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Pixel by pixel: real-time observation and quantification of passive flotation speeds of three common equine endoparasite egg types

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ABSTRACT

The efficacy of anthelmintic treatments against populations of endoparasites infecting livestock throughout the world is decreasing. To mitigate this, the use of fecal egg counts is recommended to determine both the necessity, and to ensure the appropriate choice, of anthelmintic treatment. Traditionally, and in order to facilitate easier identification and/or enumeration, samples are analysed after separating eggs from other fecal particulates by exposing them to a solution with a density higher than that of the eggs, but lower than the remaining fecal contents. While many parasite egg flotation protocols exist, little is known about the characteristics of these eggs with respect to their movement through a flotation solution. In this study, we have demonstrated a novel method for the observation and quantification of microscopic (65–100 μm) objects as they experience unassisted flotation. This also represents, to our knowledge for the first time, that the flotation of parasite eggs has been observed and their movement characteristics quantified as they float through solution. Particle tracking and video analysis software were utilised to automatically detect and track the movement of individual eggs as they floated. Three 30 s videos and one 2 min video of each egg type were analysed. If the first 30 s of video were discounted, the differences in mean flotation speed among all videos was statistically significant between egg types ($P = 0.0004$). Strongyle type eggs ($n = 201$) moved the fastest with a mean 51.08 μm/s (95% confidence interval: 47.54–54.62). This was followed by *Parascaris* spp. ($n = 131$) and *Anoplocephala perfoliata* eggs ($n = 322$), with mean speeds of 44.43 μm/s (95% confidence interval: 39.47–49.4) and 31.11 μm/s (95% confidence interval: 29.6–32.61), respectively. This method for evaluating the mean speed of passive flotation may represent a first step towards further optimizing fecal egg flotation and be of interest to parasitologists and veterinary practitioners.

1. Introduction

The ability to separate parasite eggs from feces for detection and enumeration is a fundamental tool of veterinary parasitology, and fecal egg count reduction tests are the ‘gold standard’ by which anthelmintic treatment efficacy is measured in equine parasitology (Nielsen et al., 2013). The well documented occurrence of decreasing treatment efficacy (Kaplan, 2004; Falzon et al., 2014) has led to the recommendation that regular fecal egg counts should be performed (Kaplan and Nielsen, 2010; Kaplan and Vidyashankar, 2012; Reinemeyer and Nielsen, 2017), and spurred our interest in improving the understanding of the movement of parasite eggs during flotation. Many protocols utilise a flotation solution with a specific gravity (SG) that exceeds that of the eggs, but not that of the majority of the fecal debris, and so separates the eggs from the feces via their subsequent flotation either under gravity or in a centrifugal field (Ballweber et al., 2014; Becker et al., 2016; Paras et al., 2018). The difference between the SG of the solution and the density of the eggs must be great enough to facilitate expedient flotation while maintaining an osmolarity that is not so great as to exert traumatic osmotic forces on the eggs, potentially causing rupture or collapse. The time suggested to allow eggs to be released from the feces and float to the surface varies widely by protocol and by the SG of the flotation solution (Roepstorff and Nansen, 1998; Barda et al., 2013), and the determination of these parameters have been historically based on trial and error (Bass, 1906; Sheather, 1923). More recently, the effectiveness of flotation protocols has been evaluated by spiking negative fecal samples with a known number of eggs and determining their recovery via egg count (Noel et al., 2017; Scare et al., 2017; Bosco et al., 2018; Papaiakovou et al., 2018; Paras et al., 2018). However, the eggs of

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various species of endoparasites can have dramatically different specific gravities (David and Lindquist, 1982; Harnnoi et al., 1998) and, due to this, numerous modifications have been made to flotation protocols. We have recently estimated the mean specific gravities of *Anoplocephala perfoliata*, *Parascaris*, spp., and strongyle type eggs as 1.064, 1.090, and 1.045, respectively (Norris et al., 2018).

The spherical shape and rough outer membrane of *Parascaris* spp. eggs are unique among the eggs assessed in this study, although this proteinaceous outer layer can also sometimes be shed to produce a decorticated egg with a smooth surface (Donoghue et al., 2015). The presence of this uneven outer layer could have an impact on the drag experienced by these eggs as they move through liquid. In comparison, strongyle type eggs appear with a smooth surface, and it can be hypothesised that their ellipsoid shape might facilitate movement through flotation media. The eggs of *A. perfoliata*, on the other hand, are irregularly-shaped, ridged flattened trigonal pyramids, and an approximation of their shape produced using the Blender open source 3D model creation suite (Blender Online Community, 2019. *Blender – a 3D modelling and rendering package*. Blender Foundation, Blender Institute, Amsterdam.) (Supplementary S1).

