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

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1 Long live the worms: Methods for maintaining and assessing the viability of intestinal stages of

2 *Parascaris* spp. *in vitro*

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

9

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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 i*n 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 CO_2 did not significantly affect worm viability
49 - Female worms had significantly better viability than males and L_4s

50 **1. Introduction**

51 *Parascaris* spp*.* is a clinically important helminth parasite infecting foals (Clayton and Duncan,
52 1978; Cribb *et al.* 2006; Tatz *et al.* 2012) with anthelmintic resistance reported world-wide (Peregrine *et*
53 *al.* 2014). Anthelmintic resistance has not been described for other mammalian ascarid species. The
54 ability to maintain gastro-intestinal helminths *in vitro* would enhance the experimental tractability of
55 nematode parasites by facilitating the application of a range of molecular and biochemical tools and
56 analyses in clinically relevant species. Such an advance would prompt a paradigm shift in parasitology
57 research permitting progress in key areas including evaluation of anthelmintics and natural products
58 with anthelmintic properties , (Rapson *et al.* 1985; Brownlee *et al.* 1997; O'Grady and Kotze, 2004;
59 Jansen *et al.* 2013), application of transcriptomics to investigate the genetic mechanisms driving
60 anthelmintic resistance (Jansen *et al.* 2013), induction of RNAi interference for the identification of
61 novel drug targets (McCoy *et al.* 2015) , analysis of excretory and secretory products (Young *et al.* 1995;
62 Geldhof *et al.* 2000, Islam *et al.* 2004; Cribb *et al.* 2006; Burk *et al.* 2014; Thomas *et al.* 2016), and
63 interrogation of host-parasite interactions (Kotze and McClure, 2001).

64 Most of the literature on *in vitro* culture and maintenance of ascarid parasites has focused on
65 the pig nematode, *Ascaris suum,* where a variety of culture conditions have been employed. Some
66 reports describe *in vitro* maintenance of larval stages (Douvres and Urban, 1983, 1986), however the
67 size and nutrient requirements of the intestinal stages introduce new challenges to *in vitro*
68 maintenance. Chehayeb *et al.* (2014) maintained adult *A. suum* collected from the small intestine of
69 pigs for 24 hours in Locke's solution where glucose was provided as the main nutrient. Weisblat and
70 Russel (1976) described culturing *A. suum* in artificial perienteric fluid (APF), and Brownlee *et al.* (1997)
71 maintained worms in APF for five days. Islam *et al.* (2004) maintained adult *A. suum* under both aerobic
72 and anaerobic conditions to observe changes in proteome expression patterns. Worms were maintained
73 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 (Eguale *et al.* 2007*a,*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

98 set amount of time for each observation was not described. Marcellino *et al.* (2012) developed the
99 WormAssay, a high throughput screening method to assess the anthelmintic efficacy against
100 macroparasites based on motility. The WormAssay uses an open source computer software program
101 and a camera to automatically assess worm movement and provide a quantitative measurement.
102 Worms must be placed in microtiter plates, and the system is compatible with plates of either 6, 12, 24,
103 48, or 96 wells. The *Parascaris* species, however, are still too large for the well plates used in this
104 system. Even the largest wells (6-well plate) measuring approximately 3.48 cm in diameter are not large
105 enough for a mature Parascaris spp., which are commonly over 10 cm long (Clayton and Duncan, 1978).
106 The Worminator uses a similar method but is specifically designed for determining the motility of
107 microscopic nematode stages (Storey *et al.* 2014).
108 The purpose of this study was to characterize appropriate *in vitro* conditions for maintaining
109 intestinal *Parascaris* spp*.,* and to establish a scoring system to monitor worm viability over several time
110 points.
111

112 **2. Materials and Methods**

113 *2.1 Parasite sources*

114 The study took place over the course of eight foal necropsies from October 2016 to October
115 2017. The foals were born in a herd housed at the University of Kentucky that has not been treated with
116 any anthelmintics since 1979 and has been documented to harbor a variety of equine parasites through
117 natural infection (Lyons *et al.* 1990). The foals employed in the study consisted of five colts and three
118 fillies. Foals were humanely euthanized when they reached 4.5-5 months old and subsequently
119 necropsied. The research was conducted following approval from the University of Kentucky's
120 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 (CO_2 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 425µ 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, L_4) 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 $L_{4,}$ 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 (L_4) 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 cm^2, 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 CO_2 (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 L_4 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 L_4 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*

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

178

179 *2.4.3 Environmental conditions*

180 The environmental conditions assessed were the use of a 5% CO_2 incubator and platform rocker.
181 The number of flasks assigned to each condition can be found in Table 1. Pre-assigned flasks were placed
182 in a 5% CO_2 incubator at 37˚C for the entirety of their survival. Flasks assigned to the platform rocker
183 (Hofer Scientific Instruments, San Francisco, CA model PR70) were maintained at approximately 60 rpm
184 within the air-only incubator at 37˚C for the entirety of their survival.

185

186 *2.5 Longevity and viability assessment of Parascaris* spp.

