Relative egg extraction efficiencies of manual and automated fecal egg count methods in equines

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Summary

The World Association for the Advancement of Veterinary Parasitology recently released new recommendations for the design of fecal egg count (FEC) reduction tests for livestock. These provide suggestions as to the number of animals to be sampled and the minimum number of eggs that must be counted to produce statistically meaningful results.
One of the considerations for study design is the multiplication factor of the FEC method to be used; methods with lower multiplication factors require fewer animals to be sampled because they are presumed to count more eggs per test. However, multiplication factor is not the sole determinant of the number of eggs counted by any given method, since different techniques use very different sample extraction methodologies that could affect the number of eggs detected beyond just the amount of feces examined.
In this light, we compared three commonly used manual FEC methods (mini-FLOTAC, McMaster and Wisconsin) and two automated methods (Imagyst and Parasight All-in-One) with respect to how many equine strongylid and ascarid eggs they counted in the same samples.
McMaster and mini-FLOTAC (multiplication factors of 25x and 5x, respectively) produced the most accurate results of the methods tested but mini-FLOTAC counted approximately 5-times more eggs than McMaster. However, Wisconsin and Parasight (multiplication factor = 1x) counted 3-times more ova than mini-FLOTAC, which was less than the 5-fold difference in their multiplication factors. As a result, these tests perform with multiplication factors more akin to 1.6x relative to mini-FLOTAC. Imagyst, due to its unique sample preparation methodology, does not have a traditional multiplication factor but performed similarly to McMaster with respect to egg recovery.
Keywords: Fecal egg count; McMaster; mini-FLOTAC; Wisconsin; Parasight; Imagyst

Introduction

Fecal egg counts (FECs) are a mainstay of parasite management programs for pasture animals (Kaplan, 2013; Nielsen, 2021b), including equines (Nielsen, 2021a). In horses these tests primarily serve two purposes: the identification of high egg-shedding individuals for targeted treatment to reduce environmental infection pressure (Nielsen *et al*., 2019); and their use in fecal egg count reduction testing for the detection and quantification of anthelmintic drug resistance in a population (Kaplan *et al*., 2023).
Most FEC methods utilize the fact that densities of many parasite ova are lower than the majority of the fecal material. Suspension of feces in a high-density flotation medium therefore facilitates the separation of parasite products from the bulk of the feces, easing

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identification of the ova that would otherwise by obscured by the fecal matrix.

Traditionally, ova have been quantified manually by scrolling through the sample with a microscope while counting individual ova, but more recently systems have been developed that automate the counting process by utilizing deep-learning computer vision algorithms (Bucki et al., 2023; Cain et al., 2020; Cringoli et al., 2021; Elghryani et al., 2020; Nagamori et al., 2020; Nagamori et al., 2021; Scare et al., 2017; Slusarewicz et al., 2016). The number of ova counted using these methods are usually normalized to a metric expressed as eggs per gram (EPG) of feces by multiplying by a specific value, the multiplication factor. The multiplication factor may vary between FEC methods; more specifically it is the reciprocal of the number of grams (usually less than one) examined under the microscope, which itself is a function of the concentration of the feces in the flotation medium slurry and the volume of the slurry examined.

Until recently, the multiplication factor has not necessarily been a priority consideration when selecting a FEC method, a decision that could equally have been made based on ease-of-use, the time taken to conduct a test, or equipment availability. Recently, the World Association for the Advancement of Veterinary Parasitology (WAAVP) introduced new recommendations for the design of Fecal Egg Count Reductions Tests (FECRTs) to monitor anthelmintic resistance in livestock (Kaplan et al., 2023) that may lead to a reevaluation of these decision processes. These recommendations now include suggestions for the minimum number of eggs that must be counted in a FECRT protocol to produce statistically meaningful results, which has ramifications with respect to the number of animals to be sampled and the number of counts that need to be conducted on those samples. One of the considerations in the decision tree is the multiplication factor of the FEC method to be used; methods with lower multiplication factors facilitate the sampling of fewer animals and potentially the counting of fewer subsamples i.e., in general tests that count more eggs from the same samples result in less effort in conducting FECRTs.

It is generally considered that tests with lower multiplication factors are more sensitive because they count more eggs in a sample (by analysing more feces); in other words, methods that examine more fecal material are more likely to encounter an egg when counts are low. This attitude, however, is based on the faulty assumption that all tests perform equally well with respect to recovering eggs from the feces (Nielsen, 2021b). In reality, the actual performance of any given test is dependent not only on the amount of sample analyzed, but also on the methodology used to extract the ova from the fecal matrix prior to counting; this can only be determined empirically.

The primary purpose of this study was to compare the relative egg-extraction efficiencies of various FEC methods with respect to each other in order determine whether their relative multiplication factors can be considered as proxies for relative numbers of eggs counted when a test method is selected under the new guidelines.

These five methods entail four radically different sample preparation methodologies that could significantly affect the number of eggs counted beyond merely considering their individual multiplication factors.

Three commonly used manual methods were selected for comparison; these were mini-FLOTAC (Barda et al., 2013) and the McMaster and Wisconsin protocols described in the American Association of Equine Practioners' (AAEP) Parasite Control Guidelines (Nielsen et al., 2019). The automated methods were Imagyst (Zoetis Inc., 2023) and the Parasight System (Scare et al., 2017; Slusarewicz et al., 2016). In the latter case, we used a second-generation device, Parasight All-in-One (AIO), which has significantly improved imaging capabilities and a significantly different sample preparation process. A secondary aim of this study was therefore to evaluate Parasight AIO's performance with respect to the other methods.

Material and Methods

Fecal Samples
Samples were collected from a mixture of foals and mares in the University of Kentucky's parasite research herd. Samples were placed into sealable plastic bags and air was removed by squeezing prior to sealing. Samples were stored for no more than 1 week at 4°C prior to analysis.
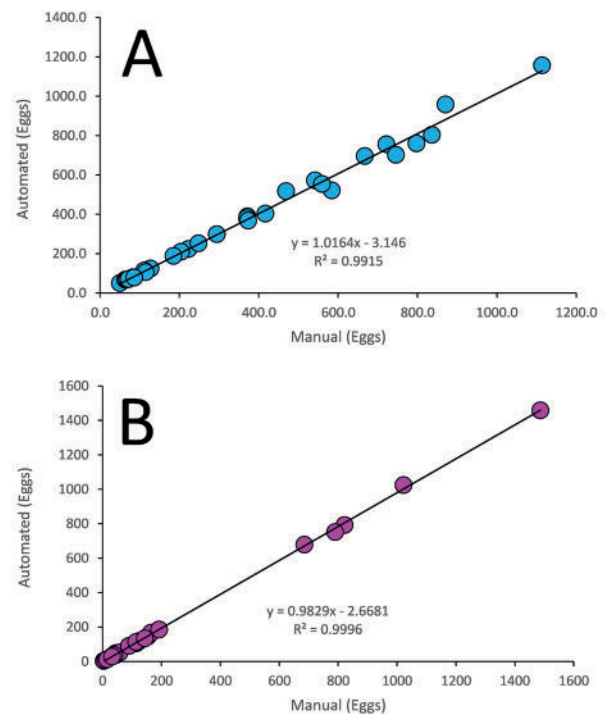

Fig. 1. Comparison of Parasight AIO counting algorithm to human analyst enumeration for both strongylid (A) and ascarid (B) ova. Triplicate images for each sample were analyzed both computationally and manually and the averages of each result plotted against each other.

