table 1. Pre-assigned flasks were placed in a 5% CO₂ incubator at 37°C for the entirety of their survival. Flasks assigned to the platform rocker (Hofer Scientific Instruments, San Francisco, CA model PR70) were maintained at approximately 60 rpm within the air-only incubator at 37°C for the entirety of their survival.

2.5 Longevity and viability assessment of *Parascaris* spp.

For the first phase of the study (necropsies 1-3), worms were monitored on an alive or dead basis and the number of worms surviving per flask at each time point/media change was recorded (*i.e.* longevity). Worms were considered dead when they became flaccid and/or displayed signs of decay. Flaccidity was determined by placing the worm over a pair of forceps at midpoint and carefully lifting it out of the medium. If the worm draped loosely over the forceps and appeared as an acute angle, it was considered flaccid. Decay was noted visually and determined as breakdown of the exterior cuticle. The second phase of the study (necropsies 4-8) began with the development of an objective scoring system to monitor worm viability. Prior to each medium change, worm viability was assessed and awarded a score according to the descriptions in Table 3. Each worm was observed for 15 seconds for movement while remaining in the flask. If no movement occurred during the 15 second observatory period, forceps were used to gently stimulate the worm in an attempt to initiate movement. If still no movement was observed, the forceps were used to assess flaccidity and check for decay as previously described. Dead worms were removed from the flask and discarded.

2.6 Statistical Analyses

2.6.1 Phase one: Longevity

For the first phase of the study (necropsies 1-3), a percent reduction in the number of worms in each flask was calculated at each time point. The final time of longevity was considered when all worms in a flask had died. Mean longevity with 95% confidence intervals (CI), and the range for media, nutrient, and incubator type were calculated using Microsoft Excel 2016 (Redmond, WA, USA). These values can be found in Table 4.

Further statistical analyses were performed using SAS software (version 9.4, SAS Institute, Cary, North Carolina, USA). Here, four mixed linear models with repeated measures across time were

constructed to determine which media, nutrient supplementation profile, and incubator type significantly affected worm longevity. 'Percent loss' was the response variable for all analyses. The first model assessed the longevity of worms maintained in the different media types without nutrient supplementation or CO₂ incubator. The covariates were 'Time' and the interaction term 'media ID*none', where 'none' implied an air incubator and no nutrients were used. 'Necropsy date' was kept as a random effect. The second analysis was used to analyze the supplementation with glucose in all types of media because it was the only nutrient tested across all media types. The interaction term 'Media ID*glucose' was the covariate analyzed and 'necropsy date' was kept as the random effect. The third model examined worm longevity when maintained in ARS media supplemented with either glucose, gelatin, *E. coli*, yeast, FBS, cholesterol, or gelatin and glucose. ARS was the only medium supplemented with all the nutrients and therefore was the only medium examined in this model. 'Nutrient' and 'time' were the covariates examined. 'Necropsy date' and 'CO₂' were kept as random effects. The fourth model examined the use of the CO₂ incubator across all media and nutrient supplements. The covariates examined were 'time' and 'CO₂'. 'Necropsy date', 'Media ID' and 'nutrient' were kept as random effects. The fifth analysis analyzed the stage (L₄ or adult) and sex (adult worms only) over time, regardless of media, nutrients used, or the use of the CO₂ incubator. The covariates analyzed were 'stage' and 'sex'. 'Media ID' and 'necropsy date' were kept as random effects. Any time a significant covariate ($\alpha=0.05$) was observed, a 'least squares means' analysis was performed for a Tukey's pair-wise comparison.

2.6.2 Phase two: Viability

For the second phase of the study (necropsies 4-8), the scoring system (see Table 3) was used to monitor worm viability. Mean worm viability per flask at each time point was calculated. Worms that

had died continued to receive a score of zero and were included in the mean calculation until all the worms within the same flask had died. Mean values and 95% confidence intervals (CI) were calculated using Microsoft Excel 2016 (Redmond, WA, USA). The percent viability per flask was calculated in Microsoft Excel for each time point using the following formula, where 'X' refers to each time point:

$$\% Viability = 100 - \left(\frac{(initial\ score - score\ at\ time\ 'X')}{initial\ score} \times 100\% \right)$$