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

200

201 *2.6 Statistical Analyses*

202 *2.6.1 Phase one: Longevity*

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

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

210 constructed to determine which media, nutrient supplementation profile, and incubator type
211 significantly affected worm longevity. 'Percent loss' was the response variable for all analyses. The first
212 model assessed the longevity of worms maintained in the different media types without nutrient
213 supplementation or CO_2 incubator. The covariates were 'Time' and the interaction term 'media
214 ID*none', where 'none' implied an air incubator and no nutrients were used. 'Necropsy date' was kept
215 as a random effect. The second analysis was used to analyze the supplementation with glucose in all
216 types of media because it was the only nutrient tested across all media types. The interaction term
217 'Media ID*glucose' was the covariate analyzed and 'necropsy date' was kept as the random effect. The
218 third model examined worm longevity when maintained in ARS media supplemented with either
219 glucose, gelatin, *E. coli*, yeast, FBS, cholesterol, or gelatin and glucose. ARS was the only medium
220 supplemented with all the nutrients and therefore was the only medium examined in this model.
221 'Nutrient' and 'time' were the covariates examined. 'Necropsy date' and 'CO_2' were kept as random
222 effects. The fourth model examined the use of the CO_2 incubator across all media and nutrient
223 supplements. The covariates examined were 'time' and 'CO_2'. 'Necropsy date', 'Media ID' and 'nutrient'
224 were kept as random effects. The fifth analysis analyzed the stage (L_4 or adult) and sex (adult worms
225 only) over time, regardless of media, nutrients used, or the use of the CO_2 incubator. The covariates
226 analyzed were 'stage' and 'sex'. 'Media ID' and 'necropsy date' were kept as random effects. Any time a
227 significant covariate (α=0.05) was observed, a 'least squares means' analysis was performed for a
228 Tukey's pair-wise comparison.

229

230 *2.6.2 Phase two: Viability*

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

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

237 $$\%\,Viability = 100 - \left(\frac{(initial\ score - score\ at\ time\ \ 'X')}{initial\ score} \times 100\%\right)$$

238 Further statistical analyses were performed using SAS software (version 9.4, SAS Institute, Cary,
239 North Carolina, USA). Here, a total of six mixed linear models with repeated measures across time were
240 performed to determine which media, nutrients, and environmental conditions significantly affected
241 worm viability. For all models, 'percent viability' was the response variable. The first model assessed the
242 viability of worms maintained in the different media without nutrient supplementation, CO_2 incubator,
243 or platform rocker. The covariates were 'time' and the interaction term 'media ID*none', where 'none'
244 implied that no nutrients or environmental conditions were implemented. 'Necropsy date' was kept as a
245 random effect. The second model analyzed worm viability when maintained in one of the saline-based
246 media (*i.e.* ARS, APF, ARS 3x Tris, APF 2x NaCl, PS, HM PS) with glucose compared to worm viability
247 maintained in the same saline-based media without glucose. Glucose was the only nutrient added across
248 all saline-based media types and therefore was the only nutrient analyzed in this model. The covariates
249 examined were 'time' and the interaction term 'media ID*glucose'. 'Necropsy date' was kept as a
250 random effect. The third model examined worm viability when maintained in APF media supplemented
251 with either glucose, FBS, cholesterol, a combination of FBS and cholesterol, Tween only control, or as a
252 no nutrient control. APF was the only medium supplemented with all the nutrients and therefore was
253 the only medium examined in this model. 'Nutrient' and 'time' were the covariates examined. 'Necropsy
254 date' and 'environment' (i.e. CO_2 incubator or platform rocker) were kept as random effects. The fourth
255 model examined the use of the platform rocker and CO_2 incubator across all media and nutrient

256 supplements. The covariates examined were 'time' and 'environment'. 'Necropsy date', 'Media ID' and
257 'nutrient' were kept as random effects. The fifth model analyzed the use of RPMI against all media,
258 nutrients, and environmental conditions. The covariate tested was 'RPMI,' and 'necropsy date' was kept
259 as random effect. The last model analyzed the stage (L_4 or adult) and sex (adult worms only) over time,
260 regardless of media, nutrients used, or the use of the CO_2 incubator or platform rocker. The covariates
261 analyzed were 'stage' and 'sex'. 'Media ID', and 'necropsy date' were kept as random effects. Any time a
262 significant covariate (α=0.05) was observed, a 'least squares means' analysis was performed for a
263 Tukey's pair-wise comparison.

264

265 **3. Results**

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

269

270 *3.1 Phase one: Longevity*

271 For the first phase of the study pertaining to worm longevity (necropsies 1-3), a total of 210
272 worms were used consisting of 98 adult males, 54 adult females, and 58 L_4s. During this phase of the
273 study, the worms lived a maximum of 84 hours. The media type employed when considered without
274 nutrient supplementation or CO_2 did have a significant effect on worm longevity (p=0.0100), however
275 the least squares means pairwise comparison did not identify any significant differences between
276 media. ARS was the only media type significantly affecting worm viability with the addition of glucose.
277 Worms maintained in ARS supplemented with glucose lived significantly longer than worms maintained

278 in ARS alone (p<0.0001). There were no significant differences observed in any of the other media types
279 supplemented with glucose compared to when glucose was not added. Regarding the various types of
280 nutrient supplementation with the ARS media, worms maintained with glucose (p<0.0006) or a
281 combination of glucose and gelatin (p<0.0001) had significantly better longevity than worms maintained
282 without any nutrient. Worms maintained with glucose had significantly better longevity than worms
283 maintained with *E. coli* (p=0.0008), yeast (p<0.0001), FBS (p=0.0013), or cholesterol (p=0.0279).
284 Similarly, worms maintained with a combination of glucose and gelatin had significantly better longevity
285 than those maintained with gelatin only (p=0.0484), *E. coli* (p<0.0001), yeast (p<0.0001), FBS (p<0.0001),
286 or cholesterol (p=0.0008). The mean longevity, 95% confidence intervals, and range of longevity for the
287 different nutrients and incubator type can be found in Table 4. The use of a CO_2 incubator did not
288 significantly affect worm longevity (p=0.2854). 'Adult male (p=0.0021) and female (p<0.0001) worms
289 had significantly better longevity than immature worms, however there was no significant difference
290 between males and females (p=0.5780). The mean longevity, 95% confidence intervals, and range of
291 longevity for immatures, males, and females can be found in Table 4.

