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Evaluating faecal egg count reduction using a specifically designed package "eggCounts" in R and a user friendly web interface

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Abstract

The seemingly straightforward task of analysing faecal egg counts resulting from laboratory procedures such as the McMaster technique has, in reality, a number of complexities. These include Poisson errors in the counting technique which result from eggs being randomly distributed in well mixed faecal samples. In addition, counts between animals in a single experimental or observational group are nearly always over-dispersed. We describe the R package "eggCounts" that we have developed that incorporates both sampling error and over-dispersion between animals to calculate the true egg counts in samples of faeces, the probability distribution of the true counts and summary statistics such as the 95% uncertainty intervals. Based on a hierarchical Bayesian framework, the software will also rigorously estimate the percentage reduction of faecal egg counts and the 95% uncertainty intervals of data generated by a faecal egg count reduction test. We have also developed a user friendly web interface that can be used by those with limited knowledge of the R statistical computing environment. We illustrate the package with three simulated data sets of faecal egg count reduction experiments.

Keywords:

Faecal egg count reduction test; Anthelmintic resistance; Mathematical techniques; Statistical analysis; Bayesian hierarchical model

1. Introduction

Simple techniques to quantify the numbers of eggs in a faecal sample are standard tools for diagnostic parasitologists. The McMaster technique has long been in use (Gordon and Whitlock, 1939) whilst more recent techniques such as FLOTAC have been developed (Cringoli et al., 2010). These techniques process a sample of faeces and enumerate the numbers of eggs observed, followed by a simple mathematical manipulation to estimate the numbers of eggs per gram of faeces (epg). The random distribution of eggs within a faecal sample will conform to a Poisson process and thus repeat calculations of eggs per gram from the same faecal sample will be subject to Poisson errors (Torgerson et al., 2012). Therefore there is inevitable variability in evaluating faecal egg counts even with a highly precise laboratory technique due to this random variation.

There is normally considerable over-dispersion of faecal egg counts between animals within an experimental or observational group. This is partly due to dilution or detection limits magnifying Poisson errors and, importantly, due to aggregation of parasite infection between hosts. This means that standard parametric statistical techniques to compare the egg counts in animals between different treatment groups are inappropriate. Often the egg counts have been logarithmically transformed to create a probability distribution closer to the normal distribution. A very obvious issue with this is the treatment of data sets where some individuals have zero counts. Usually a constant, most likely 1, is added to all counts to avoid the $-\infty$ that arises when taking the logarithm of 0 (Cox et al., 2000). However, adding 1 to all counts is no more rational than adding 0.1, 10, 100 etc. and the choice of constant can effect the results. In addition the use of logarithmic transformation can result in bias when conducting a faecal egg count reduction test (Dobson et al., 2009). The issue of over-dispersion of egg counts between animals can be more rigorously analysed by using appropriate statistical techniques that embrace the skewed statistical distributions observed. The over-dispersed distribution of egg counts can be modelled with the negative binomial distribution (Torgerson et al., 2005) or other skewed or zero inflated distributions. Similarly there is now software available that can analyse such data using generalized linear modelling (GLM) techniques (Wilson and Grenfell, 1997) with an appropriate link function and hence it should no longer be necessary to log transform or make any other mathematical manipulation of raw data in an attempt to transform it into a normal distribution.

A further important issue to address is the results of analysis when all observed post treatment egg counts are zero. Previously methods such as Coles et al. (1992) and Lyndal-Murphy et al. (2010) give a 100% reduction with 95% uncertainty intervals (UI) as 100%-100%. This is clearly incorrect as zero epg observed following treatment does not prove there are no eggs; it merely indicates that no eggs were seen. This problem is discussed further in Dobson et al. (2012). Appropriate statistical methodology is required to estimate UI in this case which will depend upon the sample size or number of replicates and the dilution factor used in the analysis when zero eggs

were observed. This is analogous to the estimation of confidence limits for prevalence when there are no diseased individuals observed in the sample. In this case an upper 95% UI of the prevalence will depend upon the sample size taken.

To improve egg count analysis and hence reduce the likelihood of errors, it is necessary to incorporate the Poisson errors that arise from enumerating egg counts and the over-dispersed distribution that occurs between animals. We have developed a statistical package for R, a free and powerful software environment for statistical computing and graphics (http://www.R-project.org/), that both encompasses these Poisson errors that inevitably occur in the enumerations of eggs in faeces and the over-dispersion of counts between animals . We have also developed a user friendly web interface where egg counts can be analysed by the non-statistical specialist, or those with no knowledge of R. This software will also give rational UI even when all observed samples have an observed epg of zero.