Further statistical analyses were performed using SAS software (version 9.4, SAS Institute, Cary, North Carolina, USA). Here, a total of six mixed linear models with repeated measures across time were performed to determine which media, nutrients, and environmental conditions significantly affected worm viability. For all models, 'percent viability' was the response variable. The first model assessed the viability of worms maintained in the different media without nutrient supplementation, CO₂ incubator, or platform rocker. The covariates were 'time' and the interaction term 'media ID*none', where 'none' implied that no nutrients or environmental conditions were implemented. 'Necropsy date' was kept as a random effect. The second model analyzed worm viability when maintained in one of the saline-based media (*i.e.* ARS, APF, ARS 3x Tris, APF 2x NaCl, PS, HM PS) with glucose compared to worm viability maintained in the same saline-based media without glucose. Glucose was the only nutrient added across all saline-based media types and therefore was the only nutrient analyzed in this model. The covariates examined were 'time' and the interaction term 'media ID*glucose'. 'Necropsy date' was kept as a random effect. The third model examined worm viability when maintained in APF media supplemented with either glucose, FBS, cholesterol, a combination of FBS and cholesterol, Tween only control, or as a no nutrient control. APF was the only medium supplemented with all the nutrients and therefore was the only medium examined in this model. 'Nutrient' and 'time' were the covariates examined. 'Necropsy date' and 'environment' (*i.e.* CO₂ incubator or platform rocker) were kept as random effects. The fourth model examined the use of the platform rocker and CO₂ incubator across all media and nutrient

supplements. The covariates examined were 'time' and 'environment'. 'Necropsy date', 'Media ID' and 'nutrient' were kept as random effects. The fifth model analyzed the use of RPMI against all media, nutrients, and environmental conditions. The covariate tested was 'RPMI,' and 'necropsy date' was kept as random effect. The last model analyzed the stage (L₄ or adult) and sex (adult worms only) over time, regardless of media, nutrients used, or the use of the CO₂ incubator or platform rocker. The covariates analyzed were 'stage' and 'sex'. 'Media ID', and 'necropsy date' were kept as random effects. Any time a significant covariate ($\alpha=0.05$) was observed, a 'least squares means' analysis was performed for a Tukey's pair-wise comparison.

3. Results

A total of 212 cultures were performed and a total of 1045 *Parascaris* spp. worms were used. The number of cultures and worms per media type, nutrient supplementation, and environmental condition (incubator type and/or platform rocker) can be found in Table 2.

3.1 Phase one: Longevity

For the first phase of the study pertaining to worm longevity (necropsies 1-3), a total of 210 worms were used consisting of 98 adult males, 54 adult females, and 58 L₄s. During this phase of the study, the worms lived a maximum of 84 hours. The media type employed when considered without nutrient supplementation or CO₂ did have a significant effect on worm longevity ($p=0.0100$), however the least squares means pairwise comparison did not identify any significant differences between media. ARS was the only media type significantly affecting worm viability with the addition of glucose. Worms maintained in ARS supplemented with glucose lived significantly longer than worms maintained

in ARS alone ($p < 0.0001$). There were no significant differences observed in any of the other media types supplemented with glucose compared to when glucose was not added. Regarding the various types of nutrient supplementation with the ARS media, worms maintained with glucose ($p < 0.0006$) or a combination of glucose and gelatin ($p < 0.0001$) had significantly better longevity than worms maintained without any nutrient. Worms maintained with glucose had significantly better longevity than worms maintained with *E. coli* ($p = 0.0008$), yeast ($p < 0.0001$), FBS ($p = 0.0013$), or cholesterol ($p = 0.0279$). Similarly, worms maintained with a combination of glucose and gelatin had significantly better longevity than those maintained with gelatin only ($p = 0.0484$), *E. coli* ($p < 0.0001$), yeast ($p < 0.0001$), FBS ($p < 0.0001$), or cholesterol ($p = 0.0008$). The mean longevity, 95% confidence intervals, and range of longevity for the different nutrients and incubator type can be found in Table 4. The use of a CO₂ incubator did not significantly affect worm longevity ($p = 0.2854$). 'Adult male ($p = 0.0021$) and female ($p < 0.0001$) worms had significantly better longevity than immature worms, however there was no significant difference between males and females ($p = 0.5780$). The mean longevity, 95% confidence intervals, and range of longevity for immatures, males, and females can be found in Table 4.