The overall aim of this study was i) to seek explanation of the notorious paucity of anoplocephalid eggs in equine fecal egg flotation, ii) to determine if a denser egg type such as the equine ascarid egg will be slower to float than the more hydrodynamic appearing strongyle type eggs, and iii) to aid future research in improving egg flotation protocols. We therefore sought to develop and validate a method for observing and analyzing the movement of equine parasite eggs as they move through a flotation solution in real time. With this method, we have estimated the flotation speed of the eggs of three common, yet morphologically diverse, equine endoparasite categories. Finally, a secondary aim was to determine a method for measuring the mean angle of strongyle type eggs in flotation media.

2. Materials and methods

2.1. Egg isolation – Parascaris spp. and strongyle type

Parasite egg-positive feces were collected from multiple members of an equine research herd maintained at the University of Kentucky, USA and known to harbor a large diversity of equine endoparasite species (Lyons et al., 1990). Approximately 30 g of feces were homogenised in tap water and filtered through two-ply cheese cloth into a plastic receptacle. Polypropylene centrifuge tubes (50 mL) were filled to 45 mL with this slurry and centrifuged for 10 min at 1000*g*. The supernatant was then carefully removed using a 25 mL serological pipette and 45 mL of flotation solution (glucose and sodium chloride – SG of 1.27) were added (Roepstorff and Nansen, 1998), and the pellet resuspended using a vortex homogeniser. The tubes were centrifuged as before and the top 25 mL of liquid removed using a disposable bulb pipette, filtered through pluriStrainer® cell strainers (pluriSelect Life Science, Leipzig, Germany) arranged, in decreasing pore size, from 400, 200, 100, to 27 μm (Norris et al., 2018). Eggs were recovered from the 100 and 27 μm strainer surfaces using tap water with a 1000 μl laboratory pipette and placed into a 15 mL centrifuge tubes, which were stored at 4 °C for up to 1 month.

2.2. Egg isolation – A. perfoliata

Due to their scarcity in fecal egg counts, equine tapeworm eggs were harvested from adult individuals that were collected from their location at or near the ileocecal junction during equine necropsy (approved by the University of Kentucky Institutional Animal Care and Use Committee under protocol number 2012-1046). The most distal proglottids obtained from gravid specimens were manually dissected with the aid of dissection microscope and forceps. Eggs were collected via a laboratory pipette, placed in distilled water in a 15 mL centrifuge tube, and kept at 4 °C for up to 1 month before use.

2.3. Viewing chamber and camera setup

To produce a receptacle in which movement of eggs could be visualised, one opening of a McMaster egg counting slide was sealed with two-part epoxy resin (Fig. 1A) and turned so that the sealed side faced downward. This modified McMaster slide was attached to the front of a Nanoha X5 (Yasuhara Co., Ltd., Setagaya Ku, Tokyo, Japan) macrophotography lens fitted to an Olympus Air 001 digital camera (Shinjuku, Tokyo, Japan) attached to a tripod. This digital camera was controlled remotely using the "OA. Central for Olympus Air" (Ver. 1.0.2 – Olympus Corporation) application installed on an Apple iPhone 5C running iOS10 (Cupertino, California, USA; Fig. 1B).

2.4. Image processing and data generation

Video of the flotation of each individual egg type was recorded. Mean velocities of these eggs were determined in pixels per frame of video using FIJI image analysis software 2.0.0-rc-69 running on Java 1.8.0_172 (Schindelin et al., 2012, 2015) in conjunction with TrackMate 3.8.0, a video analysis plug-in included with FIJI (Tinevez et al., 2017). To be processed by this software, the videos needed to be transcoded into uncompressed audio video interleave video file (AVI) utilizing the FFMPEG open-source video converter 4.0.4 (FFmpeg Team, 2000). This was achieved with the command "ffmpeg -i [ORIGINAL].avi -pix_fmt nv12 -f avi -vcodec rawvideo [OUTPUT].avi". Five min (8993 frames) of video were imported into FIJI as raw AVI files and 3375 frames (~2 min of each video) were selected from each original video, which represented the approximate lengths of the longest contiguous sequences of each video exhibiting only egg movement unaltered by camera shake. The videos were converted to virtual stacks of greyscale images and duplicate stacks were then produced (Image > Duplicate). To impose a binary mask to the truncated stacks of images, an automatic threshold was applied with "Dark Background" unchecked (image > threshold > adjust).