2. Materials and Methods

We used a Bayesian approach to model the egg counts and the egg count reduction. The first stage is to model the probability distribution of true (but unknown) egg counts given the observed egg counts. This is subject to Poisson errors multiplied by the dilution or detection rate. Here we use an uniform gamma prior with the raw observed count. From this a probability distribution can be constructed that gives the probability of the true egg count in that sample given the observed egg count. For example if the observed egg count on a McMaster slide is calculated at 200 epg, with a 50 epg detection limit (i.e. four eggs were counted on the slide), this results in the probability distribution of the true egg count illustrated in Fig. 1. Thus, the unknown egg count can take a range of values with the mean being approximately 200 epg, but the 95% UI is 54-435 epg. This distribution was calculated using an uniform gamma prior of gamma(1, 0.001). First the posterior gamma distribution was calculated with four events in one time period, giving a posterior distribution of gamma(5, 1.001), Fig.1. We use four events as the observed frequency as four eggs would have been counted on the McMaster's slide before multiplication by the correction factor in order to obtain the estimate of epg. The resulting gamma distribution is then rescaled by the dilution factor of 50. If this is expanded to a group size of 10 animals, it would be expected that the measured epg would be over-dispersed. Typical data sets of three faecal egg count reduction (FECR) trials, each involving 10 animals with inconclusive, susceptible and resistant outcomes is given in Table 1. By the same reasoning, each of these 10 egg counts is a sample from 10 unknown egg counts and each is subject to the same random sampling error. Thus 10 probability distributions can be constructed to represent the 10 distributions of the unknown egg counts. This is illustrated in Fig. 2, which illustrates the probability distributions of the pre-treatment epg for each of the 10 animals involved in the inconclusive trial. To construct a probability distribution of the mean of the

10 animals, Bayesian techniques are used which essentially repeatedly sample across these 10 distributions. Descriptive statistics of interest such as the mean and 95% UI of the epg can then be calculated. A Poisson-Gamma model (negative binomial) is used to model aggregation between animals when constructing the probability distribution of the arithmetic mean of the 10 counts (Fig. 3). For interested users, the probability distribution of the negative binomial constant k can also be illustrated. For more advanced users, other distributions such a zero inflated model can be used to model the between animal variability. For this illustration, the mean epg using this Bayesian technique is 410 epg with 95% UI = 330-496 epg. For these calculations we used the shape/rate parameterisation of the Gamma distribution. The prior of gamma (1, 0.001) was used as it is approximately uniform in its distribution. In practice this means that before the eggs are counted on the McMaster slide, we assume that any egg count is equally likely.

3. Results

Suppose the 10 animals described previously are treated with anthelmintic drugs and the faecal egg counts are recorded following treatment, giving the results in Table 1. For the inconclusive data set the arithmetic mean of the observed counts, prior to treatment, was 420 epg. The investigator may wish to calculate the reduction in egg counts. Calculation of the observed mean reduction was straightforward. The arithmetic mean of the observed data, prior to treatment, was 420 epg. Following treatment it had been reduced to 20 epg. This gives a reduction in the epg of 95.2%. Hence the investigator might conclude that the anthelmintic drug is effective. However, this simplistic calculation ignores random sampling error and over-dispersion between hosts, both of which add considerable complexity to a seemingly straightforward calculation. The package "eggCounts " (http://cran.r-project.org/web/packages/eggCounts/index.html) will calculate the probability distribution of the post-treatment egg counts, the probability distribution of the percentage reduction and from these the 95% UI of all parameters (all in the sense of posterior probabilities and highest probability density intervals). The mean epg post-treatment of the inconclusive data set is calculated at 21.1 epg with a 95% UI = 7.8-55.1 epg (Fig. 3). The mean percentage reduction in faecal egg counts is 94.4% with 95% UI of 88.1-98.1% (Fig. 4). In comparison the FECR in the susceptible data set is 98.4% (UI = 96.0%-99.6%), whilst that of the resistant data set is 90.2% (UI = 83.1%-94.2%). Finally, if all of the observed epgs post-treatment in the susceptible data set were zero – ie an arithmetic mean of zero, the estimated FECR is 99.6% with UI of 98.0% to 100%. The code for implementing this is in the R package "eggCounts" which is given in Supplementary Data S1. Full details of the mathematical and statistical theory of the calculations used in eggCounts is given in Paul et al. (2014).