Study Design

Samples were pre-screened using mini-FLOTAC and assigned to one of three groups based on eggs/gram content of strongyles. The groups were assigned as Low (5 – 200 EPG), Medium (201 – 650 EPG) and High (>650 EPG). Ten individual samples were assigned to each group.

Of these 30 samples, 22 were also infected with ascarids, which were also counted. Five of the ascarid-positive samples fell into the High group, 7 into the Medium and 10 into the Low.

Each sample was counted in triplicate using each method, with each count being performed on a separate independent subsample of the parent fecal sample. All counts for each sample were performed on the same day.

Counting Methods

Unless indicated otherwise, the flotation medium used in this study was sodium nitrate (Fecamed, Vedco Inc. St. Joseph MO) with, again unless indicated otherwise, a specific gravity of 1.25 g/L. Mini-FLOTAC counts were performed as described previously (Barda *et al.*, 2013), while McMaster and Wisconsin counts were performed as described in the AAEP's Parasite Control Guidelines (Nielsen *et al.*, 2019) using Sheather's flotation medium and a fixed-angle centrifuge for Wisconsin centrifugation. All manual counts were conducted using Nikon Eclipse E200 microscopes at a magnification of 100x.

Parasight AIO (Parasight System Inc., Lexington KY) samples were prepared by suspending 6 g of feces into the silicone bottle of a Sample Preparation Tool that had previously been filled with 54 mL of flotation medium (diluted to a density of 1.18 g/L). The sample was suspended with 12 rapid depressions of the spring-loaded plunger integrated into the handle and then 10 mL was dispensed though the filter cap into a 15 mL centrifuge tube. The sample was then spun at 2000 *g* for 1 min in a CF-800-1 fixed-angle centrifuge (Hardware Factory Store Inc., Azusa, CA). A single-use egg separator tool consisting of a hollow cylinder fitted at the distal end with a 130 µm filter and a rubber sealing gasket was inserted into the tube. This device allowed floated ova to pass through the mesh while preventing debris in the pellet from dislodging and contaminating the sample in the subsequent step. The sample was then poured into an egg chamber placed on the device and suctioned through the mesh with a vacuum. Once pouring had been completed the device automatically bleached, stained, washed, imaged and analyzed the sample.

To separately assess the performance of the Parasight AIO algorithm compared to manual counting, the same images were counted both using the algorithm and by manual inspection of the same raw images generated by the device. The algorithm-generated results exhibited extremely high agreement with manual counts of the same images (Fig. 1) as assessed using Lin's concordance correlation for both strongyles (R^2 = 0.996, 95% CI = 0.991-1.0) and ascarids (R^2 = 0.999, 95% CI = 0.999-1.0). A portion of a representative Parasite AIO image used to generate these data is shown in Figure 2.

Imagyst samples were processed as directed by the manufacturer (Zoetis Inc., Kalamazoo, MI). Briefly, 4 g of feces was suspended in 26 mL of flotation medium in a 5 oz. paper cup, mixed thoroughly, and then filtered into a second cup through 2-ply cheesecloth. The suspension was left to sit for 10 minutes. The entire surface was then skimmed with an Imagyst sample loop to harvest floated eggs and the loop dabbed onto a glass slide. The sample was then
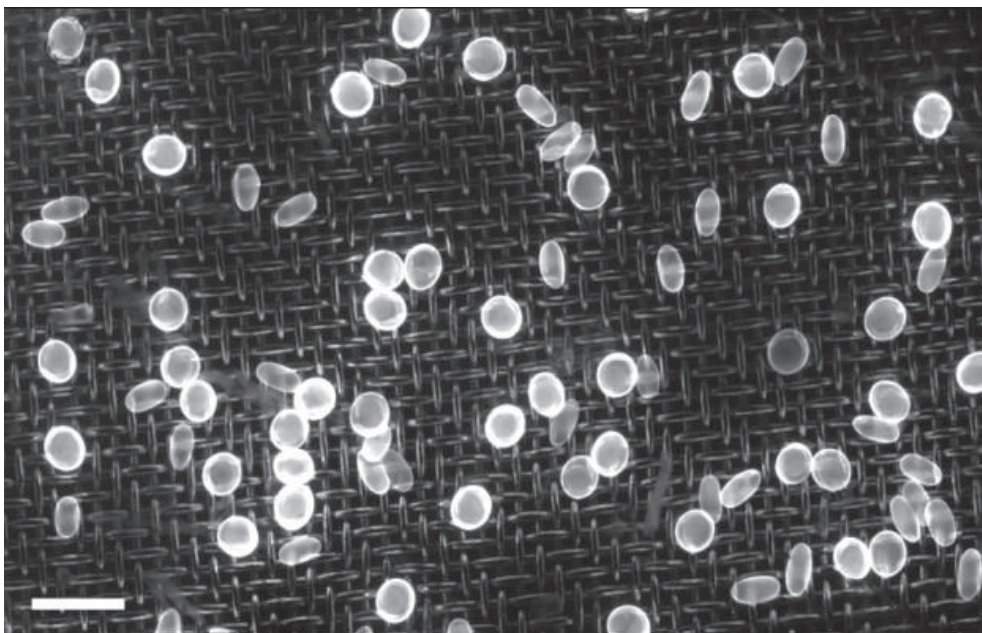

Fig. 2. Fluorescence imaging of parasite ova. A portion from a representative image taken with the Parasight AIO unit showing both strongylid and ascarid ova. The mesh of the egg chamber is visible in the background. Bar = 200 µm.

Table 1. Mean numbers of strongylid and ascarid ova counted by each method in each count group and for all groups combined. n = number of positive samples in the indicated group.

Parasite	Group	n	McMaster	mini-FLOTAC	Wisconsin	Imagyst	Parasight
Strongylids	H	10	48.9	220.8	822.4	84.4	730.8
	M	10	18.5	92.2	303.4	31.6	339.7
	L	10	4.4	25.1	87.0	8.7	82.5
	All	30	23.9	112.7	404.3	41.6	381.2
Ascarids	H	5	42.1	304.4	923.7	51.3	941.4
	M	7	10.4	70.7	171.0	16.3	134.6
	L	10	3.2	13.5	38.8	2.4	37.6
	All	22	14.3	97.8	282.0	17.9	273.9

H = High EPG (>650), M = Medium EPG (201-650), L = Low EPG (5-200).

overlaid with a coverslip and counted manually. Since we were unable to purchase an Imagyst microscope unit from the manufacturer, we were unable to assess the automated counting capability of the system but rather only the sample preparation aspect of the method. However, it has been shown that Imagyst algorithms perform comparably to counting by a parasitologist (Nagamori *et al.*, 2020; Nagamori *et al.*, 2021). In particular, the equine model generates Lin's concordance correlation coefficients of 0.978 and 0.944 for strongylid and ascarid ova, respectively (Zoetis Inc.), indicating that manual counting would serve as a suitable proxy when comparing to the other methods. However, in order to remove the potential confounding factor of algorithm performance from the comparison between both automated and manual method sample preparation methods, we used manual Parasight AIO counts when analyzing the data.