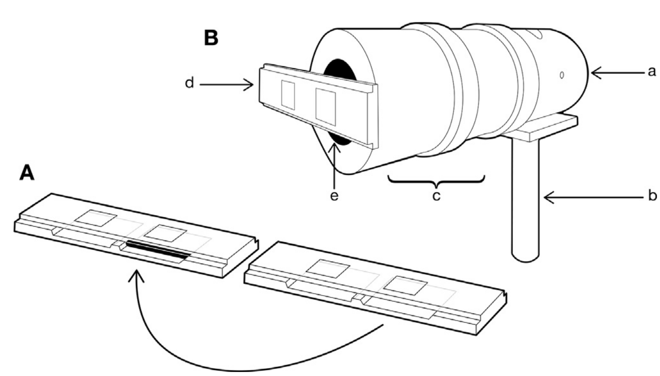

Fig. 1. Illustration of egg movement observation setup including a McMaster egg counting slide modification. Epoxy glue was used to seal the entrance to one well of the egg counting chamber and allowed to dry. This was then affixed to the front of the macro lens using general purpose laboratory labeling tape (A). Olympus Air A01 digital camera (a) with tripod (b) to which a Yasuhara Nanoha ×5 macrophotographic lens (c) was mounted and to which a modified McMaster egg counting slide (d) was attached. One of the egg counting chambers of the McMaster slide was sealed on one side with epoxy (e) and a parasite egg + flotation solution mixture was added to this chamber for viewing (B).

The TrackMate plug-in settings were adjusted using an estimated blob diameter where only eggs in the video were selected (31 pixels for *A. perfoliata* and 33 pixels for the other two egg types). Mean track quality is the mean of a unitless quality score assigned as TrackMate interprets objects in subsequent frames of the video. The quality value imposed upon an egg is calculated based on brightness (difference form the black background) and its closeness to the specified circular blob diameter. A quality threshold of 2.00 was imposed on all bitmasked videos. Simple Lap Tracker was used to track objects throughout their duration in the field of view. Linking max distance and gap-closing max distance were kept at 15 pixels. The gap closing max frame gap was also kept at 2 pixels.

In order to observe trends related to the effects of time on the flotation of the eggs, this was also repeated with 928 frames (30 s) of video from the beginning, middle, and end of each original 8993 frame video. These videos did not overlap with the longest 2 min in the previous analysis. Utilizing these 938 frame videos, the impact of the imposition of the bitmask was also assessed by replacing this step with the inversion of the greyscale image stacks (edit > invert), with all other steps unchanged except for the necessary reduction of quality threshold to 0.50.

Because no apparent automated method exists within FIJI for measuring the mean angle of an ellipsoid object while in motion, manual angle measurements of strongyle type eggs were made using the angle function in FIJI. A single frame of the aforementioned 3375 frame video was analysed every 225 frames, producing 15 time points at which angle measurements were made.

2.5. Data processing and statistical analysis

Comma separated value files generated from 3375 frames of (Supplementary Data S1–S3) and 30 s segments (928 frames) of video output from TrackMate (Tinevez et al., 2017) were processed using R Studio 1.1.456 (RStudio Team, 2015. *RStudio: Integrated Development for R.* RStudio, Inc., Boston, MA) and R 3.5.1 (R Core Team, 2017. *R: A Language and Environment for Statistical Computing.* R Foundation for Statistical Computing, Vienna, Austria.) together with the reshape2 1.4.3 (Wickham, 2007) plug-in for R. Tracks with a duration of less than 100 out of the 3375 or 928 total frames were removed from subsequent analysis. The Shapiro-Wilk normality test was performed on all data. A simple linear model was constructed with track mean speed as the outcome variable and egg type as the covariate using the R package 'lsmeans' 2.30-0 (Lenth, 2016). Whenever differences between egg types were found to be statistically significant, a pairwise least square means analysis was carried out with a Tukey adjustment. Figs. 2 and 3 were produced using GGplot2 3.1.1 for R (Wickham, 2016). To ascertain whether mean speed was statistically different between egg types among all time points, a multivariate analysis of variance was performed with egg type as a fixed effect and keeping the time