We have also developed an easy to use web interface: http://www.math.uzh.ch/as/?calc. This will facilitate those that have limited or no knowledge of R or statistical programming. The user can simply enter the data into the interface and choose one of three options. The first is for the analysis of one sample – ie to estimate the mean egg count and 95% UI for a series of egg counts from a single group of animals (or people) without any treatments. The second is a paired design. The third is an unpaired design would be used for two groups of animals, an experimental or treatment group and a control group. For the data in Table 1, we have used the paired sample as the epgs were measured in the same animals before and after treatment. The web interface will then give the results as mean epgs for pre- and post-treatment with their 95% UI and the faecal egg count reduction and its 95% UI. See Fig. 5 for a snapshot of the web interfaces. The web interface assumes a negative binomial distribution to model over-dispersion between animals.

Table 1. Observed faecal eggs counts from three faecal egg count reduction trials, each of 10 sheep calculated using the McMaster technique with a detection limit of 50 eggs per gram , before and after anthelmintic treatment

	Faecal Eggs Counts (eggs per gram)					
	Inconclusive		Susceptible		Resistant	
	Before Treatment	After Treatment	Before Treatment	After Treatment	Before Treatment	After Treatment
	0	0	1000	0	900	100
	200	0	400	0	600	50
	450	50	450	0	650	50
	150	0	350	0	450	50
	550	0	550	0	1250	150
	700	100	700	50	750	50
	100	0	1250	0	250	0
	150	0	1500	0	1450	150
	550	0	850	0	700	50
	1350	50	1350	50	1150	100
Arithmetic mean	420	20	840	10	815	75
% Reduction (Uncertainty Interval)		94.4 (88.1-98.1)		98.4 (95.7-99.7)		90.4 (82.9-94.7)

4. Discussion

We have developed mathematically and statistically rigorous software to estimate parasite egg counts in faecal samples and their 95% UI. It will also calculate the percentage reduction in a faecal egg count reduction test. Of great importance is that the software incorporates both (random) sampling errors and aggregation between individual hosts in a group. A user friendly web interface has been developed to enable users with limited mathematical or statistical knowledge to undertake such calculations in a straightforward manner.

Egg counts can never be estimated with complete accuracy, even with precise instrumentation, due to the random distribution of eggs in faeces. Hence enumerating eggs in any faecal sample only gives information from which a probability distribution of the egg counts can be

calculated. This is well illustrated when considering a faecal sample that has been examined by the McMaster technique and zero eggs are observed in such a sample. It would be wrong to conclude there are no parasite eggs in the faeces as even quite substantial positive real counts can result in zero eggs being observed on the McMaster slide due to Poisson errors. For a fuller explanation see Torgerson et al. (2012)). For example, with a dilution factor of 50, even if the true egg count is 100 epg, there is still a probability of 13.5% of observing zero eggs. This is the reason that the probability distribution of the true egg count, given an observed egg count of zero included the possibility of non-zero egg counts (see Fig. 2, '0 epg'). Unless there are infinite samples from the same faecal sample, all giving zero epgs, there can never be absolute certainty the true epg is zero. It is for this reason that the mean epgs given by our software can be higher than the arithmetic mean of the observed epgs, especially in data sets where there are several observed zero counts and a high dilution factor. Thus "eggCounts" calculates the post-treatment epg as 23.4 epg compared with the 20 epg mean of the observed data. The closer the arithmetic mean of the observed data is to zero, the greater will be this deviation. Our software models the egg counts on the assumption that a zero count means eggs were not observed, and thus could be present, rather than assuming eggs were absent. This is further illustrated when all of the observed epgs in the post-treatment susceptible group are all set to zero epg. In this case the UI of the percentage reduction are 98.0% to 100%. The method previously described by Dobson et al. (2012) attempts to address the problem of uncertainty intervals for such observations with a mean of zero epg, an issue earlier techniques such as the RESO method cannot address (Excel version of RESO available at http://***sydney***.*edu.au/vetscience/sheepwormcontrol/****software/FECR4*****.xls).**

The proposed solution of Dobson et al. (2012) uses the raw observed egg count and the inverse beta distribution. However this method is unable to generate rational UI for the FECR when the efficacy is poor or if the observed post-treatment egg counts are higher than pre-treatment counts. Our method both rigorously embraces the low sensitivity that may occur with, for example, the McMaster technique when the true egg count is low, estimates UI when all post-treatment egg counts are zero and estimates rational UI when post-treatment egg counts are higher than pre-treatment counts.

