

A comparison of two methods for quantifying parasitic nematode fecundity

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Abstract Accurate measures of nematode fecundity can provide important information for investigating parasite life history evolution, transmission potential, and effects on host health. Understanding differences among fecundity assessment protocols and standardizing methods, where possible, will enable comparisons across different studies and host and parasite species and systems. Using the trichostrongyle nematode *Cooperia fuelleborni* isolated from wild African buffalo (*Syncerus caffer*), we compared egg recovery and enumeration between two methods for measuring the fecundity of female worms. The first method, in utero egg count, involves visual enumeration of the eggs via microscopic inspection of the uterine system. The second method, ex utero egg count, involves dissolving the same specimens from above in a sodium chloride solution to release the eggs from the female's uterus, then enumeration under an inverted microscope. On

average, the ex utero method resulted in 34% more eggs than the in utero method. However, results indicate that the two methods used to quantify parasitic nematode fecundity are highly correlated. Thus, while both methods are viable options for estimating relative nematode fecundity, we recommend caution in undertaking comparative studies that utilize egg count data collected using different methods.

Keywords Fecal egg count · Helminth fecundity · *Cooperia fuelleborni* · Trichostrongyle nematode

Introduction

Parasitic nematodes infect the gastrointestinal (GI) tracts of nearly all vertebrates (Poulin 1995; Grencis et al. 2014), and prevalence can be high in wild populations (Shaw and Dobson 1995; Koprivnikar et al. 2012). In addition to causing direct mortality (Stjernman et al. 2008), GI nematodes can also have a number of sub-lethal effects on hosts that impact fitness. For example, infection with *Ostertagia gruehneri* Skrjabin, 1929, led to reductions in host body mass, back fat depth, and the probability of pregnancy in Svalbard reindeer *Rangifer tarandus platyhyinchus* Vrolik, 1829, with ultimate effects on host fitness (Stien et al. 2002; Albon et al. 2002). Often, the fitness costs of GI nematode infection are a direct result of the number of worms harbored by a host (Hudson et al. 1992; Stjernman et al. 2008; Benesh 2011). However, fitness costs to the host have also been linked to the number of eggs produced by nematodes, i.e., their total fecundity (Rowe et al. 2008; Holand et al. 2015). For example, Holand et al. (2015) found that the number of gapeworm *Syngamus trachea* (Montagu, 1811) eggs in the parent bird's feces was negatively correlated with the proportion of successful fledglings in house sparrows (*Passer domesticus*, Linnaeus, 1758). Quantifying nematode fecundity, in addition to adult

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worm burdens, can therefore provide valuable insight into the costs of infection, patterns of dissemination in the environment, and the dynamics of local transmission.

From the parasite perspective, fecundity is central to determining how many offspring each individual will contribute to the next generation (Godfray et al. 1991). Correspondingly, parasite fecundity may be a trait under strong selection (Skorping et al. 1991; Poulin 1995, 1997). Indeed, the lifecycle of GI nematodes typically require that infective stages (eggs or larvae) leave the host in order to generate new infections. Thus, transmission of GI nematodes depends on the production of viable eggs, their development in the environment, dispersal of infective stages and potential for exposure of appropriate hosts. Production of eggs can depend on factors such as parasite body size, the availability of host resources (i.e., space and nutrients), and host immunity, that affect how much energy female nematodes can devote to egg production (Poulin 1997; Singleton et al. 2011; Romeo et al. 2014). Quantifying nematode fecundity can provide valuable insight into transmission potential, the strength of helminth competition, parasite evolutionary ecology, and the life history strategies of parasites.