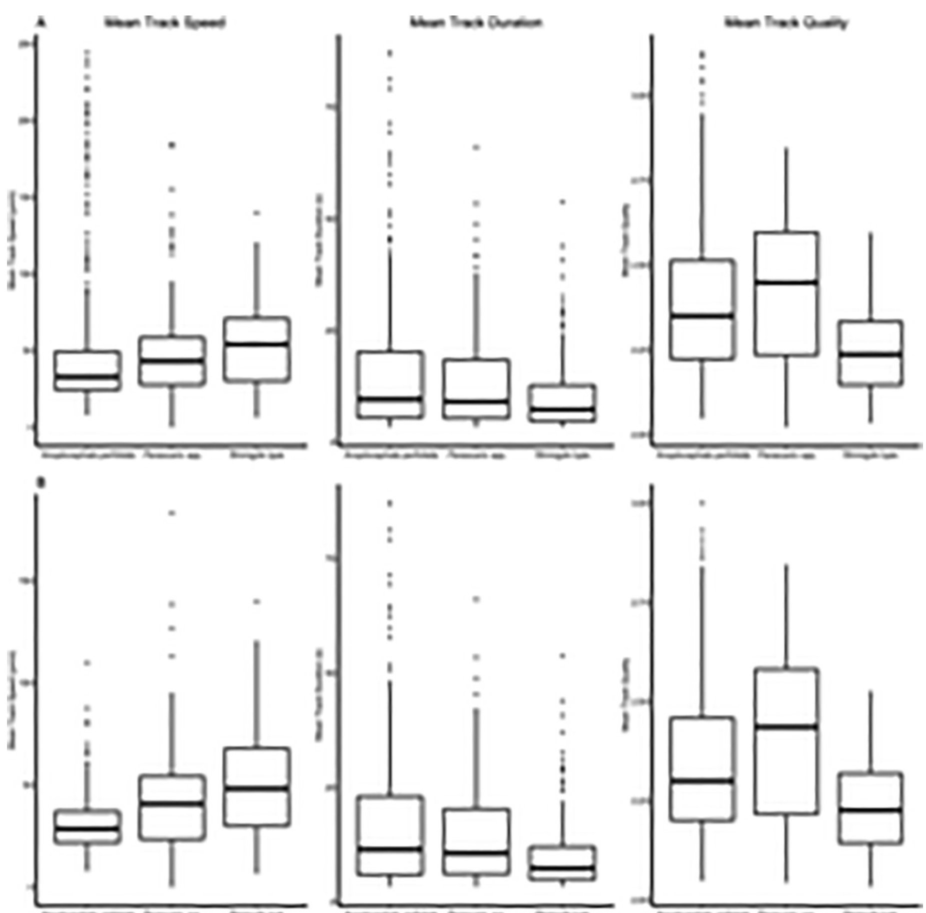

Fig. 2. Parasite egg type versus mean track velocity, duration, and quality – with and without the first 30 s videos. Box plot of the comparison of mean velocity per track between egg types in μm per s mean track duration and quality. (A) Values with all available videos used in the analysis. (B) Data from all videos with the first 30 s excluded. Track mean velocity is the mean of the movement of each track produced using TrackMate converted from pixels/frame to μm/s. Track duration is defined as the number of frames over which the TrackMate plug-in was able to detect the object converted to s. Track quality is a unitless numeric scoring system automatically computed by the TrackMate plug-in using both the brightness of an object and this object's similarity in diameter to the diameter supplied to the TrackMate plugin as an "estimated blob diameter."

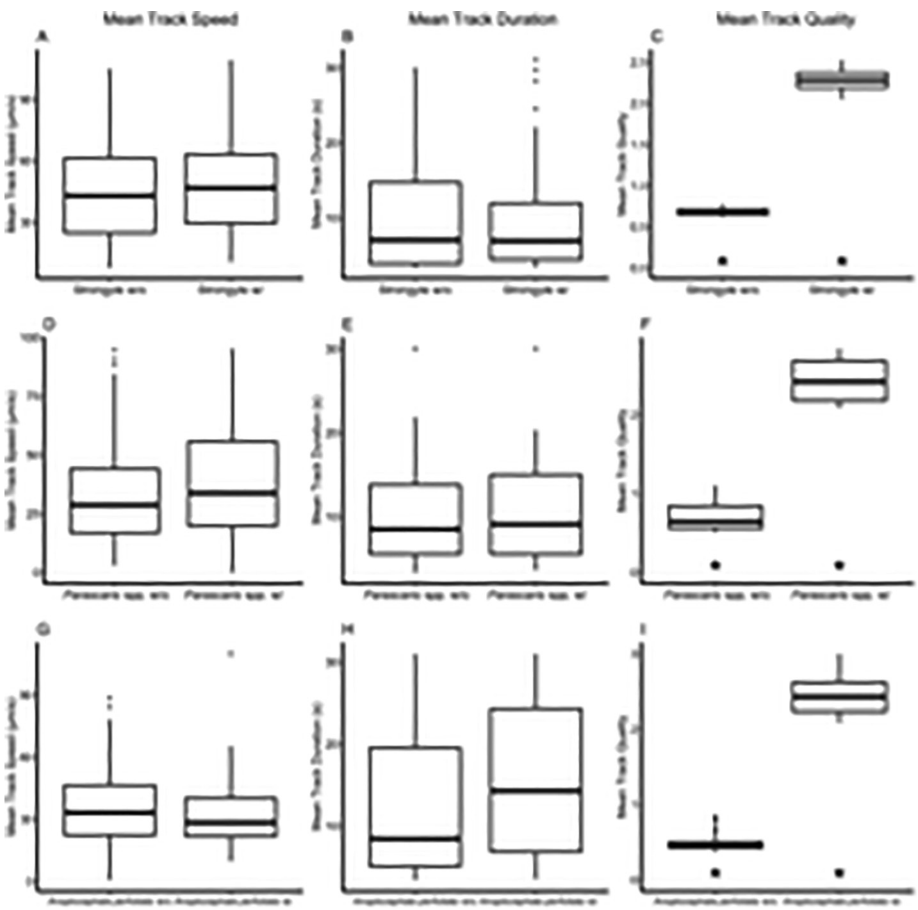

Fig. 3. Impact of bitmasking on three egg types during the middle 30 s time period of videos. (A, D, G) The mean track speed per egg type both with (w/) and without (w/o) the bitmasking step. (B, E, H) The duration over which the eggs were able to be tracked, given each egg type as impacted by the aforementioned bitmasking step in s. (C, F, I) The impact of bitmasking on quality. Plots A–C represent data involving strongyle type eggs, while plots D–F and G–I represent those of *Parascaris* spp. and *Anoplocephala perfoliata*, respectively. Asterisks indicate statistical significance ($P \leq 0.05$) results between replicates with or without the bitmasking step.

point as a random effect. A value of $P \leq 0.05$ was considered sufficient for significance.