3.2 Phase two: Viability

For the second phase of the study pertaining to worm viability (necropsies 4-8), a total of 835 worms were used, consisting of 350 adult males, 215 adult females, and 270 L₄s. The RPMI-1640 media resulted in significantly better worm viability than any of the other media ($p < 0.0001$) (Figure 1). APF 2x NaCl had significantly better viability than ARS ($p = 0.0002$). APF ($p = 0.0005$), ARS 3x Tris ($p = 0.0169$), and APF 2x NaCl ($p < 0.0001$) had significantly better viability than the homemade physiological saline. The addition of glucose to the saline-based media did not significantly affect worm viability compared to those maintained in the saline-based media without glucose ($p = 0.3048$). The addition of a nutrient to

the APF medium did significantly decrease worm viability ($p=0.0413$), however the least squares means pairwise comparison did not identify any significant differences (Figure 2). The use of the platform rocker resulted in significantly better worm viability than worms maintained without the rocker ($p=0.0305$), while there were no significant differences in worm viability between the use of an air or CO₂ incubator ($p=1.0000$) (Figure 3). Overall, worms maintained in RPMI-1640 had significantly better viability than worms maintained with any other method regardless of media, nutrient, or environmental condition ($p<0.0001$) (Figures 1 and 2). In regards to worm stage and sex, adult worms regardless of sex had significantly better viability than L₄s ($p<0.0001$) and females had significantly better viability than males ($p<0.0001$) across all media types, nutrient supplementation, and environmental conditions.

4.0 Discussion

This is the first study to determine the preferred *in vitro* conditions for the intestinal stages of *Parascaris* spp., and to describe a reliable and objective method for assessing their viability. Worm motility and the presence of muscle tone appears to be reliable indicator for assessing *in vitro* conditions. This study is the first to report a difference in *in vitro* worm viability for *Parascaris* spp. between L₄ and adult stages, as well as between male and female adult worms.

Intestinal stages of *Parascaris* spp. must be active swimmers against the flow of intestinal contents in order to maintain their position in the host and avoid being expelled by peristalsis (Drudge and Lyons, 1983). Therefore, worm responses to *in vitro* conditions should be judged based on activity level, where a decrease in activity likely reflects a decrease in overall worm viability. Other scoring systems for gastrointestinal nematodes have been developed, but these did not provide strict parameters of movement per score (Richards *et al.* 1995; O'Grady and Kotze, 2004). While *Parascaris* spp. intestinal stages are not compatible with the current size restrictions of the WormAssay (Marcellino

et al. 2012), a modification of this technique to accommodate larger macroparasites should be a target for future research.

The use of RPMI-1640 media resulted in significantly better worm viability than all other media types regardless of nutrient supplementation and/or environmental condition (Figures 1 and 2). Worms lived a maximum of 168 hours in RPMI-1640 (Figures 1 and 2), which is well above the 84 and 96 hours achieved in phase 1 and phase 2, respectively, with the addition of glucose (Table 3 and Figure 2). At this time, it is unknown which components of the RPMI-1640 media caused this improvement in viability and longevity, but it is likely due to the combination of vitamins and amino acids that were missing from the other media evaluated. This finding is in agreement with Urban *et al.* (1984) who found improved growth and survival of L₄ *A. suum* when cultured in RPMI-1640 rather than a saline medium supplemented with glucose.

The use of sugar (glucose or dextrose) as a nutrient is reported in several other studies maintaining adult stages of *A. suum* (Weisblat and Russel, 1976; Brownlee *et al.* 1997; Chehayeb *et al.* 2014), and one study used dextrose for maintaining adult *P. equorum* (Jansen *et al.* 2013). While it is assumed that sugar is necessary for the *in vitro* cultivation of *Ascaris* and *Parascaris* species, this had not previously been evaluated in a published study. In phases one and two of this study, *Parascaris* spp. survived a maximum of 84 and 96 hours, respectively, when glucose was added as a nutrient and it did not significantly affect worm viability. The success of the RPMI-1640, but not the glucose provides evidence that *Parascaris* spp. intestinal stages require different and/or additional nutrients beyond glucose for sustainment *in vitro*. It is interesting that *A. suum* can be maintained for eight days in ARS without any nutrient supplementation (McCoy *et al.* 2015). In the current study, *Parascaris* spp. did not live more than 168 hours in any of the media regardless of the media type or nutrient provided. This may suggest that adult *A. suum* and *Parascaris* spp. worms have very different nutrient and metabolic

requirements, however direct conclusions cannot be made at this time. A comparative study could be performed to determine the viability of *Parascaris* spp. and *A. suum* when supplemented with different nutrients, and analyses of the media after a nutrient has been provided could determine if the worms successfully ingested the nutrient. If so, the effectiveness of the worm to generate energy from the given nutrient could be assessed using metabolic techniques. Such findings would provide significant advances toward *in vitro* techniques of the parasitic stages.