292

293 *3.2 Phase two: Viability*

294 For the second phase of the study pertaining to worm viability (necropsies 4-8), a total of 835
295 worms were used, consisting of 350 adult males, 215 adult females, and 270 L_4s. The RPMI-1640 media
296 resulted in significantly better worm viability than any of the other media (p<0.0001) (Figure 1). APF 2x
297 NaCl had significantly better viability than ARS (p=0.0002). APF (p=0.0005), ARS 3x Tris (p=0.0169), and
298 APF 2x NaCl (p<0.0001) had significantly better viability than the homemade physiological saline. The
299 addition of glucose to the saline-based media did not significantly affect worm viability compared to
300 those maintained in the saline-based media without glucose (p=0.3048). The addition of a nutrient to

301 the APF medium did significantly decrease worm viability (p=0.0413), however the least squares means
302 pairwise comparison did not identify any significant differences (Figure 2). The use of the platform
303 rocker resulted in significantly better worm viability than worms maintained without the rocker
304 (p=0.0305), while there were no significant differences in worm viability between the use of an air or
305 CO_2 incubator (p=1.0000) (Figure 3). Overall, worms maintained in RPMI-1640 had significantly better
306 viability than worms maintained with any other method regardless of media, nutrient, or environmental
307 condition (p<0.0001) (Figures 1 and 2). In regards to worm stage and sex, adult worms regardless of sex
308 had significantly better viability than L_4s (p<0.0001) and females had significantly better viability than
309 males (p<0.0001) across all media types, nutrient supplementation, and environmental conditions.

310

311 **4.0 Discussion**

312 This is the first study to determine the preferred *in vitro* conditions for the intestinal stages of
313 *Parascaris* spp., and to describe a reliable and objective method for assessing their viability. Worm
314 motility and the presence of muscle tone appears to be reliable indicator for assessing *in vitro*
315 conditions. This study is the first to report a difference in *in vitro* worm viability for *Parascaris* spp.
316 between L_4 and adult stages, as well as between male and female adult worms.

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

324 *et al.* 2012), a modification of this technique to accommodate larger macroparasites should be a target

325 for future research.

326 The use of RPMI-1640 media resulted in significantly better worm viability than all other media

327 types regardless of nutrient supplementation and/or environmental condition (Figures 1 and 2). Worms

328 lived a maximum of 168 hours in RPMI-1640 (Figures 1 and 2), which is well above the 84 and 96 hours

329 achieved in phase 1 and phase 2, respectively, with the addition of glucose (Table 3 and Figure 2). At this

330 time, it is unknown which components of the RPMI-1640 media caused this improvement in viability and

331 longevity, but it is likely due to the combination of vitamins and amino acids that were missing from the

332 other media evaluated. This finding is in agreement with Urban *et al.* (1984) who found improved

333 growth and survival of L_4 *A. suum* when cultured in RPMI-1640 rather than a saline medium

334 supplemented with glucose.

335 The use of sugar (glucose or dextrose) as a nutrient is reported in several other studies

336 maintaining adult stages of *A. suum* (Weisblat and Russel, 1976; Brownlee *et al.* 1997; Chehayeb *et al.*

337 2014), and one study used dextrose for maintaining adult *P. equorum* (Jansen *et al.* 2013). While it is

338 assumed that sugar is necessary for the *in vitro* cultivation of *Ascaris* and *Parascaris* species, this had not

339 previously been evaluated in a published study. In phases one and two of this study, *Parascaris* spp.

340 survived a maximum of 84 and 96 hours, respectively, when glucose was added as a nutrient and it did

341 not significantly affect worm viability. The success of the RPMI-1640, but not the glucose provides

342 evidence that *Parascaris* spp. intestinal stages require different and/or additional nutrients beyond

343 glucose for sustainment *in vitro.* It is interesting that *A. suum* can be maintained for eight days in ARS

344 without any nutrient supplementation (McCoy *et al.* 2015). In the current study, *Parascaris* spp. did not

345 live more than 168 hours in any of the media regardless of the media type or nutrient provided. This

346 may suggest that adult *A. suum* and *Parascaris* spp. worms have very different nutrient and metabolic

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

353 Douvres and Urban (1983, 1986) described methods for culturing larval stages of *Ascaris* species
354 utilizing various gaseous stages, including 5% CO_2. Several studies report the maintenance of adult *A.*
355 *suum* worms without CO_2 (Weisblat and Russel, 1976; Brownlee *et al.* 1997; Chehayeb *et al.* 2014;
356 McCoy *et al.* 2015). Jansen *et al.* (2013) maintained *P. equorum* adult worms without 5% CO_2 while Burk
357 *et al.* (2014) cultured second and third larval stages of *P. equorum* under 5% CO_2 conditions, but not the
358 adult worms. Based on these reports, it appears that adult worms may not require CO_2, but this had not
359 been specifically evaluated for *Parascaris* spp*.* The current study did not find the use of 5% CO_2 to
360 significantly affect worm longevity or viability (Figure 3). However, this study did not investigate the
361 impact of CO_2 on worms maintained in RPMI-1640 and this should be evaluated in future studies.