There are several methods for measuring the fecundity of female nematodes, each having advantages and disadvantages in terms of cost, training, and sample preservation. In cases of non-invasive sampling of the host, total fecundity of the community of worms infecting a host can be quantified by counting worm eggs in host fecal samples (e.g., fecal flotation or sedimentation (Faust et al. 1938; Coles et al. 1992). Fecal egg counting methods, however, do not provide information on faunal diversity (species cannot usually be identified) and adult worm burdens, so per capita estimates of fecundity cannot be calculated, which limits the value of this approach for studies focused on understanding worm fecundity from a parasite perspective. When hosts are sampled through field-based collection and adult worms are isolated from the GI tract, there are two common protocols for measuring individual worm fecundity. The first method involves clearing the worm specimen (a process that renders the external cuticle transparent) and visual enumeration of the eggs via inspection of the uterine system (single, paired, or multiple tracts, depending on the taxon involved) under magnification (hereafter, *in utero* count) (Karvonen et al. 2006). Notably, this *in utero* method is non-destructive, which allows specimens to be retained for archival storage, but requires a skilled professional; further, applicability may be limited by the specific structure of the uterus and whether it is narrow and tubular with relatively few discrete eggs or broad and voluminous with numerous and overlapping eggs. A modified version of this technique involves removing the reproductive tract and extracting the eggs before counting under light microscopy (Kuzmina et al. 2012). This modified technique enhances the observer's ability to accurately count eggs, but results in the destruction of the specimen. The second method of assessing fecundity

involves the chemical digestion of the female nematode's tissues to isolate the eggs (hereafter, *ex utero* count) (Kanobana et al. 2002). This *ex utero* method allows for easy visualization and enumeration of eggs, but results in the destruction of both the specimen and the eggs. In this study, we compared egg recovery and enumeration between the *in utero* (Karvonen et al. 2006) and *ex utero* (Kanobana et al. 2002) methods of fecundity assessment. We focused on *Cooperia fuelleborni* Hung, 1926, isolated from wild African buffalo *Syncerus caffer* Sparman, 1779.

Methods

Sample collection

Adult nematode specimens were collected from African buffalo in Kruger National Park, South Africa, between June and August 2012. At the end of a 4-year study on parasite interactions (Ezenwa and Jolles 2015), buffalo were euthanized following the South African National Parks Standard Operating Procedure for Lethal Population Control. Aliquots representing 2.5% of the contents of the small intestine were collected following standard procedures (Wood et al. 1995) and preserved in 5% phosphate buffered formalin as described in Budischak et al. (2015). Adult nematode specimens were isolated by rinsing the samples through 250 and 44 μm sieves. Specimens were stored in 70% ethanol and identified at the USDA Agricultural Research Service, US National Parasite Collection (USNPC), and Animal Parasitic Disease Laboratory. We chose to focus on *C. fuelleborni* since it was the most prevalent (94% of hosts) and abundant (96% of specimens) GI nematode in the study population (Budischak et al. 2015). *In utero* and *ex utero* methods were applied to between 4 and 10 (mean \pm se 8.2 \pm 0.7) female *C. fuelleborni* specimens collected from each of ten buffalo. In total, 82 individual parasite specimens were examined.

In utero fecundity

To quantify female fecundity *in utero*, we visually inspected and counted the number of eggs within the uterine tract of each *C. fuelleborni* specimen. To enhance visualization, specimens were first cleared using a phenol-alcohol mixture consisting of 80 parts melted phenol crystals and 20 parts absolute ethanol. *In utero* counts were done at 400 \times in differential interference contrast on a Zeiss Axiophot scope. Counts were based on observations from single intact specimens starting with the first fully shelled, free, eggs in the anterior and posterior uterine limbs of each specimen, from the respective ovarian limb and including the anterior and posterior ovejector and vestibule, consistent with structure of the female system in these trichostrongyline nematodes.