Douvres and Urban (1983, 1986) described methods for culturing larval stages of *Ascaris* species utilizing various gaseous stages, including 5% CO₂. Several studies report the maintenance of adult *A. suum* worms without CO₂ (Weisblat and Russel, 1976; Brownlee *et al.* 1997; Chehayeb *et al.* 2014; McCoy *et al.* 2015). Jansen *et al.* (2013) maintained *P. equorum* adult worms without 5% CO₂ while Burk *et al.* (2014) cultured second and third larval stages of *P. equorum* under 5% CO₂ conditions, but not the adult worms. Based on these reports, it appears that adult worms may not require CO₂, but this had not been specifically evaluated for *Parascaris* spp. The current study did not find the use of 5% CO₂ to significantly affect worm longevity or viability (Figure 3). However, this study did not investigate the impact of CO₂ on worms maintained in RPMI-1640 and this should be evaluated in future studies.

The use of a platform rocker for *in vitro* maintenance of ascarid parasites had not been evaluated prior to this study. In this study, the use of the rocker significantly improved worm viability (Figure 3), however no firm conclusions can be made at this time. The platform rocker could not be tested simultaneously with CO₂ due to limited space in the incubator. Furthermore, this study did not evaluate RPMI-140 media with the use of the rocker, and this should be investigated in future studies.

It is also known that nematodes are unable to synthesize cholesterol *de novo* (Dutky *et al.* 1967; Cole and Krusberg, 1968), however this study did not find the addition of cholesterol to improve worm longevity or viability. Additionally, the addition of FBS did not significantly improve viability. These

findings are interesting because Urban *et al.* (1984) found the addition of cholesterol (50 µg/mL) and serum (10%) to RPMI-1640 to have an additive effect on the growth of L₄ *A. suum*. Urban *et al.* (1984) also found that an increase in cholesterol concentration to 250 µg/mL from 50 µg/mL reversed this effect. While the aforementioned study examined the development of larval stages, it is possible that a similar scenario was observed in the current study where the *Parascaris* spp. intestinal stages were negatively impacted by the cholesterol concentration examined herein. Future studies should investigate varying concentrations of cholesterol to determine if there is an optimum concentration and/or a tolerance threshold.

The varying sample sizes between the nutrient trials are a limitation to this study, particularly in regards to the number of worms used for evaluating the RPMI-1640 media and the saline-based medias supplemented with cholesterol, FBS, yeast, and *E. coli* (Table 1). Variations occurred due to the number of worms harvested at each necropsy. While the results of this study clearly support the recommendation for using RPMI-1640 for maintaining intestinal stages of *Parascaris* spp., the conclusions should be interpreted with caution and warrant further investigation. The effects of stocking density and keeping male, female, and immature worms together would also provide interesting points for future studies.

It is important to note that the *in vivo* immune responses exhibited by the foal prior to necropsy may also affect worm viability *in vitro*. Foals typically gain immunity to *Parascaris* spp. worms around nine months of age (Clayton and Duncan, 1979). Some response by the immune system to the present parasites is expected and it is unknown how the parasites were affected prior to harvest and culturing. This variability was controlled for by using foals which were all born into the same herd, and harvesting the worms when the foals were between 4.5-5 months of age which is the peak age for *Parascaris* spp. burden (Fabiani *et al.* 2016) and thus minimizing the potential influence of host immunity.

In summary, the scoring system proved to be a useful method for monitoring L₄ and adult worm viability *in vitro*, and should be considered for future studies. This study found RPMI-1640 media to significantly improve worm viability. The use of a 5% CO₂ incubator did not significantly affect worm viability, but a platform rocker significantly increased viability. The viability of adult worms was also significantly better than that of L₄s. Further investigations should be performed to examine the effects of a platform rocker and CO₂ incubator when RPMI-1640 is used as the culture media.

Conflict of Interest Statement

The authors declare no conflict of interest.

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Figure 1. A graphical representation of mean viability of *Parascaris* spp. intestinal stages when maintained in various media types (ARS: *Ascaris* Ringer's solution; APF: artificial perienteric fluid; ARS 3x Tris; ARS with triple the amount of Tris buffer; APF 2x NaCl: APF with double the amount of NaCl; PS: physiologic saline; HM PS: homemade physiologic saline, and RPMI: Roswell Park Memorial Institute). Error bars represent 95% confidence intervals ($\alpha=0.05$).