362 The use of a platform rocker for *in vitro* maintenance of ascarid parasites had not been
363 evaluated prior to this study. In this study, the use of the rocker significantly improved worm viability
364 (Figure 3), however no firm conclusions can be made at this time. The platform rocker could not be
365 tested simultaneously with CO_2 due to limited space in the incubator. Furthermore, this study did not
366 evaluate RPMI-140 media with the use of the rocker, and this should be investigated in future studies.

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

370 findings are interesting because Urban *et al.* (1984) found the addition of cholesterol (50 µg/mL) and
371 serum (10%) to RPMI-1640 to have an additive effect on the growth of L_4 *A. suum.* Urban *et al.* (1984)
372 also found that an increase in cholesterol concentration to 250 µg/mL from 50 µg/mL reversed this
373 effect. While the aforementioned study examined the development of larval stages, it is possible that a
374 similar scenario was observed in the current study where the *Parascaris* spp. intestinal stages were
375 negatively impacted by the cholesterol concentration examined herein. Future studies should
376 investigate varying concentrations of cholesterol to determine if there is an optimum concentration
377 and/or a tolerance threshold.

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

386 It is important to note that the *in vivo* immune responses exhibited by the foal prior to necropsy
387 may also affect worm viability *in vitro*. Foals typically gain immunity to *Parascaris* spp*.* worms around
388 nine months of age (Clayton and Duncan, 1979). Some response by the immune system to the present
389 parasites is expected and it is unknown how the parasites were affected prior to harvest and culturing.
390 This variability was controlled for by using foals which were all born into the same herd, and harvesting
391 the worms when the foals were between 4.5-5 months of age which is the peak age for *Parascaris* spp.
392 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 L_4 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% CO_2 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 L_4s. Further investigations should be performed to examine the effects of a platform rocker and CO_2 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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413 **References**

414 **Bolla, RI, Weinstein, PP, Lou, C** (1972) *In vitro* nutritional requirements of *Nippostrongylus brasiliensis*- I.
415 Effects of sterols, sterol derivatives, and heme compounds on the free-living stages.
416 *Comparative Biochemistry and Physiology.* **43B**, 487-501.

417 **Brownlee, DJA, Holden-Dye, L, Walker, RJ** (1997) Actions of the anthelmintic ivermectin on the
418 pharyngeal muscle of the parasitic nematode, *Ascaris suum*. *Parasitology*. **115**, 553-561.

419 **Burk, SV, Dangoudoubiyam, S, Brewster-Barnes, T, Bryant, UK, Howe, DK, Carter, CN, Vanzant, ES,**
420 **Harmon, RJ, Kazacos, KR, Rossano, MG** (2014) In vitro culture of *Parascaris equorum* larvae
421 and initial investigation of parasite excretory-secretory products. *Parasitology Research.* **113**,
422 4217-4224. DOI: 10.1007/s00436-014-4097-0.

423 **Chehayeb, JF, Robertson, AP, Martin, RJ, Geary, TG** (2014) Proteomic analysis of adult *Ascaris suum*
424 fluid compartments and secretory products. *PLoS Neglected Tropical Diseases*. **8**:6, e2939. DOI:
425 10.1371/journal.pntd.0002939.

426 **Clayton, HM and Duncan, JL** (1978) Clinical signs associated with *Parascaris equorum* infection in worm-
427 free pony foals and yearlings. *Veterinary Parasitology.* **4**, 69-78. DOI:10.1016/0304-
428 4017(78)90037-7.

429 **Clayton, HM and Duncan, JL** (1979) The development of immunity to *Parascaris equorum* infection in
430 the foal. *Research in Veterinary Science*. **26**, 383-384.

431 **Cole, RJ and Krusberg, LR** (1968) Sterol metabolism in *Turbatrix aceti*. *Life Sciences*. **7**, 713-724.
432 DOI:10.1016/0024-3205(68)90315-9.

433 **Cribb, NC, Corté, NM, Bouré, LP, Peregrine, AS** (2006) Acute small intestinal obstruction associated with
434 *Parascaris equorum* infection in young horses: 25 cases (1985-2004). *New Zealand Veterinary*
435 *Journal*. **54**, 338-343. DOI: 10.1080/00480169.2006.36721.

436 **Dmitryjuk, M, Łopieńska-Biernat, E, Zaobidna, EA** (2014) The *In vitro* effect of ivermectin on the activity
437 of trehalose synthesis pathway enzymes and their mRNA expression in the muscle of adult
438 female *Ascaris suum* (Nematoda). *The Scientific World Journal*. 2014, 936560. DOI:
439 10.1155/2014/936560

440 **Douvres, FW and Urban, JF Jr.** (1983) Factors contributing to the in vitro development of *Ascaris suum*
441 from second-stage larvae to mature adults. *Journal of Parasitology*. **69**, 549-558. DOI:
442 10.2307/3281369.

443 **Douvres, FW and Urban, JF Jr.** (1986) Development of *Ascaris suum* from In Vivo-derived Third-stage
444 Larvae to Egg-laying Adults In Vitro. *Proceedings of the Helminthological Society of Washington.*
445 **53**, 256-262.