Table 1 With the outliers included, ex utero egg count was still a significant predictor of the percent difference between ex utero and in utero fecundity methods. The random effect of buffalo identity explains a larger (15%), but still minimal, fraction of variance in percent difference

	Estimate	SE	df	<i>t</i> value	<i>p</i> value
Ex utero count	0.73	0.14	79.5	5.08	<0.0001
	Variance	SD			
Buffalo ID	75.3	8.7			
Residual	423.0	20.6			

Ex utero fecundity

To quantify female fecundity using the ex utero method, we dissolved the same specimens from above in bleach following the methods of Kanobana et al. (2002). Specimens were processed in 50 μ L of 5% sodium hyperchlorite (Sigma Aldrich, USA) to release the eggs from the female's uterus. After approximately 4 min in solution, the specimen tissue dissolved and the eggs were released. Because of their protective shells, eggs remained stable for an additional \sim 2 min, allowing an observer to perform egg counts before dissolution of the eggs. A 5% sodium hypochlorite concentration was selected after preliminary trials, which is a modification of the methods used by Kanobana et al. (2002). We used an inverted stage microscope (Olympus CKX41) at 100 \times power, to visualize the eggs. Each set of eggs was counted three times by a single observer. The best estimate of the replicate counts for a specimen was used as the ex utero egg count.

Statistical analysis

First, the correlation between egg counts derived from the in utero and ex utero measurement techniques was examined using a Pearson's correlation test. To explore whether host identity influenced the degree of variability between the two

methods, buffalo identity was tested as a predictor of the difference in the ex utero versus in utero egg counts using an ANOVA. Next, a general linear mixed model (GLMM) that accounted for the sampling of multiple female worms per host individual was used to test for an effect of number of eggs on the magnitude of the difference between counts (while controlling for host identity). The percent difference between ex utero and in utero egg count for each specimen was the response variable in the GLMM, ex utero egg count was included as a fixed effect, and host identity was included as a random effect.

Three outliers were excluded from all of the analyses. The outliers occurred in worms counted on the first/early days of data collection and were likely the result of a newly trained technician. Thus, a sample was considered appropriate to remove if it was not only a statistical outlier but was also counted on an early observation day. Removing these outliers allowed model residuals to meet normality assumptions but did not qualitatively change results (Table 1).

Results

The ex utero and in utero methods for assessing fecundity were strongly and positively correlated ($n = 79$, $r = 0.80$, $p < 0.0001$, Fig. 1). However, for a vast majority of specimens (95%), the ex utero count was higher than the in utero count. On average, the ex utero method resulted in 34.2% (18.5 ± 1.14) more eggs than the in utero method. Minimum, maximum, and mean egg counts are presented in Table 2. Differences in egg count between methods did not vary by host (ANOVA $F = 1.09$, $df = 9,69$, $p = 0.38$; Fig. 2). However, the percent difference between counting methods was affected by ex utero nematode fecundity (GLMM: Est \pm SE = 0.45 ± 0.12 , $t = 3.61$, $df = 51$, $p = 0.00069$;

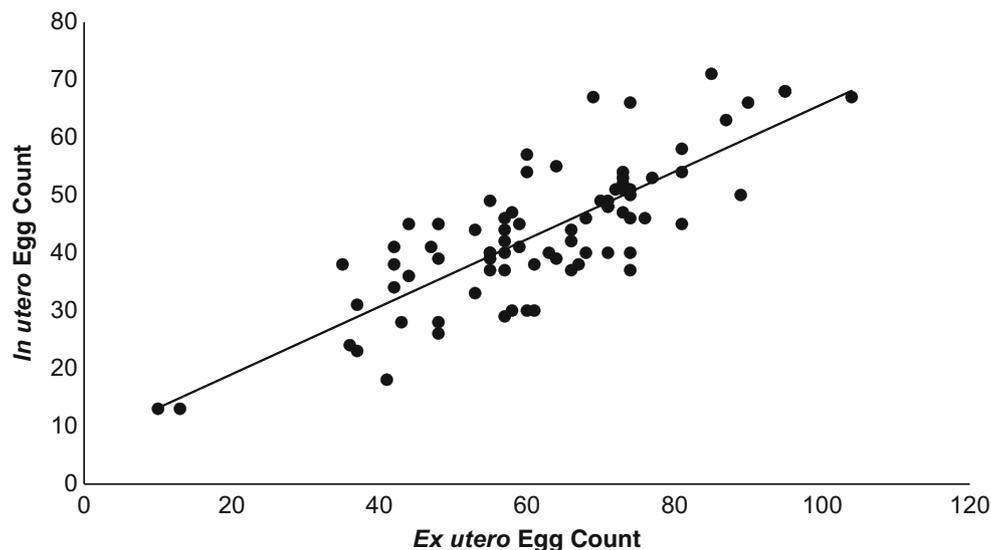


Fig. 1 Estimates of fecundity for each *C. fuelleborni* female were correlated between the two counting methods