Statistical Analysis
Comparison of regression line slopes was performed using the "SlopesTest" function of the Real Statistics Resource Pack add-in (https://real-statistics.com/) for Microsoft Excel (Microsoft, Redmond, WA). The same add-in was used for calculation of Lin's concordance correlation coefficients using the "LINCCC" function. Significance testing of differences between EPGs and counted eggs by the various methods was conducted using a linear mixed model with a Bonferroni correction in SPSS build 1.0.0.1447 (IBM, Armonk, NY). Counted eggs or EPGs were set as the dependent variable, test-type and sample group (i.e., High, Medium, or Low) were selected as fixed effects and sample ID was selected as a random effect. Counted eggs were normalized by log-transformation prior to analysis.
All differences were considered significant at the $p<0.5$ level.

Ethical Approval and/or Informed Consent

Approval and consent were not required for this study because it did not utilize human subjects or vertebrate animals.

Results

Average results expressed as the mean of the total eggs counted by the triplicate counts of each method were plotted against the same results generated using mini-FLOTAC (Fig. 3). Least-square linear regressions were fitted for each egg type (strongylids and ascarids) individually. In some cases, there were substantial differences in the slopes of the lines for each egg type depending on the methods being compared with the slope ratios (strongylid/ascarid) being Imagyst (2.41)>McMaster (1.58)>Wisconsin (1.21)>Parasight AIO (1.04). These differences were all statistically significant

Table 2. Summary of the average number of eggs counted (EC) across all samples in the dataset for both strongyles and ascarids. Eggs counted were converted to eggs/gram (EPG) values by multiplication of EC by the appropriate multiplication factor (MF) for the method. The magnitude of the difference of EC and EPG relative to the mini-FLOTAC (mFT) method are also shown (EC vs. mFT and EPG vs. MFT).

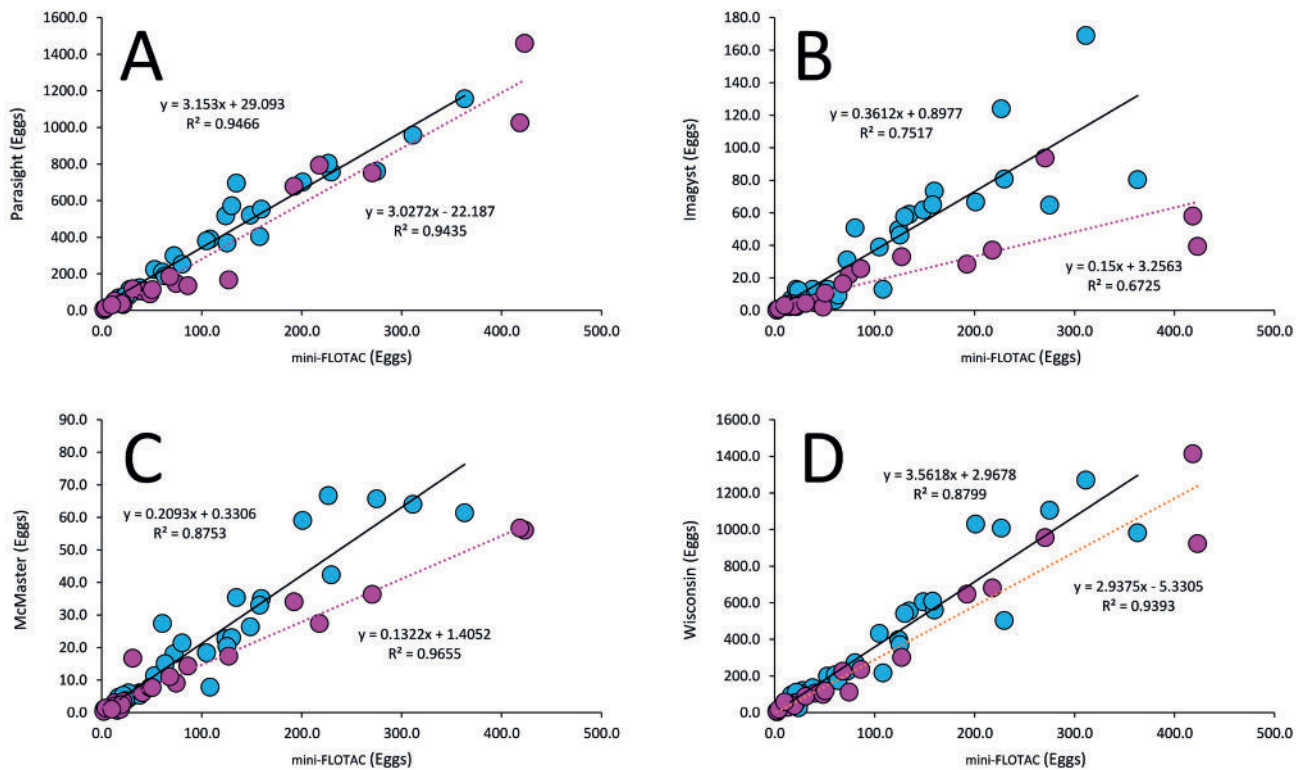| Method	MF	Strongyles				Ascarids			
		EC	EPG	EC vs mFT	EPG vs mFT	EC	EPG	EC vs mFT	EPG vs mFT
Wisconsin	1	404.3	404.3	3.59	0.72	282.0	282.0	2.88	0.58
Parasight	1	381.2	381.2	3.38	0.68	273.9	273.9	2.80	0.56
mini-FLOTAC	5	112.7	563.3	1.00	1.00	97.8	489.0	1.00	1.00
McMaster	25	23.9	597.8	0.21	1.06	14.3	358.3	0.15	0.73
Imagyst	N/A	41.6	N/A	0.37	N/A	17.9	N/A	0.18	N/A

Fig. 3. Comparison of mini-FLOTAC counts against counts of the same samples by Parasight AIO (A), Imagyst (B), McMaster (C) and Wisconsin (D). Each point is the average of three counts of the same sample by each method. Strongyle counts (blue) and ascarid counts (magenta) were plotted separately and fitted by linear regression to provide two lines whose equations and coefficients of determinations are displayed next to them.

except for that of Parasight AIO. Similar plots showing comparisons of all the methods against each other are presented in supplemental figures 1-4. In these cases, differences were also all significant except for the comparison of Parasight AIO vs Wisconsin (ratio= 0.83) and Imagyst vs McMaster (ratio = 0.79).