2.6. Converting between pixels per frame and micrometers per second

The approximate distance between the middle of two lane markers visible during flotation was measured using the pixel measurement function in Fiji. The recording speed of the camera was reported by the manufacturer to be 29.9 frames per s. The mean track speed in frames per s of movement per egg type were reported by the TrackMate plug-in and was averaged. Without adjusting for the effect of parallax, the following formula (where (A) is the reported mean speed per egg type in pixels per frame, (B) is the measured pixel width per lane, and (C) is the number of lanes per McMaster 1 cm grid) was used to produce an average mean track speed in μm/s:

$$\text{Mean Track Speed}\,(\mu\text{m/s}) = \frac{A * 29.9}{\left(\frac{B*C}{10{,}000}\right)}$$

3. Results

Our initial 3375 frame (~2 min) videos indicated that differences between strongylid, ascarid and anoplocephalid ova in relation to mean movement speed was statistically significant ($P < 0.05$) and were 51.86 μm/s (95% confidence interval (CI): 47.81–55.9, n = 126), 48.3 μm/s (95% CI: 39.93–56.68, n = 63), and 32.43 μm/s (95% CI: 30.54–34.32, n = 195), respectively (Table 1). With the inclusion of the shorter 30 s videos from the three separate time points (Fig. 2), mean track speed was not statistically different ($P \geq 0.25$) between egg types and changed to 53.44 μm/s (95% CI: 50.30–56.59, n = 274), 49.07 μm/s (95% CI: 44.22–53.92, n = 184), and 50.11 μm/s (95% CI: 45.50–54.73, n = 418). According to a separate least squared means test, the first 30 s video was statistically different compared with all other videos, in particular among the anoplocephalid ($P \leq 0.01$) and strongylid ($P = 0.03$) egg types. When the first 30 s video was excluded from analysis, mean speeds among all other time points changed to 51.08 μm/s (95% CI: 47.54–54.62, n = 201), 44.43 μm/s (95% CI: 39.47–49.40, n = 131), 31.11 μm/s (95% CI: 29.60–32.61, n = 322) and were again found to be statistically different from one another ($P \leq 0.025$). Disregarding measurements taken in the first 30 s, mean track qualities among strongylid, ascarid and anoplocephalid egg types were also significantly different ($P \leq 0.0001$) between all egg types and were 2.24 (95% CI: 2.22–2.25), 2.42 (95% CI: 2.39–2.46), 2.35 (95% CI: 2.33–2.37), respectively (Table 2). The difference in mean track duration, the first 30 s excluded, were 10.19 s (9.08–11.31), 14.93 s (12.9–16.95), and 16.69 s (15.04–18.35), respectively, and was only statistically significant between strongyle type and the other two egg types ($P \leq 0.01$, Table 2).

3.1. Strongyle type egg flotation angle

Strongyle type eggs (n = 152) were measured to have an overall mean angle of 84.53° (95% CI: 77.78–91.28°), a minimum of 6.30°,

Table 1
A comparison of mean track speeds with 95% confidence intervals where relevant for the three equine egg types involved in the study.

	Strongyle type			*Parascaris* spp.			*Anoplocephala perfoliata*		
	A	B	C	A	B	C	A	B	C
n	274	126	201	184	63	131	418	195	322
Mean Speed (μm/s)	53.44	51.86[a]	51.08[b]	49.07	48.30[a]	44.43[b]	50.11	32.43[a]	31.11[b]
	(50.30–56.59)	(47.81–55.9)	(47.54–54.62)	(44.22–53.92)	(39.93–56.68)	(39.47–49.40)	(45.50–54.73)	(30.54–34.32)	(29.60–32.61)

A, measurements from the full complement of videos.
B, measurements which used the longest (~2 min) video.
C, measurements after the first 30 s video was removed from analysis.
Superscripts represent statistical significance ($P < 0.05$).

Table 2
Egg movement data with 95% confidence intervals where relevant for the three equine egg types involved in the study.