Table 2 Minimum, maximum, and mean egg counts and standard deviation for the ex utero and in utero egg counting methods, with outliers excluded

	Ex utero	In utero
Minimum	10	13
Maximum	104	71
Mean	62.04	43.57
Standard deviation	16.81	12.19

Fig. 3). The random effect of host identity explained less than 3% of the variance in the model and was not significant (GLMM: buffalo ID: variance \pm SE = 9.4 ± 3.1 ; residual: variance \pm SE = 319 ± 18).

Discussion

Results indicate that the two methods used to quantify parasitic nematode fecundity are highly correlated, suggesting that both methods are viable options for estimating relative nematode fecundity within a study. However, the ex utero method consistently recovered a higher number of eggs. The ex utero method resulted in approximately 18.5 (34.2% difference) more eggs on average than the in utero method, which suggests that in utero counts may underestimate nematode fecundity. Moreover, percent difference was positively correlated with ex utero egg count, indicating a greater discrepancy between techniques for individuals with high fecundity. Due to these differences between techniques, comparisons across studies using these different fecundity assessment methodologies are likely to be biased.

The lower egg counts observed in the in utero counts may be due, in part, to the protocol we used for counting eggs in utero. In utero counting began by looking upstream of the ovejectors for the point between adherent eggs (i.e., shelled but not fully developed) and independent eggs. Since it is difficult to distinguish adherent eggs from developing ova visually, only independent eggs from that point to the vulva were counted. By contrast, the ex utero counts included both adherent and independent eggs because, unlike developing ova, both have shells resistant to the bleach solution. The inclusion of adherent eggs in ex utero, but not in utero counts, could account for some of the difference between the two methods.

The appropriate nematode fecundity assessment method to use in any given situation depends on the advantages and disadvantages of each method as well as study logistics, including personnel training, sample preservation or archiving needs, and time. For example, the in utero method requires highly trained personnel to count eggs while they are still in utero, but allows for the archival preservation of specimens. This method might be better suited for use in studies that prioritize specimen preservation and involve species with a relatively restricted and strongly tubular uterus, and in which the eggs are organized in a linear fashion, rather than in layers, or with a large number of overlapping eggs. For example, good candidate taxa among ungulate nematodes for the in utero method include Trichostrongylina such as species among the Cooperiinae, Trichostrongylinae, Ostertagiinae, and some Nematodirinae (e.g., Skrjabin et al. 1954; Hoberg et al. 2005, 2012). Conversely, the ex utero method could provide more reliable counts for species whose eggs are difficult to count in utero, including taxa among the Trichostrongyloidea such as Ostertagiinae with species of *Africanastromylus* Hoberg et al., 2008, and *Robustostromylus* Hoberg et al., 2009, and