The results for the average number of each egg-type counted in each group by each method as well as across the entire dataset are summarized in Table 1. For both strongylids and ascarids, the differences between test methods were significant between all methods except for Wisconsin and Parasight AIO and followed the order Wisconsin=Parasight>mini-FLOTAC>Imagyst>McMaster.

Compared to mini-FLOTAC, Wisconsin and Parasight AIO counted approximately 3.5 times more strongylid ova while Imagyst and McMaster counted approximately one third and one fifth as many respectively (Table 2). When absolute counts were converted to EPG using each method's respective multiplication factor, McMaster and mini-FLOTAC produced the highest counts, which were not significantly different from each other. They were, however, significantly higher than EPGs for both Wisconsin and Parasight AIO, which, in turn were not significantly different but approximately 30 % lower than mini-FLOTAC/McMaster. EPG could not be calculated for Imagyst for either ascarids or strongyles, since the unique sample preparation method for this test does not produce a traditional multiplication factor.

In the case of ascarids, Wisconsin and Parasight AIO counted approximately 3 times more eggs than mini-FLOTAC while Imagyst and McMaster both counted approximately one sixth as many respectively (Table 2). As with strongylids, McMaster and mini-FLOTAC produced the highest EPGs for ascarids, but unlike with strongylids the difference between them was significant, with mini-FLOTAC producing EPG values approximately 40 % greater than McMaster. The mini-FLOTAC EPGs were significantly higher by approximately 40 % compared to Wisconsin and Parasight AIO while the McMaster EPGs were not significantly different from Wisconsin and Parasight AIO.

All of the tests produced positive results in all strongyle counts. This was also the case for ascarids with Wisconsin, mini-FLOTAC and Parasight AIO; however, both McMaster and Imagyst produced some false negative results in some low-count ascarid samples. The complete dataset for ascarid absolute counts in the Low group for these two methods are shown in Table 3. The EPG counts of each sample as calculated using the average mini-FLOTAC count multiplied by its multiplication factor (5x) are also shown.

As a whole, McMaster generated 7 false negatives in the 30 counts of the Low group, while Imagyst generated 9. Both McMaster and Imagyst generated at least one negative count in 5 of the ten samples but not all in the same samples. Negatives from both tests were generated in 3 samples with EPGs ranging between

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Table 3. Number of ascarid ova observed in each replicate count from the Low group samples for both the McMaster and Imagyst methods.

Sample	Count (EPG)	McMaster			Imagyst		
		Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
A	12	0	2	1	0	0	0
B	10	0	0	1	0	1	0
C	65	7	0	2	4	2	1
D	93	3	0	0	3	2	7
E	107	3	4	3	2	0	5
F	155	16	18	16	0	6	7
G	20	3	1	0	3	0	0
H	65	2	2	2	5	5	1
I	98	3	1	3	4	2	2
J	50	1	1	1	2	2	5

10 and 20, while the remaining mutually exclusive counts (where either McMaster or Imagyst produced at least one negative) were distributed among 4 samples with EPGs ranging between 65 and 155. Only Imagyst produced negative in all three replicates of a single sample (Table 3, sample A), whose EPG (as assessed by the sample's averaged mini-FLOTAC counts) was 12.

Discussion

With respect to the secondary aim of this study, the Parasight AIO algorithm performed with very high concordance compared to manual counting of the same images (Fig. 1) and so produced almost identical results. Furthermore, Parasight AIO results produced strong correlations with all the other methods tested here (Supplementary Fig. 1). The strongest correlation (as assessed by coefficients of determination – R^2) was with mini-FLOTAC ($R^2 \sim$ 0.94) followed by Wisconsin/McMaster ($R^2 \sim$ 0.84 - 0.92) and then Imagyst ($R^2 \sim$ 0.56 – 0.75).

Most of the FEC methods compared in this study utilize disparate methodologies to process samples prior to counting, which could influence the number of ova counted by each method independently of its multiplication factor. The multiplication factor for Wisconsin and Parasight AIO are 1 (since both count ova derived from 1 g of feces), that of mini-Flotac is 5 and that of the McMaster version used here is 25. The multiplication factor of Imagyst is unknown and has not, to our knowledge, been reported by the manufacturer, most likely because its unique sample preparation approach precludes calculation of a traditional multiplication factor (see below). The two methods whose sample preparations are relatively similar are mini-FLOTAC and McMaster, the main difference being in the filtration system for the fecal slurry (a plastic injection-molded filter vs. cheesecloth, respectively) and in the concentration of the fecal slurry (0.1 g/mL vs. 0.133 g/mL, respectively). Essentially both methods involve the suspension of the sample in flotation medium,

filtration, and loading of a portion of the slurry into a specialized counting slide. The major difference here, which is conferred by the design and geometry of the respective counting slides, is that mini-FLOTAC counts a total of 0.2 g of feces while McMaster counts 0.04 g. As a result, the multiplication factor of mini-FLOTAC is 5 whereas that of McMaster is 25 (though variants of the latter exist where the multiplication factor can be 50 or even 100 depending on the concentration of the fecal slurry and the number of slide chambers counted).

The third manual method, the Wisconsin, counts a full 1 g of feces at the expense of a radically different sample preparation method. The 1 g of sample is first suspended in water and centrifuged. The supernatant is replaced by flotation medium and centrifuged again, at which point more flotation medium is added to the tube to the point of forming a meniscus. A glass coverslip is overlaid on the tube and then removed after sufficient time has elapsed to allow the ova to float to the surface under gravity and adhere to the coverslip. The advantage of this method is its ability to process more fecal material (and thus potentially count more eggs) at the expense of possibly losing some ova during the more convoluted sample processing.

All-but-one current automated FEC methods utilize high-magnification visible light imaging systems coupled to automated moving stages to produce large, high-resolution files for subsequent computational analysis. In contrast, the Parasight System utilizes fluorescence imaging following labeling of helminth ova with a recombinant chitin-binding protein that attaches to the surface of the eggs. The high contrast and binding specificity of this system means that samples can be imaged at lower magnification. One consequence of this approach is that ova are captured and imaged on a filter, which further serves to facilitate staining and washing of the sample, which in turn results in a unique sample preparation methodology. The second-generation device used here has a higher magnification (1x vs. 0.5x) to improve image quality, and

as a consequence a smaller filter area to facilitate imaging in a single exposure. In order to prevent clogging of this smaller area, the sample is first filtered, centrifuged briefly in flotation medium to remove excess fecal particulates, and then filtered again post-spin to prevent sedimented material from falling onto the mesh and obscuring the ova. Both centrifugation and the filtration steps are potential sources of egg loss that could adversely affect egg recovery. Of all the methods tested here, Imagyst is unique in that it does not sample from a homogenous slurry of suspended fecal material. Instead, after preliminary filtration through cheesecloth, the slurry containing the entire 4 g sample is allowed to sit to facilitate separation of eggs from feces under gravity; ova are then transferred to a slide using a circular plastic sample loop skimmed over the entire surface of the slurry. Thus, Imagyst does not analyze a fixed volume of an evenly mixed slurry, and so does not have a traditional multiplication factor. However empirical data such as those presented here can provide an estimate of the "effective multiplication factor" of this system relative to others.