	Strongyle Type	*Parascaris* spp.	*Anoplocephala perfoliata*
n	201	131	322
Mean speed (μm/s)	51.08	44.43	31.11
	(47.54–54.62)	(39.47–49.40)	(29.60–32.61)
Mode of speed (μm/s)	47.46	51.76	22.47
Median speed (μm/s)	48.20	40.71	28.51
Mean duration (s)	10.19	14.93	16.69
	(9.08–11.31)	(12.90–16.95)	(15.04–18.350)
Mean quality	2.24	2.42	2.35
	(2.22–2.25)	(2.39–2.46)	(2.33–2.37)

and a maximum of 177.46° from the longitudinal axis being horizontal. A distribution of these angles and a representation of this data is shown in Fig. 4.

3.2. Bitmask versus no-bitmask + inversion

Overall, the imposition of a bitmask did not significantly impact findings except for a higher mean track quality, which was always significantly higher with the inclusion of the bitmask ($P < 0.0001$, Fig. 3). Track speeds were lower in ascarid eggs without bitmask during the last 30 s of video ($P = 0.017$, Table 3).

4. Discussion

Here we present the first known study to use live video microscopy and computational image analysis to estimate flotation speeds of common parasite eggs. Furthermore, the system established and described herein facilitates observation and description of movement patterns of eggs in a flotation medium, which is a topic that has not received much attention despite almost a century of utilizing flotation as a tool in veterinary parasitology (Bass, 1906; Sheather, 1923). This system could easily be applied to ova from other parasite and host species, and to other commonly used flotation media. Taking these findings into consideration, together with the significant differences in specific gravities between egg types, future studies could work to improve egg flotation protocols. Furthermore, it now seems likely that wait times for many fecal egg flotation protocols may be extraneously long and an effort should be made towards revision and optimisation of these protocols.

It should be noted that the inclusion of the glucose and NaCl flotation solution used in this study is only one flotation solution recommended for fecal egg flotation (Roepstorff and Nansen,

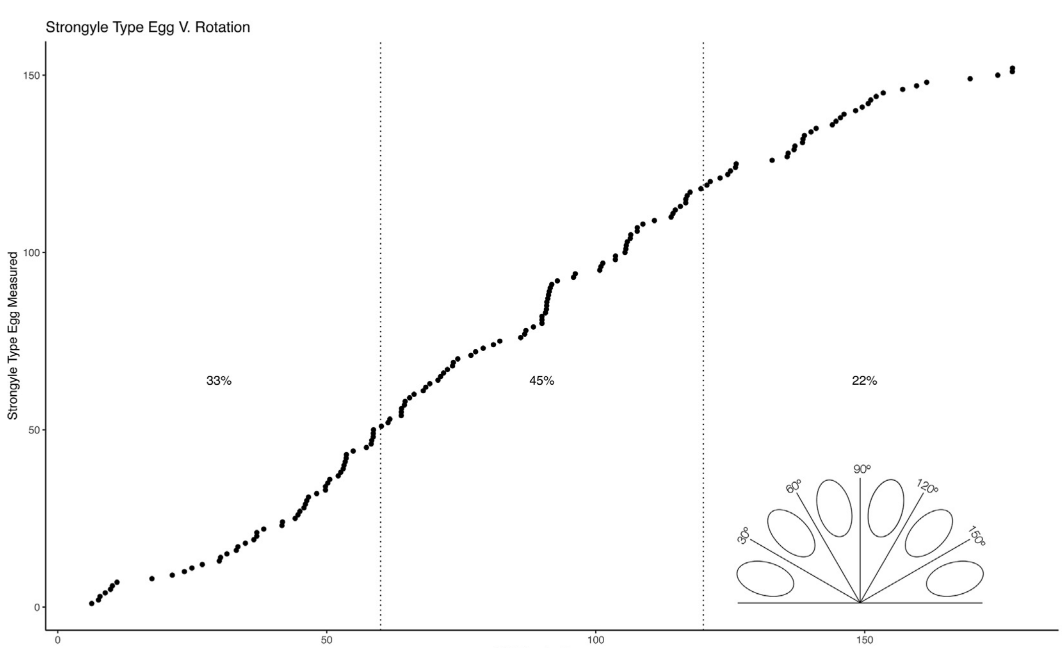

Fig. 4. Strongyle type egg versus manually measured angle of movement. A summary of manually measured angles at which strongyle type eggs were observed during their movement. Over the entirety of the analysed video, still images were measured every 225 frames and summarised. Fig. 3 represents the manual angle measurement criteria used and displays the detailed range (6.3–177.46°) over which angles were observed. Percentages were calculated using the categorisations (<60°), (≥60° to ≤120°), and (>120°). Dotted lines are drawn at 60° and 120°.

Table 3
Shorter videos with and without bitmask at different points during the flotation. Egg movement data with 95% confidence intervals where relevant for the three equine egg types involved in the study compared across three time points. Track qualities were always statistically different between bitmask and invert-only replicates.