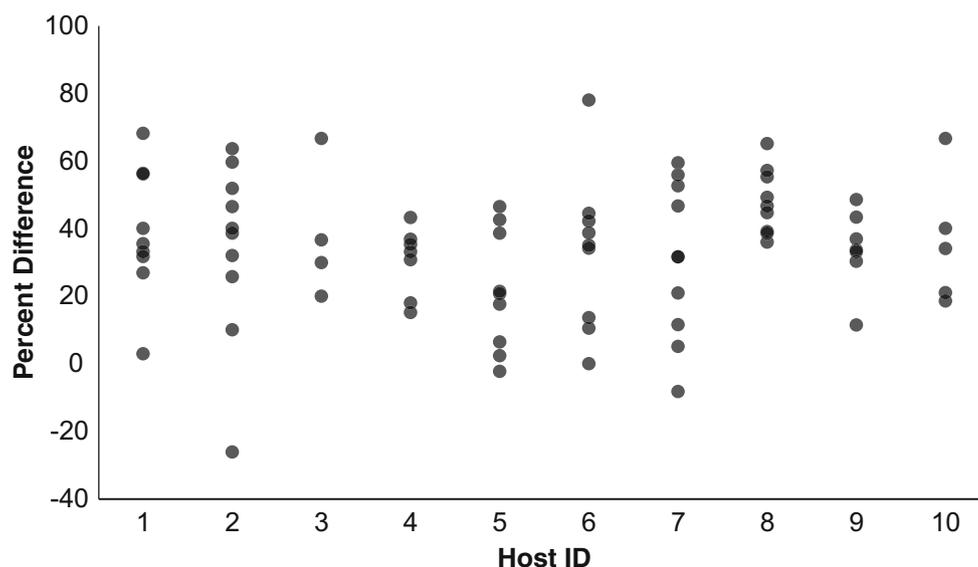
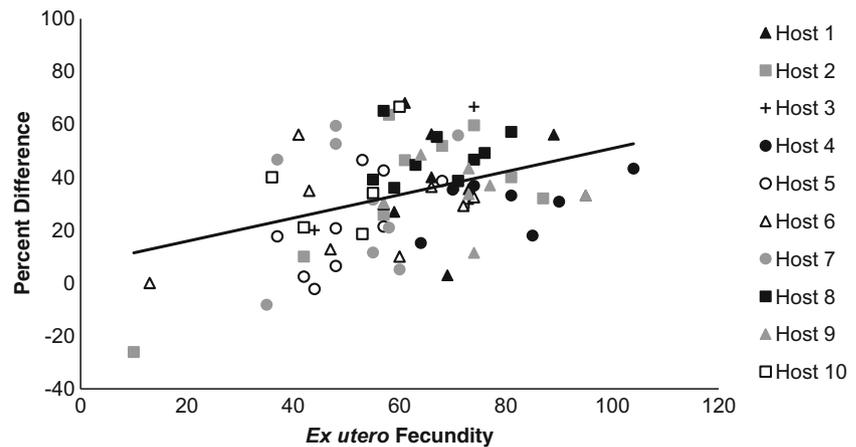


Fig. 2 Percent difference between counting methods did not vary among hosts

Fig. 3 Percent difference between fecundity methods was positively associated with ex utero fecundity



Haemonchinae with species of *Haemonchus* Cobb, 1898, and *Ashworthius* Le Roux, 1930, (Chauhan et al. 1972; Lichtenfels et al. 2002).

In contrast to the in utero method, the ex utero method requires minimal personnel training, but specimens are destroyed in the process. As such, this method might only be appropriate for studies where tissue and specimens do not have to be archived or used for other purposes or where species identity is considered to be unequivocal; in these situations, it remains a critical point to retain voucher specimens held in a museum repository. In addition, accuracy in the ex utero methods depends on performing counts within a short window of time. Counts must occur not too soon or too late after exposure to sodium hypochlorite solution (i.e., during an approximately 2-min window), which could pose a challenge for novice technicians, as demonstrated by some initial counts in our analyses that resulted in outliers.

Accurate measures of nematode fecundity can provide important information for investigating parasite life history evolution (Skorping et al. 1991), transmission potential (May and Anderson 1979), and effects on host health (Stien et al. 2002; Albon et al. 2002; Holand et al. 2015). This study compared two methods for assessing fecundity of a trichostrongyle nematode, finding that both were good options, although each had its unique advantages and disadvantages. However, because of the large discrepancy in egg counts between methods, we recommend caution in undertaking comparative studies and meta-analyses that utilize egg count data from multiple studies. Understanding the correspondence between different protocols for assessment of nematode fecundity, and standardizing methods, where possible, will enable comparisons across different studies and host and parasite species and systems.

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