Since mini-FLOTAC lay in the middle of the five methods with respect to the number of strongyles recovered and counted from the samples, and since it is a method that is widely adopted by research parasitologists, we primarily compared the other methods to it, although full comparisons of each method against the others are presented in the Supplemental Figures.

One unexpected finding from this study was that in some cases there were significant differences in the recovery of strongylid versus ascarid ova from the same samples. As assessed by the ratio of the slopes of strongylid:ascarid lines of best-fit for different test comparisons, this difference was negligible and insignificant when comparing mini-FLOTAC to Parasight AIO (1.04), small but significant (1.21) for Wisconsin, moderate and significant (1.58) for McMaster and large and significant (2.41) for Imagyst.

It is unclear what phenomenon or phenomena might underly these differences. Interestingly, the three methods with the largest (and significant) deviations from mini-FLOTAC all utilized cheesecloth as a filter material in the preliminary steps to remove the bulk of the fecal material. In contrast mini-FLOTAC used an injection molded pre-filter while Parasight AIO uses a combination of molded and stainless-steel filters. One possibility, therefore, is that ascarid ova could exhibit differential adhesion/adsorption compared to strongylids to the cellulosic fibers of the cloth, and less so to the filters in the other two systems. It has been previously reported that filtering though the mini-FLOTAC filter vs. cheesecloth results in relatively higher counts of bovine trichostrongylids, possibly due to such a phenomenon (Paras *et al.*, 2018). However, simple filter-effects alone do not explain the largest difference observed, i.e., with Imagyst, which also uses a cheesecloth filtering step. The Imagyst method samples floated ova from the flotation medium surface using circular plastic sample loop and so relies on the flotation medium's surface tension to produce films of liquid in the four quadrants of the loop; it is these films that are dabbed onto the microscope slide for analysis. One possible explanation of the larger

magnitude of the mini-FLOTAC-Imagyst difference, therefore, is that there is a differential efficiency in the transfer of strongylid vs. ascarid ova to the loop films, perhaps due to differences in egg geometry or physico-chemical surface characteristics such a hydrophobicity or charge density.

Some of these hypotheses could be tested experimentally, and further work will be required to determine the nature of this differential extraction. In any case, this observation indicates that it may be prudent not to assume that all ova types are extracted equally well by any given FEC sample preparation method (as is the case when single global multiplication factors are applied to counts to generate EPGs). Many published reports comparing egg counting techniques have focussed on only counting one egg type as a proxy for test performance for all parasite genera; the data presented here suggest a reconsideration of this generalized approach to qualifying/validating tests for host species that carry numerous distantly related parasites.

Historically, the multiplication factor of a method has also been referred to as the "sensitivity" or the "detection limit" (Bagley *et al.*, 2014; Ballweber *et al.*, 2014; Dias de Castro *et al.*, 2017; Levecke *et al.*, 2012). Thus, a test such as the Wisconsin (multiplication factor=1), is considered five-times more sensitive than mini-FLOTAC (multiplication factor=5) because the former counts 1 g of feces while the latter counts only 0.2 g. This nomenclature is misleading, however, because it is based on the assumption that only the amount of feces from a sample that makes it to the microscope determines the sensitivity of a test, but this is almost certainly never the case (Nielsen, 2021b). The equivocation of multiplication factor and sensitivity/detection limit assumes that each test quantitatively extracts either all or the same proportion of the ova in a fecal sample and that there are no differences between sample preparations methodologies in this respect. The data presented here (and in many other studies (Bosco *et al.*, 2014; Cain *et al.*, 2020; Godber *et al.*, 2015; Levecke *et al.*, 2012; Shifaw *et al.*, 2021)) clearly demonstrate that this is not the case, and that it even may not be the case between different egg types in the same fecal sample. Thus, multiplication factor should not be considered to be synonymous with "sensitivity" and rather only regarded as a "theoretical sensitivity" i.e., as a guideline to the possible upper limit of test performance; in reality, the true sensitivity of a test can only be determined by spiking studies, while the relative sensitivities of multiple tests can only be assessed empirically by studies such as the one presented here.

Wisconsin and Parasight both recovered significantly more strongylid ova (3- to 3.5-times) than mini-FLOTAC (tables I and II), but not to the degree (five-times) that would have been expected based simply on multiplication factors, presumably due to a lower degree of egg recovery during sample preparation. Of these three methods, mini-FLOTAC and McMaster utilize the least amount of manipulation prior to counting (see above). These simple steps minimize the opportunity for egg loss, although possibilities such as entrapment of eggs in fecal debris, the presence of non-float-

ing ova or egg loss during filtration cannot be eliminated. In fact, despite this simplicity, spiking studies have shown that mini-FLO-TAC can recover 40 – 90 percent of ova (Noel *et al.*, 2017; Paras *et al.*, 2018; Shifaw *et al.*, 2021). Nevertheless, in this study mini-FLOTAC was still more efficient with respect to egg recovery. In the case of Wisconsin, additional egg loss could occur during decanting of the aqueous supernatant, entrapment in the centrifugation pellets, adhesion to the sides of the centrifuge tubes and the non-quantitative transfer of ova from the tube to the slide via the cover slip (including loss of ova in the liquid exuded from the coverslip after it is placed on the slide). Similarly, egg loss in the Parasight AIO method could occur due to centrifugal entrapment/ adhesion as well as in the non-quantitative recovery of ova during the post-centrifugal filtration step. Furthermore, Parasight AIO could also miss ova that are occluded by fecal material on the capture filter. Despite their dissimilarities in sample preparation, however, the relative losses in the Wisconsin and Parasight AIO sample preparation methods appear to be very similar as evidenced by the lack of significant differences in the number of ova recovered by either. Furthermore, despite their reduced egg recovery rate both tests were significantly more sensitive (i.e., counted more eggs) than mini-FLOTAC by virtue of being able to analyze five-times more sample albeit with a reduced recovery rate.

In contrast, McMaster, whose multiplication factor is 5 times higher than mini-FLOTAC, also detected approximately five-times fewer strongylid ova. This congruence may have been due to the essentially similar sample preparation of the two methods. However, previous comparisons of McMaster and mini-FLOTAC using both naturally infected and spiked samples have yielded contradictory results, with some studies reporting higher recovery with McMaster (Class *et al.*, 2023; Das *et al.*, 2020; Dias de Castro *et al.*, 2017; Shifaw *et al.*, 2021), other showing the opposite (Noel *et al.*, 2017; Paras *et al.*, 2018; Scare *et al.*, 2017) and yet another showing no difference (Alowanou *et al.*, 2021). Some of these incongruencies may have resulted from subtle variations in the McMaster methodologies employed, such as the flotation medium or specific filtration material used, while others could be due to the use of feces from other host-species and therefore also differential extraction of different parasite ova such as that described here between strongylids and ascarids.