Figure 2. A graphical representation of mean viability of *Parascaris* spp. intestinal stages when maintained in either artificial perienteric fluid (APF) medium only, APF medium supplemented nutrients (glucose, fetal bovine serum (FBS), cholesterol, cholesterol and FBS, tween), or Roswell Park Memorial Institute- 1640 (RPMI-1640) medium only. Error bars represent 95% confidence intervals ($\alpha=0.05$).

Figure 3. A graphical representation of mean viability of *Parascaris* spp. intestinal stages maintained with environmental conditions of a platform rocker or a 5% CO₂ incubator across all media and nutrient types. 'None' implies stationary culture flasks in an air incubator. Error bars represent 95% confidence intervals ($\alpha=0.05$).

Table 1. Distribution of intestinal stages of *Parascaris* spp. specimens among the different media, nutrients, and environmental conditions (*i.e.* CO₂ incubator, platform rocker) for *in vitro* maintenance. The number of worms is listed followed by the number of cultures in parenthesis. The top table is from phase one of the study (necropsies 1-3) for initial observations regarding worm longevity. The bottom table is from phase two of the study (necropsies 4-8) when worm viability was assessed. Cultures were kept at 37°C.

Medium	None ^a	Glucose (5 mM)	Gelatin	Glucose & Gelatin*	Cholesterol (50µg/mL)	FBS (10%)	<i>E. coli</i> <i>OP50</i>	Yeast (1%)	CO ₂ (5%) Incubator	Glucose & CO ₂ *	Gelatin & CO ₂ *	Total*
ARS	30 (8)	28 (6)	10 (2)	8 (2)	15 (3)	15 (3)	10 (2)	15 (3)	17 (4)	4 (1)	5 (1)	140 (31)
APF	10 (2)	5 (1)	0	0	0	0	0	0	0	0	0	15 (3)
ARS 3x Tris	10 (2)	10 (2)	0	0	0	0	0	0	0	0	0	20 (4)
APF 2x NaCl	10 (2)	10 (2)	0	0	0	0	0	0	0	0	0	20 (4)
PS	10 (2)	5 (1)	0	0	0	0	0	0	0	0	0	15 (3)
Total	70 (16)	58 (12)	10 (2)	8 (2)	15 (3)	15 (3)	10 (2)	15 (3)	17 (4)	4 (1)	5 (1)	210 (45)

Medium	None ^a	Glucose (5 mM)	Cholesterol (50µg/mL)	FBS (10%)	Cholesterol & FBS*	Tween (5%) control	Platform rocker	CO ₂ (5%) Incubator	Glucose & CO ₂ *	Glucose & Rocker*	Total*
ARS	25 (5)	35 (7)	0	0	0	0	15 (3)	10 (2)	5 (1)	5 (1)	85 (17)
APF	65 (13)	195 (39)	60 (12)	60 (12)	30 (6)	20 (4)	15 (3)	90 (18)	85 (17)	5 (1)	505 (101)
ARS 3x Tris	25 (5)	35 (7)	0	0	0	0	15 (3)	10 (2)	5 (1)	5 (1)	85 (17)
APF 2x NaCl	15 (3)	35 (7)	0	0	0	0	15 (3)	5 (1)	5 (1)	5 (1)	70 (14)
PS	10 (2)	15 (3)	0	0	0	0	5 (1)	0	0	0	30 (6)
HM PS	10 (2)	10 (2)	0	0	0	0	5 (1)	0	0	0	25 (5)
RPMI-1640	35 (7)	0	0	0	0	0	0	0	0	0	35 (7)
Total*	185 (37)	325 (65)	60 (12)	60 (12)	30 (6)	20 (4)	70 (14)	115 (23)	100 (20)	20 (4)	835 (167)

^a 'None' implies an air incubator and no nutrient was used.

* Nutrient combinations with other nutrients or environmental conditions were not included in the total values because these were already accounted for in the individual nutrient, CO₂, and platform rocker columns.

Abbreviations: FBS, fetal bovine serum; ARS, ascaris ringers solution; APF, artificial perienteric fluid; ARS 3x Tris, ARS with triple Tris buffer concentration; APF 2x NaCl, APF with double NaCl concentration; PS, physiological saline (0.9% NaCl); HM PS, homemade physiological saline (0.9% NaCl); Roswell Park Memorial Institute- 1640, RPMI-1640

Table 2. Components of the media tested and nutrients provided for the *in vitro* maintenance for intestinal stages of *Parascaris* spp.