	Strongyle type		*Parascaris* spp.		*Anoplocephala perfoliata*	
	w/ mask	w/o mask	w/ mask	w/o mask	w/ mask	w/o mask
First 30 s (928 frames)						
n	73	104	53	67	96	103
Mean speed (μm/s)	59.95 (53.49–66.41)	52.32 (45.71–58.93)	60.52 (49.49–71.55)	59.94 (48.94–70.95)	113.87 (100.89–126.86)	90.71 (77.66–103.76)
Mean quality[a]	2.28 (2.24–2.31)	0.67 (0.65–0.69)	2.44 (2.37–2.50)	0.78 (0.73–0.83)	2.54 (2.50–2.59)	0.64 (0.63–0.66)
Middle 30 s (928 frames)						
n	45	28	24	44	71	236
Mean speed (μm/s)	50.43 (42.75–58.12)	45.81 (35.33–56.29)	40.55 (28.63–52.46)	34.39 (27.17–41.61)	31.90 (28.29–35.51)	34.76 (32.69–36.83)
Mean quality[a]	2.28 (2.24–2.32)	0.68 (0.66–0.69)	2.44 (2.33–2.55)	0.71 (0.65–0.76)	2.45 (2.39–2.51)	0.48 (0.47–0.49)
Last 30 s (928 frames)						
n	30	47	44	64	56	66
Mean speed (μm/s)	48.81 (36.72–60.91)	53.49 (40.85–66.12)	41.02[a] (35.38–46.65)	31.23[a] (25.97–36.48)	25.50 (22.61–28.38)	32.24 (26.42–38.06)
Mean quality[a]	2.21 (2.17–2.25)	0.61 (0.59–0.63)	2.50 (2.42–2.57)	0.77 (0.72–0.83)	2.26 (2.22–2.30)	0.61 (0.59–0.63)

w, with; w/o, without.
Superscripts indicate statistical significance ($P < 0.05$).
[a] Mean quality was always considered statistically significant between masked and unmasked data sets.

1998). Less or more ionizing flotation media with similar or different SGs and viscosities could also be used and may even exacerbate the differences observed between egg types (i.e. sucrose only, Zn sulfate, etc.), and these could be topics of future investigations with the approach described herein.

The movement speeds of the eggs of *A. perfoliata* were significantly slower than those of the other egg types assessed in this study, but the greater density and rough outer coat of the ascarid eggs did not result in those floating most slowly. In the 30 s videos, it should be noted that the mean speed of the anoplocephalid eggs during the first 30 s was measured to be 113.87 μm/s, but then sharply declined to 33.22 and then to 25.50 μm/s. Thus, doing repeated measurements of egg movement likely offers a more nuanced explanation than the overall trend of egg speeds observed in the longer video. This first measurement is perhaps also indicative of anoplocephalid eggs being more prone to being affected by turbulence, which may have been more pronounced at the initiation of the experiment, although mean movement speeds were always higher during this time point among all egg types. Discounting these first 30 s videos, the overall ranking of mean speeds (strongyle type, ascarid and anoplocephalid – in decreasing speed) held true regardless of replicate or chronological variation. In future studies, allowance of an appropriate amount of time for the eggs to come to a place where the turbulence imparted by the addition of flotation solution to the viewing chamber has subsided, should be considered.

Furthermore, a minority of strongyle type eggs floated with their longitudinal axes between 60° and 90° from the vertical axis, which was expected to cause the most hydrodynamic drag (Fig. 4). This surprising result suggests that these objects were likely not moving solely based on the influence of hydrodynamic forces acting upon their surfaces. As these eggs move through the ionic flotation solution, they also likely gain a slight charge on their surfaces. In other objects close to the size of these eggs, it has been shown that electrostatic forces can also have an effect on speed, such as that of microbubbles of gas between 10 μm and 1 μm diameter (Pérez-Garibay et al., 2018) as they move through liquid. Movement of eggs in a flotation medium may also be partially explained by the Derjaguin, Landau, Verwey and Overbeek (DLVO) theory, which states that disequilibrium between slightly attractive and repellent electrostatic forces near the surface of the object produces a double layer of counterions around the object, thereby altering its speed in the liquid. This difference in charge at the point where the electrical double layer (EDL) intersects with the fluid of the colloid is referred to as the zeta-potential (ζ). As the ζ increases, the size of the EDL surrounding the object decreases, and the drag experienced by the object as it moves through the medium also decreases. This would increase the object's speed (Wang et al., 2014). Sugar/salt flotation media, being ionic, has the capacity to introduce charge to particles. The more efficiently this electrokinetic layer can be built, the less resistance the surrounding liquid will impart to the object. An irregularly shaped object (such as an *A. perfoliata* ovum) may be less likely to amass a large ζ, thus producing a larger EDL and consequently increasing drag and decreasing speed. The tendency for a charge to be strongest at an edge and weaker on flat surfaces, where there is more contact between like charges, may help to explain the slower velocities of the more angular shapes and nearly planar surfaces of anoplocephalid eggs (Supplementary Movie S2). A limitation of this study was that the hydrodynamic/electrostatic forces potentially impacting the eggs during flotation were not studied. Future studies utilizing the method demonstrated in this paper for observing flotation patterns could be carried out to assess if, and by how much, these forces impact flotation of endoparasite eggs.