Finally, Imagyst counted 1.7-times more strongylids than McMaster, 2.7-times fewer than mini-FLOTAC and approximately nine-times fewer than Wisconsin/Parasight AIO, differences that were all significant. With respect to the FECRT guidelines and strongylids, Imagyst appears to perform like a test with an effective multiplication factor of approximately fifteen. These data should be useful to those wishing to use Imagyst for conducting FECs with respect to sample size selection.

Wisconsin and Parasight AIO performed similarly to mini-FLOTAC with ascarids as they did with strongylids, counting approximately 3-times more ova (tables I and II). Due to a slight reduction in relative recovery, McMaster counted 6.6-times fewer ascarid ova

than mini-FLOTAC. The larger reduction in relative ascarid recovery meant that Imagyst counted approximately the same number of these ova as McMaster and five-times fewer than mini-FLOTAC. For the same reason, Imagyst counted approximately fifteen-times fewer ascarids than Wisconsin/Parasight AIO. As a result, the effective multiplication factor of Imagyst with respect to ascarids appeared to be approximately twenty-five.

It should be noted that ascarid infection was not a criterion in sample selection for this study. Thus, not all the samples tested were ascarid-positive, and neither were the samples divided equally into three count groups. As a result, the ascarid data were biased towards the lower EPG range, where sampling variation will be larger (Torgerson *et al.*, 2012). Ideally the differences observed here should be confirmed with samples containing larger numbers of ascarid ova.

Nevertheless, the facts that some of the ascarid-infected samples had very low egg counts and that McMaster and Imagyst counted the least number of ova of the five assessed methods, meant that these two tests were the only ones that generated false-negative results in this study (Table 3). All of these false-negative counts resided in the Low group (1 – 200 EPG). Consistent with their similar recovery of ascarid ova (Table 2), these tests generated roughly the same number of false-negative counts (7/30 for McMaster vs 9/30 for Imagyst). In general, these false negatives occurred only in samples with EPGs below 100 (as assessed from mini-FLOTAC counts), although Imagyst generated 2 such negatives in samples with EPGs between 100 and 160.

The accuracy of a method is defined as how closely its results match the "true" count of a sample. In the case of FECs, this is primarily dependent on the efficiency with which the test method extracts the eggs in a sample. In an ideal world a hypothetical test would extract and count eggs with 100 % efficiency and so after application of the multiplication factor the results would be a true reflection of the sample EPG; unfortunately, no method can achieve perfect efficiency, and there is no practical way to know the "true" count of a naturally infected sample so that this efficiency can be determined. Thus studies aimed at such a goal tend to use samples spiked with known numbers of eggs, even though processing of spiked samples may not fully reflect the extraction of eggs from naturally infected counterparts (Nielsen, 2021b) and spiking studies have previously reported disparate recovery efficiencies for the same FEC method (Noel *et al.*, 2017; Paras *et al.*, 2018; Shifaw *et al.*, 2021). An alternative approach, such as the one in this study, is to identify methods that produce the highest counts after applying the multiplication factor in order to determine relative recovery efficiencies. While this does not facilitate the determination of absolute accuracy, it does provide information regarding the relative accuracies of the methods being tested and provides empirical data that can be used to interconvert results from different methods.

When the absolute counts in this study were converted to EPGs using the appropriate multiplication factors, mini-FLOTAC and Mc-

Master generated higher counts than both Wisconsin and Parasight AIO. As a result, the former can be considered to be more accurate because they produce results that are, by definition, closer to the actual count of the sample but never above it. The relative accuracy of Imagyst could not be determined due to the lack of a multiplication factor for the method, however, empirical data such as those presented here can be used to determine calibration factors to convert its result to McMaster, Wisconsin or mini-FLOTAC "equivalent" EPGs. This also applies to the methods that underestimate EPGs i.e., Wisconsin and Parasight AIO, thereby allowing analysts to select tests that are more appropriate for their needs, such as ease-of-use, sensitivity, speed etc. without compromising accuracy.

The data presented in this study show that multiplication factor is not necessarily a proxy for the sensitivity of a given FEC method since different methods extract ova from fecal samples with differing efficiencies. McMaster and mini-FLOTAC were equally effective and superior at egg extraction and so produced the most accurate results. However, Wisconsin and Parasight AIO were more sensitive by virtue of their ability to count more eggs from the same samples, although not by the 5-fold and 25-fold that might be expected when compared to mini-FLOTAC and McMaster based on multiplication factor differences alone. Relative to McMaster and mini-FLOTAC, Wisconsin and Parasight AIO performed like tests with an effective multiplication factor of 1.6 (since they counted approximately 3x or 15x more eggs than mini-FLOTAC and McMaster, respectively, as opposed to the 5x and 25x that would have been expected based solely on their multiplication factors). The other automated system tested (Imagyst) performed similarly to McMaster. These data should prove useful when designing FECRT protocols with respect to test method selection and animal sampling.

Furthermore, the observation here that some tests extract ascarids with differing efficiency to strongylids suggests that the assumption that any given test extracts all helminth ova with equal efficiency should be reconsidered. The application of a single global multiplication factor to all ova species/genera may be an oversimplification and that should be taken into account when developing new FEC methods and revisited with current established protocols.