446 **Drudge, JH and Lyons, ET** (1983) Ascariasis. In Robinson, N.E. (eds). *Current Therapy in Equine Medicine*.
447 W.B. Saunders, Philadelphia, pp. 262-264.

448 **Dutky, SR, Robbins, WE, Thompson, JV** (1967) The demonstration of sterols as requirements for the
449 growth, development, and reproduction of the DO-136 nematode. *Nematologica*. **13**:140.

450 **Eguale, T, Tilahun, G, Debella, A, Feleke, A, Makonnen, E** (2007*a*) In vitro and in vivo anthelmintic
451 activity of crude extracts of *Coriandrum sativum* against *Haemonchus contortus. Journal of*
452 *Ethnopharmacology.* **110**, 428-433. DOI:10.1016/j.jep.2006.10.003.

453 **Eguale, T, Tilahun, G, Debella, A, Feleke, A, Makonnen, E** (2007*b*) *Haemonchus contortus*: In vitro and in

454 vivo anthelmintic activity of aqueous and hydro-alcoholic extracts of *Hedra helix*. *Experimental*

455 *Parasitology.* **116**, 340-345. DOI: 10.1016/j.exppara.2007.01.019.

456 **Fabiani, JV, Lyons, ET, Nielsen, MK** (2016) Dynamics of *Parascaris* and *Strongylus* spp. parasites in

457 untreated juvenile horses. *Veterinary Parasitology.* **230**, 62-66.

458 **Geldhof, P, Claerebout, E, Knox, DP, Jagneessens, J, Vercruysse, J** (2000) Proteinases released in vitro

459 by the parasitic stages of the bovine abomasal nematode *Ostertagia ostertagi*. *Parasitology*.

460 **121**, 639-647. DOI: 10.1017/S0031182000006806.

461 **Hu, Y, Ellis, BL, Yiu, YY, Miller, MM, Urban, JF, Shi, LZ, Aroian, RV** (2013) An extensive comparison of the

462 effect of anthelmintic classes on diverse nematodes. *PLoS One* **8**:7, e70702. DOI: doi:

463 10.1371/journal.pone.0070702.

464 **Islam, MK, Miyoshi, T, Yokomizo, Y, Tsugi, N** (2004) The proteome expression patterns in adult *Ascaris*

465 *suum* under exposure to aerobic/anaerobic environments analyzed by two-dimensional

466 electrophoresis. *Parasitology Research.* **93**, 96-101. DOI: 10.1007/s00436-004-1101-0.

467 **Jansen, JI, Krücken, J, Demeler, J, Basiaga, M, Kornaś, S, von Samson-Himmelstjerna, G** (2013) Genetic

468 variants and increased expression of *Parascaris equorum* P-glycoprotein-11 in populations with

469 decreased ivermectin susceptibility. *PLoS One* **8**:4, e61635. DOI: doi:

470 10.1371/journal.pone.0061635.

471 **Kotze, AC and McClure, SJ** (2001) *Haemonchus contortus* utilizes catalase in defence against exogenous

472 hydrogen peroxide in vitro. *International Journal for Parasitology.* **31**, 1563-1571. DOI:

473 10.1016/S0020-7519(01)00303-4.

474 **Lyons, ET, Drudge, JH, Tolliver, SC** (1990) Prevalence of some internal parasites found (1971-1989) in
475 horses born on a farm in Central Kentucky. *Journal of Equine Veterinary Science.* **10**, 99-107.
476 DOI: 10.1016/S0737-0806(06)80114-0.

477 **McCoy, CJ, Warnock, ND, Atkinson, LE, Atcheson, E, Martin, RJ, Robertson, AP, Maule, AG, Marks, NJ,**
478 **Mousley, A** (2015) RNA interference in adult *Ascaris suum* – an opportunity for the development
479 of a functional genomics platform that supports organism-, tissue-, and cell-based biology in a
480 nematode parasite. *International Journal for Parasitology.* **45**, 673-678. DOI:
481 10.1016/j.ijpara.2015.05.003.

482 **Marcellino, C, Gut, J, Lim, KC, Singh, R, McKerrow, J, Sakanari, J** (2012) WormAssay: A novel computer
483 application for whole-plate motion-based screening of macroscopic parasites. *PLoS Neglected*
484 *Tropical Diseases*. **6**:1, e1494. DOI: 10.1371/journal.pntd.0001494.

485 **O'Grady, J and Kotze, AC** (2004) *Haemonchus contortus*: in vitro drug screening assays with the adult life
486 stage. *Experimental Parasitology*. **106**, 164-172. DOI: 10.1016/j.exppara.2004.03.007.

487 **Peregrine, AS, Molento, MB, Kaplan, RM, Nielsen, MK** (2014) Anthelmintic resistance in important
488 parasites of horses: Does it really matter? *Veterinary Parasitology.* **201**, 1-8. DOI:
489 10.1016/j.vetpar.2014.01.004.

490 **Rapson, EB, Jenkins, DC, Topley, P** (1985) *Trichostrongylus colubriformis*: in vitro culture of parasitic
491 stages and their use for the evaluation of anthelmintics. *Research in Veterinary Science*. **39**, 90-
492 94.