Interestingly, in the case of bubbles averaging a diameter of 2400 μm, it has been shown that those with a more spherical shape (i.e. similar to that of ascarid eggs) should have a slower terminal velocity (Kracht and Finch, 2010). Taking this, as well as the mean SGs of ascarid, anoplocephalid and strongyle egg types (1.08, 1.06, and 1.04, respectively) into consideration, the ranking of mean velocities observed in this study is surprising. In future studies, the impact of the uneven outer layer observed on most ascarid eggs could be studied by the inclusion of corticated versus decorticated ascarid eggs.

Some egg movements were not fully explained by flotation alone and eggs appeared to move toward the bottom of the viewing area after some period of flotation. Once the full videos were sped up (×12), a mixing motion was evident with the solutions over the 5 min course of the videos (Supplementary Movie S3), indicating that eggs that reached the top of the liquid may not be guaranteed to remain there. This should be considered as well when assessing the three replicates involving 928 frame (30 s) videos. Taken all together, exposure to osmotic forces, and their subsequent deformation of eggs, may not be the only concern in extending wait times beyond what is necessary. Future experiments could observe the air/liquid interface of the flotation solution to determine the dwelling time of eggs at the surface in order to inform an appropriate wait time for each egg type. Furthermore, any changes in the appearance of the eggs could also give clues as to the propriety of the choice of flotation solution.

The TrackMate plug-in uses a scale normalised gaussian filter to detect bright objects. Due to this filter, the local maxima of the image can be determined and are interpreted as sinusoidal amplitudes. The amplitudes of these curves, together with the difference between the diameter of the object and the specified diameter, are used to determine spot quality. This explains why the relatively dark (and therefore light, once threshold adjusted or inverted) and circular appearance of ascarid eggs would yield the highest mean track quality and could also help explain the higher mean track quality of anoplocephalid eggs, whose shape when viewed in a two-dimensional video appears close to circular. These egg types are both perhaps better suited to this particular particle estimation method than the strongyle type eggs, which had the overall lowest mean track quality. This method of measuring speed may therefore be biased towards superior identification of round and opaque objects. Inclusion of a thresholding step may not be necessary, as shown in Table 2. When compared with the same videos which were inverted instead of bitmasked, few significant differences could be observed, discounting radically different mean quality scores. Interestingly, the mean speed of the eggs was typically slightly lower without the bitmask. This may be due to aberrant inclusion of stationary points tracked for over 100 frames of video, which may have been mistaken to be parasite eggs, likely due to the low quality threshold imposed. In discussing the camera set-up, it should be noted that if the laboratory bench was knocked during filming, a rapid change in the movement patterns of the eggs was observed (a rapid vibration from approximately left to right). Such brief episodes were not observed in any frames of any video used for analysis.

The procedure of parasite egg isolation may have an impact on results as well. In the case of strongyle type and *Parascaris* spp. eggs, prior exposure to flotation media may have selected for eggs with faster flotation speeds than those of *A. perfoliata*, which were not exposed to flotation solution prior to being observed. This selection may therefore have impacted the rankings determined in this study. In an attempt to explain the turbulence observed over the course of filming (Supplementary Movie S4), slight differences in temperature between the bottom and top of the slide, the aforementioned DLVO theory, the movement of bubbles or debris, the movement of the eggs themselves (or some combination thereof), may have caused this phenomenon. The storage of these eggs for up to 1 month at 4 °C may have also impacted flotation, although egg counts have been reported to not decline with statistical significance in samples stored over a 28 day period (Sengupta et al., 2016). Neither morphological changes nor embryonation were noted during this study, therefore the age of the eggs was not taken into consideration during analysis; however, the impact of age or the effects of storage on egg flotation could be the aim of a future study.

Differences in the mean flotation speed of each egg type may explain why some fecal egg flotation protocols such as the standard McMaster method are less sensitive to eggs of *A. perfoliata* (Tomczuk et al., 2014; Nielsen, 2016).

We have developed a novel method for viewing and analyzing the speeds and movement patterns of eggs of endoparasites as they interact with flotation solution. This may be of use in future efforts to produce type-specific endoparasite egg flotation protocols and can aid in optimizing egg flotation protocols for reliably detecting parasite ova of clinical significance. Our results indicate that eggs of *A. perfoliata* float more slowly and with higher variability than do ascarid and strongyle type eggs, which suggests that detection and enumeration of this egg type may benefit from specific modifications to standard techniques. The significantly denser ascarid eggs exhibited a faster median speed than might be expected by their density alone, which suggests that flotation properties are more than just a matter of the specific densities of the egg and the surrounding flotation medium (Supplementary Movie S2). This technique may also be of interest in other fields pertaining to the study of flotation or the quantification of movement speed of small objects.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2019.06.004.

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