Conflicts of Interests

LB and PS are full-time employees of Parasight System Inc. (PSI), the manufacturer of Parasight AIO. BR is a contract employee of PSI. PS holds stock in PSI.
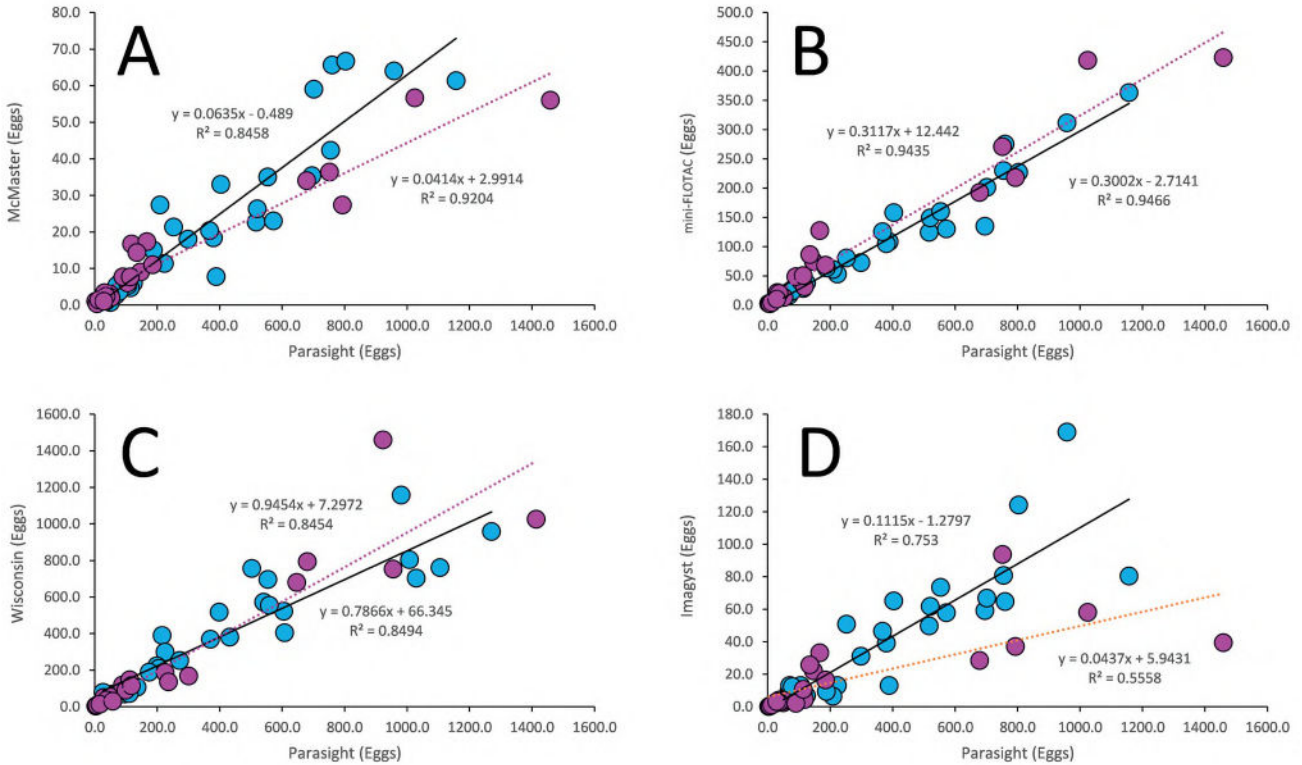
Acknowledgments

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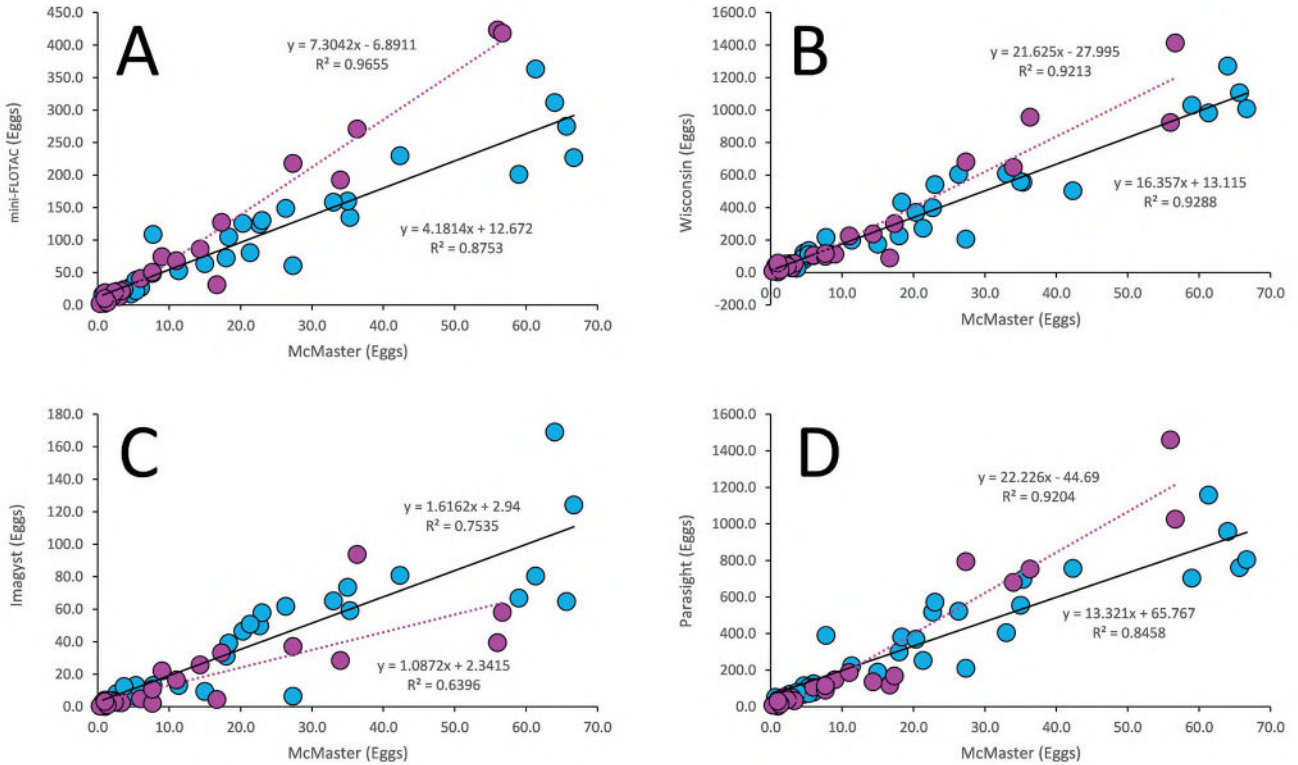ZOETIS INC. (2023): *AI Fecal Egg Count (FEC) Analysis*. Retrieved 10/11/2023 from https://www.vetscanimagyst.com/equine/ai-fec-analysis

Supplementary material
(03_paper.pdf)

Supplementary Fig. S1. Comparison of Parasight AIO counts against counts of the same samples by McMaster(A), mini-FLOTAC (B), Wisconsin (C) and Imagyst (D). Each point is the average of three counts of the same sample by each method. Strongyle counts (blue) and ascarid counts (magenta) were plotted separately and fitted by linear regression to provide two lines whose equations and coefficients of determinations are displayed next to them.

Supplementary material
(03_paper.pdf)

Supplementary Fig. S2. Comparison of McMaster counts against counts of the same samples by mini-FLOTAC (A), Wisconsin (B), Imagyst (C) and Parasight AIO (D). Each point is the average of three counts of the same sample by each method. Strongyle counts (blue) and ascarid counts (magenta) were plotted separately and fitted by linear regression to provide two lines whose equations and coefficients of determinations are displayed next to them.