493 **Richards, JC, Behnke, JM, Duce, IR** (1995) In vitro studies on the relative sensitivity to ivermectin of
494 *Necator americanus* and *Ancylostoma ceylanicum. International Journal for Parasitology.* **25**,
495 1185-1191. DOI: 10.1016/0020-7519(95)00036-2

496 **Storey, B, Marcellino, C, Miller, M, Maclean, M, Mostafa, E, Howell, S, Sakanari, J, Wolstenholme, A,**
497 **Kaplan, R** (2014) Utilization of computer processed high definition video imaging for measuring
498 motility of microscopic nematode stages on a quantitative scale: "The Worminator".
499 *Int*ernational *Journal for Parasitology.* **4**, 233-243. DOI: 10.1016/j.ijpddr.2014.08.003.

500 **Tatz, AJ, Segev, G, Steinman, A, Berlin, D, Milgram, J, Kelmer, G** (2012) Surgical treatment for acute
501 small intestinal obstruction caused by *Parascaris equorum* infection in 15 horses (2002-2011).
502 *Equine Veterinary Journal*. **44**, 111-114. doi: 10.1111/j.2042-3306.2012.00607.x

503 **Thomas, D, Jeyathilakan, N, Basith, SA, Senthilkumar, TMA** (2016) In vitro production of *Toxocara canis*
504 excretory-secretory (TES) antigen. *Journal of Parasitic Diseases.* **40**, 1038-1043. DOI:
505 10.1007/s12639-014-0630-4.

506 **Urban, JF Jr, Douvres, FW, Xu, S** (1984) Culture requirements of *Ascaris suum* larvae using a stationary
507 multi-well system: increased survival, development and growth with cholesterol. *Veterinary*
508 *Parasitology*. **14**, 33-42. DOI: 10.1016/0304-4017(84)90131-6.

509 **Weisblat, DA, Russel, RL** (1976) Propagation of electrical activity in the nerve cord and muscle
510 syncytium of the Nematode *Ascaris lumbricoides*. *Journal of Comparative Physiology*. **107**, 293-
511 307. DOI: 10.1007/BF00656739.

512 **Young, CJ, McKeand, JB, Knox, DP** (1995) Proteinases released in vitro by the parasitic stages of
513 *Teladorsagia circumcincta*, an ovine abomasal nematode. *Parasitology*. **110**, 465-471. DOI:
514 10.1017/S0031182000064805.

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517

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

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

528 **Figure 3.** A graphical representation of mean viability of *Parascaris* spp. intestinal stages maintained
529 with environmental conditions of a platform rocker or a 5% CO_2 incubator across all media and
530 nutrient types. 'None' implies stationary culture flasks in an air incubator. Error bars represent
531 95% confidence intervals (α=0.05).

532

533

Table 1. Distribution of intestinal stages of *Parascaris* spp. specimens among the different media, nutrients, and environmental conditions (*i.e.* CO_2 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%)	*E. coli OP50*	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, CO_2, 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**	**$NaC_2H_3O_2$**	**$C_4H_{11}NO_3$/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
E. coli 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

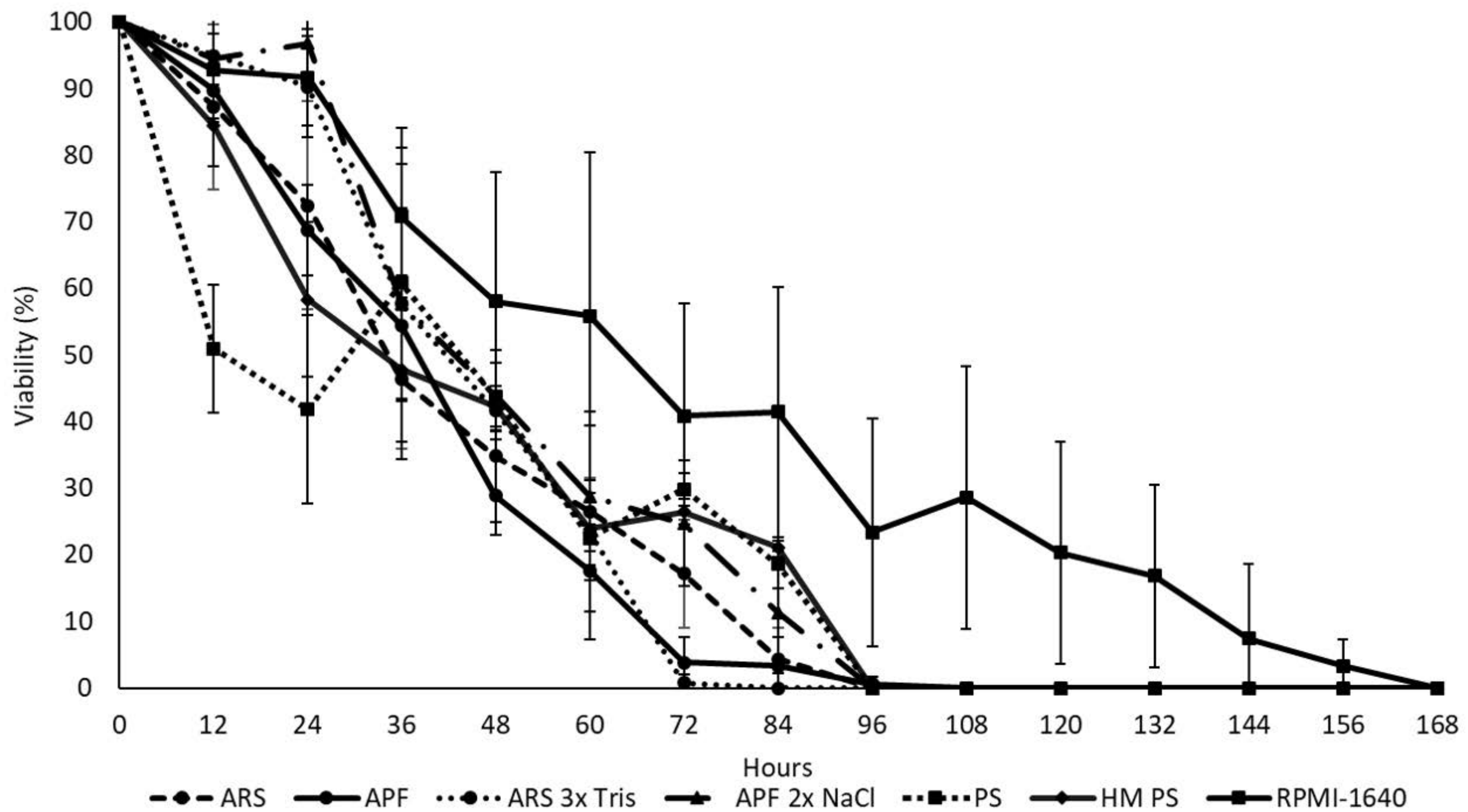
Table 3. Scoring system used to assess the *in vitro* viability of *Parascaris* spp. intestinal stages. Scores were assigned following individual observation for 15 seconds.