Concentration of media components (mM)							
Media	NaCl	CaCl ₂	MgCl ₂	KCl	NaC ₂ H ₃ O ₂	C ₄ H ₁₁ NO ₃ /Tris	pH ^c
ARS ^a	13.14	9.47	7.83	19.64	100	12.09	7.8
APF ^b	23	6	5	24	110	12.09	7.8
ARS 3x Tris	13.14	9.47	7.83	19.64	100	36	7.8
APF 2x NaCl	46	6	5	24	110	12.09	7.8
PS	154	0	0	0	0	0	NA
HM PS	154	0	0	0	0	0	NA
RPMI-1640 ^d	-	-	-	-	-	-	NA
Nutrient		Concentration		Manufacturer/Source			
(D+)-Glucose monohydrate		5 mM		Acros organics, Fischer Scientific, Hampton, NH)			
Food grade unflavored gelatin		2 g/L		Kroger, Cincinnati, OH			
<i>E. coli</i> OP ₅₀		8.55E+10 CFU/ml ^g		University of Kentucky			
FBS		10%		Millipore Sigma, St. Louis, MO			
Cholesterol ^e		50 µg/mL ^f		Millipore, Sigma, St. Louis, MO			
Yeast		1%		BD Biosciences, San Jose, CA			
Tween only control ^h							

^a McCoy et al., 2015

^b Weisblat and Russel, 1976

^c pH adjusted with Hydrochloric Acid, the pH was not adjusted for PS, HM PS, or RPMI-1640

^d The components remained as provided by the manufacturer (Millipore Sigma, St. Louis, MO)

^e Prepared as at 0.1% stock solution in 5% aqueous Tween 80 (Bolla et al. 1972)

^f Urban and Douvres, 1984

^g Average number of CFU calculated from all input concentrations

^h Included not as a nutrient, but as a control because cholesterol was prepared by dissolving it in 5% aqueous Tween 80 (Bolla et al. 1972).

Abbreviations: ARS, *Ascaris ringers* solution; APF, artificial perienteric fluid; ARS 3x Tris, ARS with triple the Tris buffer concentration; APF 2x NaCl, APF with double the NaCl concentration; PS, physiologic saline; HM PS, homemade physiologic saline; RPMI, Roswell Park Memorial Institute; CFU, colony forming units

Table 3. Scoring system used to assess the *in vitro* viability of *Parascaris* spp. intestinal stages. Scores were assigned following individual observation for 15 seconds.

Score	Description
0	Dead, no movement observed independently nor when stimulated with forceps. Lack of muscle tone/flaccid over forceps when lifted out of the solution. Signs of decay may be present.
1	No movement observed independently nor when stimulated with forceps. Muscle tone is apparent.
2	Movement only when stimulated with forceps.
3	Movement of head only without stimulation.
4	1-3 whole body movements without stimulation.
5	4-6 whole body movements without stimulation.
6	7 or more whole body movements without stimulation.

Table 4. Mean longevity of intestinal stages of *Parascaris* spp. *in vitro* with various nutrients and CO₂ incubator use, and of different stages and sex (necropsies 1-3). Worms were maintained in tissue culture flasks (300 cm²) in groups of four or five. All worms were kept in 200 mL of *Ascaris* ringer's solution and incubated at 37°C. The time of longevity was considered the hour when all worms in a flask were dead. Flasks were checked every 12 hours. 95% confidence intervals are included in parenthesis ($\alpha=0.05$).

Nutrient/Incubator	Mean Longevity (hours)	Range of longevity (hours)
None ^a	42 (34.7-49.3)	12-60
Glucose (5 mM)	72 (63.7-80.3)	48-84
Gelatin	72 (63.7-80.3)	60-84
Glucose & Gelatin	78 (69.7-86.3)	72-84
Cholesterol (50 µg/mL)	56 (49.6-62.4)	48-60
Fetal Bovine Serum (10%)	40 (33.6-46.4)	36-48
E. Coli OP50	36	36
Bacto Yeast Extract (1%)	36	36
5% CO ₂ Incubator	67.2 (54.6-79.8)	48-84
Glucose & CO ₂ Incubator	84	84
Gelatin & CO ₂ Incubator	60	60
Total	46 (42.4-49.7)	
Stage/Sex	Mean Longevity (hours)	Range of longevity (hours)
Immature	38 (34.9-41.2)	12-84
Male	43 (3.3-39.7)	24-84
Female	46.5 (42.3-50.7)	24-84

^a 'None' implies an air incubator and no nutrient was used.