Supplementary material
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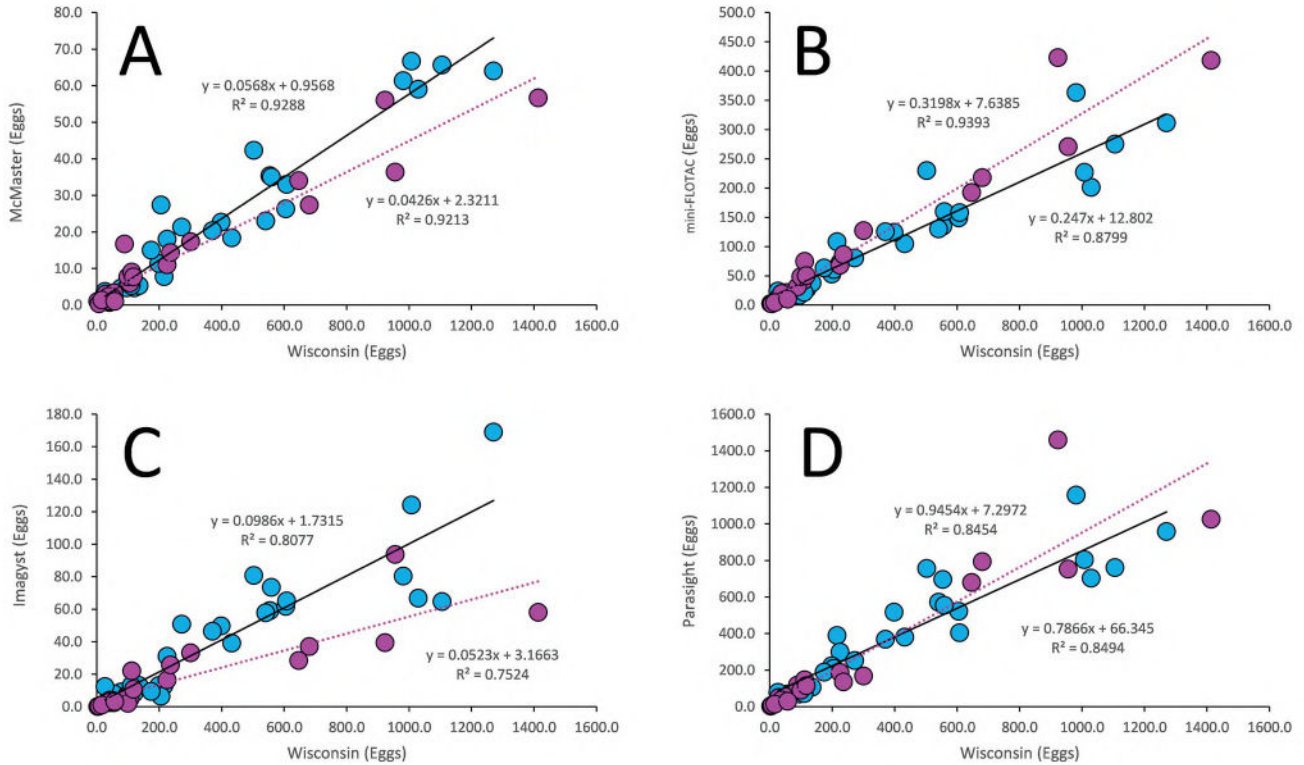
Supplementary Fig. S3. Comparison of Wisconsin counts against counts of the same samples by McMaster(A), mini-FLOTAC (B), Imagyst (C) and Parasight AIO (D). Each point is the average of three counts of the same sample by each method. Strongyle counts (blue) and ascarid counts (magenta) were plotted separately and fitted by linear regression to provide two lines whose equations and coefficients of determinations are displayed next to them.

Supplementary material
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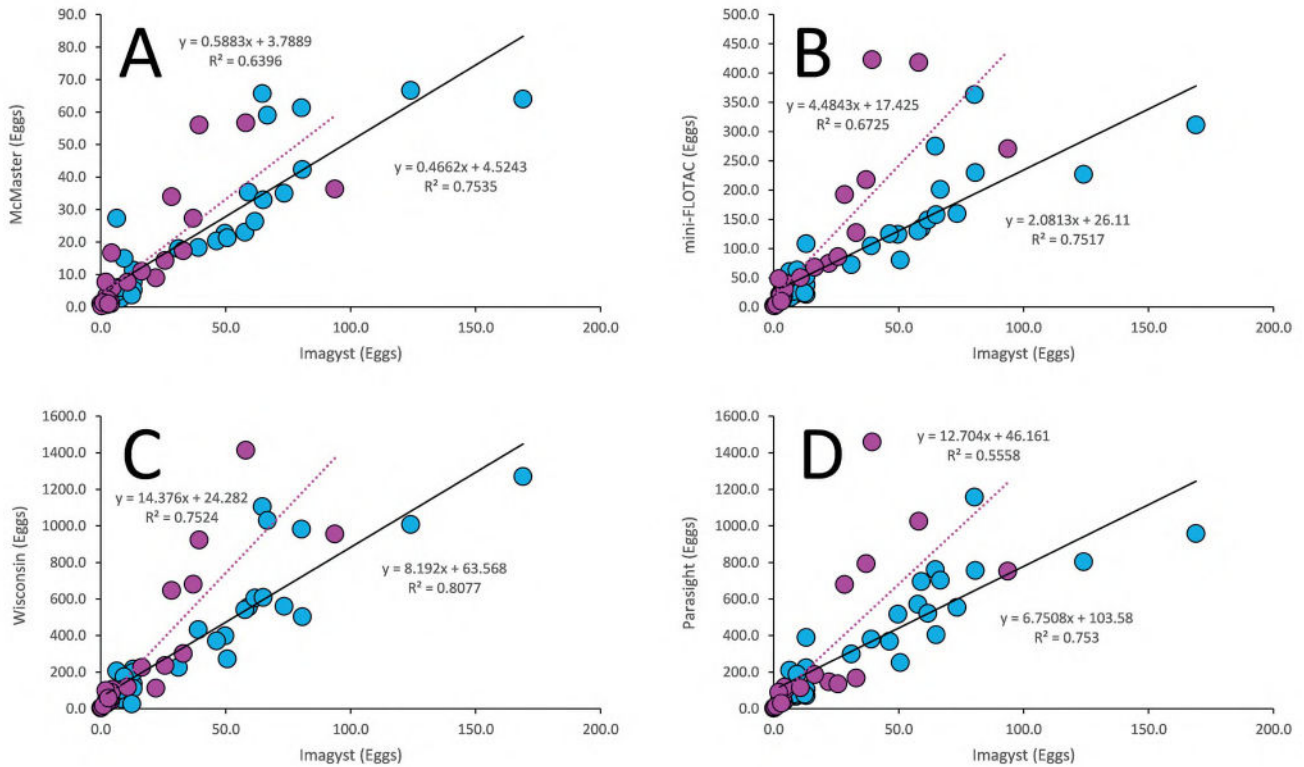
Supplementary Fig. S4. Comparison of Imagyst counts against counts of the same samples by McMaster(A), mini-FLOTAC (B), Wisconsin (C) and Parasight AIO (D). Each point is the average of three counts of the same sample by each method. Strongyle counts (blue) and ascarid counts (magenta) were plotted separately and fitted by linear regression to provide two lines whose equations and coefficients of determinations are displayed next to them.