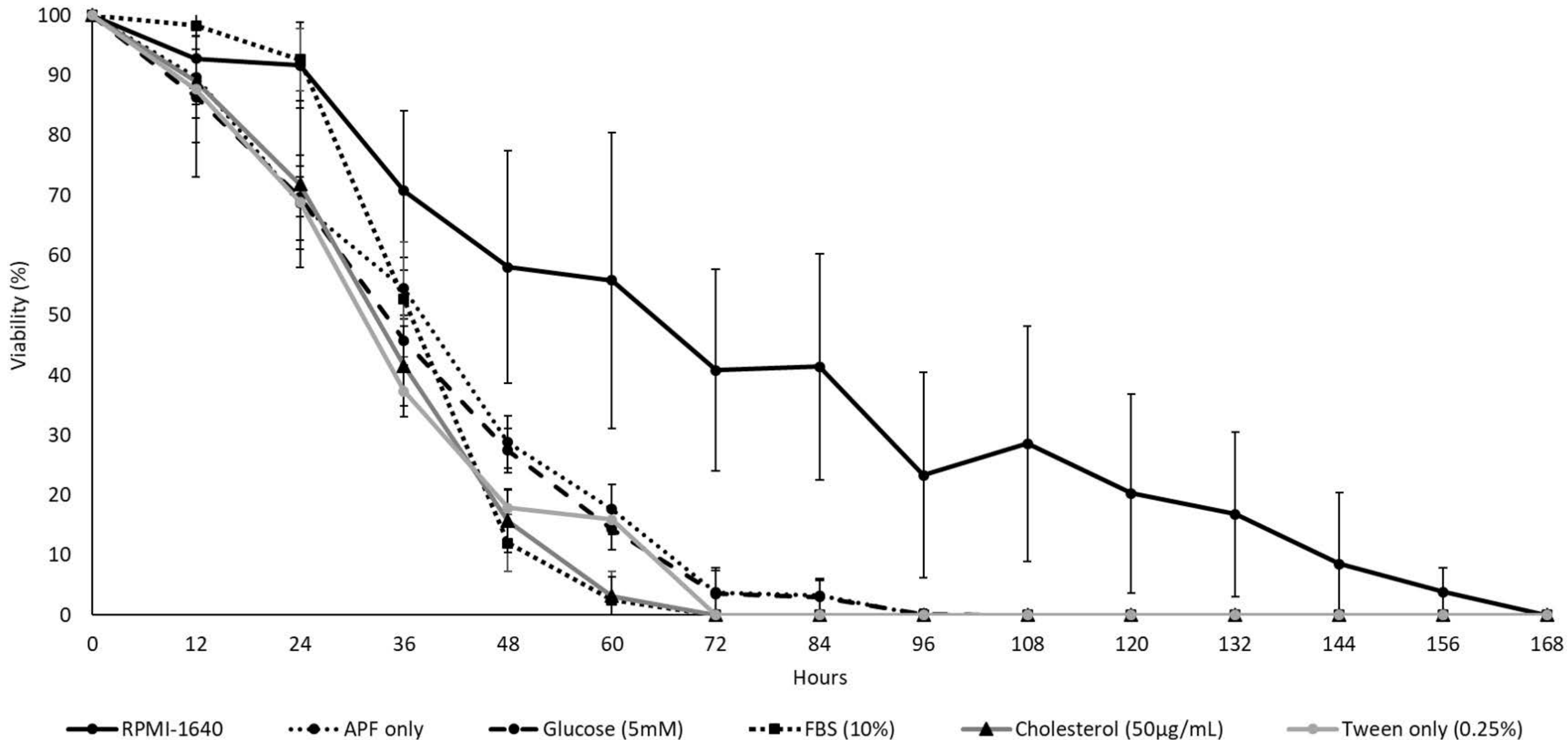
Score	Description
0	Dead, no movement observed independently nor when stimulated with forceps. Lack of muscle tone/flaccid over forceps when lifted out of the solution. Signs of decay may be present.
1	No movement observed independently nor when stimulated with forceps. Muscle tone is apparent.
2	Movement 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 CO_2 incubator use, and of different stages and sex (necropsies 1-3). Worms were maintained in tissue culture flasks (300 cm^2) 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 (α=0.05).

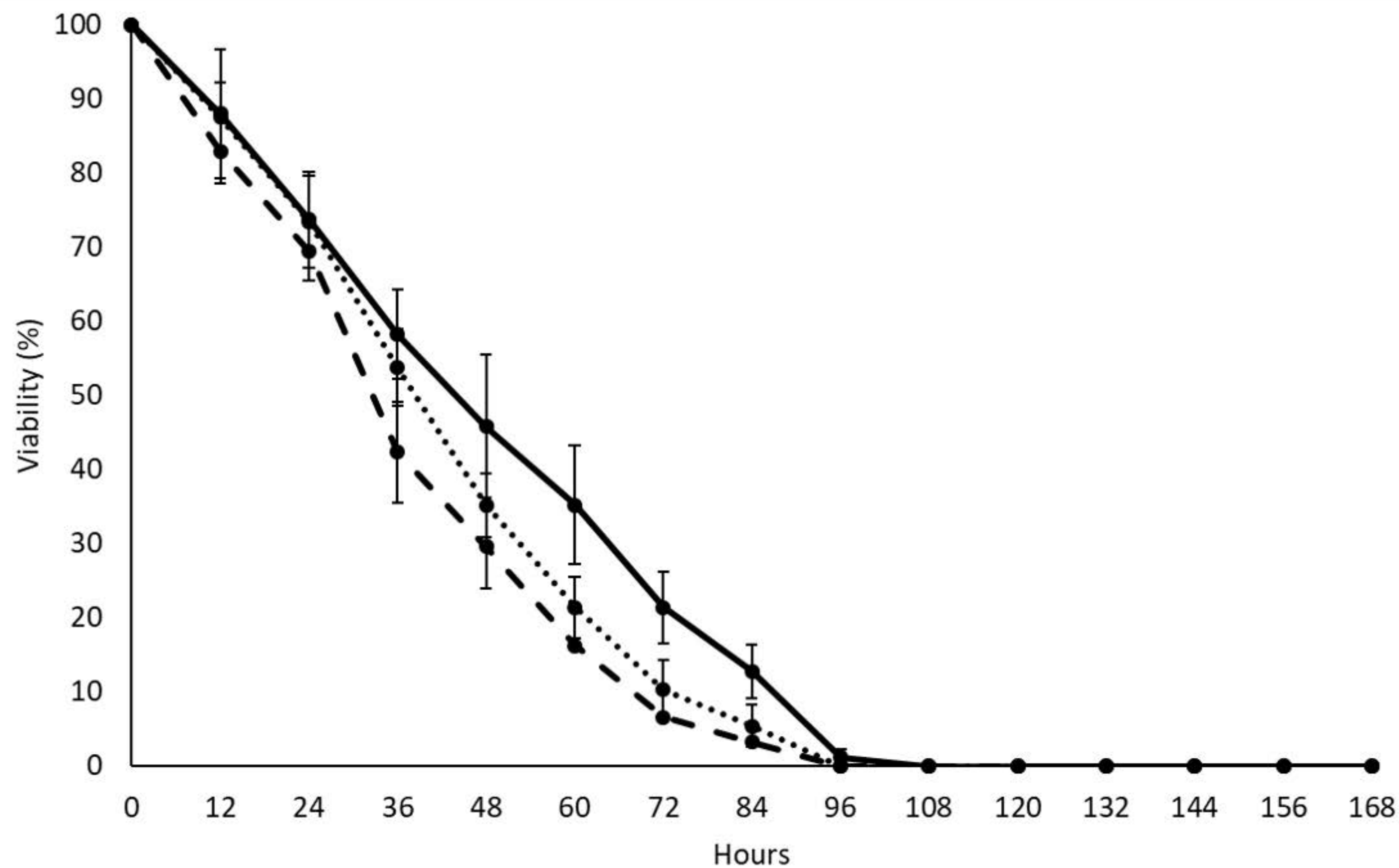
Nutrient/Incubator	Mean Longevity (hours)	Range of longevity (hours)
None[a]	42 (34.7-49.3)	12-60
Glucose (5 mM)	72 (63.7-80.3)	48-84
Gelatin	72 (63.7-80.3)	60-84
Glucose & Gelatin	78 (69.7-86.3)	72-84
Cholesterol (50 µg/mL)	56 (49.6-62.4)	48-60
Fetal Bovine Serum (10%)	40 (33.6-46.4)	36-48
E. Coli OP50	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.*


Viability (%)
100
90
80
70
60
50
40
30
20
10
0
0
12
24
36
48
60
72
84
96
108
120
132
144
156
168
Hours
ARS
APF
ARS 3x Tris
APF 2x NaCl
PS
HM PS
RPMI-1640



Viability (%)
100
90
80
70
60
50
40
30
20
10
0
0
12
24
36
48
60
72
84
96
108
120
132
144
156
168
Hours
RPMI-1640
APF only
Glucose (5mM)
FBS (10%)
Cholesterol (50µg/mL)
Tween only (0.25%)



Viability (%)
100
90
80
70
60
50
40
30
20
10
0
0
12
24
36
48
60
72
84
96
108
120
132
144
156
168
Hours
None
CO2 Incubator (5%)
Rocker