The faecal egg count reduction in the example from the inconclusive data set is a mean of 94.4% with 95% UI of 88.1% to 98.1% (Fig. 4). The question that arises is how we interpret this in the context, for example, of anthelmintic drug efficacy or defining resistance? The correct interpretation is that there is a 95% probability that the efficacy of the anthelmintic drug used in this example is somewhere between 88.1% and 98.1%. If we consider that evidence for resistance is when mean efficacy is below 95% (Coles et al., 2006) it would now be tempting to conclude that there is evidence of resistance in our example. However, the calculations were undertaken in a Bayesian framework. With this data set, close to 50% of the probability distribution gives an

efficacy of greater than 95% (Fig. 4). Thus the correct interpretation of this analysis is that there is an approximate probability of 50% that the parasites are resistant and 50% probability that they are susceptible. Thus perhaps the best conclusion is that there is insufficient evidence to conclude that resistance is present. Similarly the corollary is true: there is insufficient evidence to conclude the parasites are fully susceptible. Therefore we might consider repeating the experiment where the sample size is larger and/or the pre-treatment egg counts are higher, either of which might give more conclusive results. This tool, therefore, can be used to easily analyse egg count data in this context. For example anthelmintic drug resistance could be defined as when there is a 95% probability that the mean efficacy is less than 95%, and no resistance when there is similarly a 95% probability of at least 95% efficacy. Table 1 and Fig. 4 give examples of data sets that would be consistent with such conclusions.

Our software is not limited to analysing data generated by the McMaster technique. The results of any technique that quantitatively enumerates eggs in faecal samples can be analysed by this software, providing the dilution or detection limit is known. For example results obtained from the FLOTAC technique (Cringoli et al., 2010) which has a detection limit of one epg can also easily be analysed, with the correction factor being 1 in this case. Any technique with a small correction factor will minimize, but not eliminate, errors that arise from the Poisson distribution of eggs in faeces.

In conclusion we have developed software that is mathematically rigorous to analyse the results of faecal egg counts. For routine use by the non-specialist, a web interface is available. For those with a more advanced statistical knowledge, data can be analysed using the R package "eggCounts".

Acknowledgements

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Legends for Figures

Fig. 1. Posterior probability distribution of the true egg count, given a calculated egg count of 200 eggs per gram of faeces (epg) using the McMaster technique with a dilution factor of 50 and a prior of gamma (1. 0.001) for the raw unadjusted counts

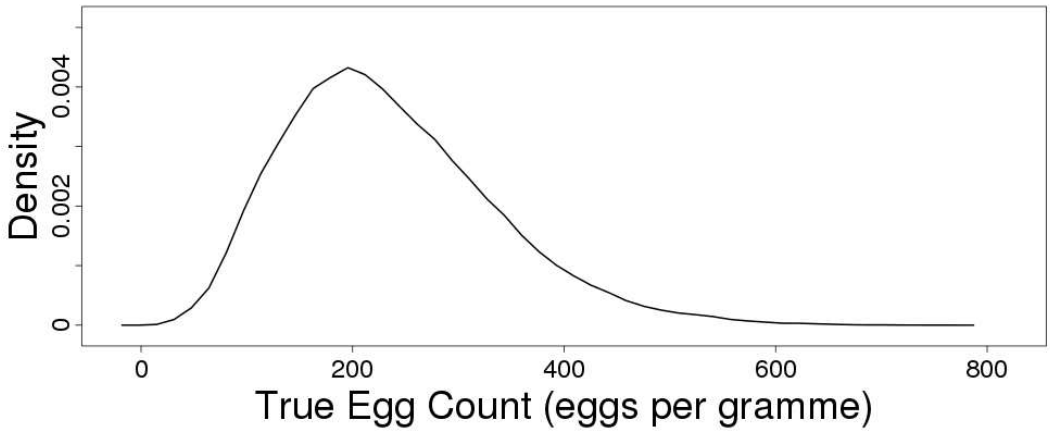
Fig. 2. Posterior probability distributions of the true egg counts of 10 faecal samples examined before anthelmintic drug treatments. All observed egg counts (labelled in the strip above each panel) were calculated using the McMaster technique with a dilution factor of 50 and a prior of gamma (1.0, 0.001) for the raw unadjusted counts.

Fig. 3. Probability distribution of the true mean egg count of the 10 samples before and after treatment.

Fig. 4. Probability distribution of the percentage of faecal egg count reduction following analysis of the three sample data sets. In the inconclusive faecal egg count reduction (FECR) (A), close to half the probability distribution is above 95% efficacy (blue, susceptible) and half below 95% (red, resistant). With the FECR that indicates anthelmintic susceptibility (B), over 95% of the probability distribution is above 95% FECR. With the FECR indicating anthelmintic resistance (C), over 95% of the probability distribution is less than 95% FECR.

Fig. 5. Screen shot of the web interface with the actual data (inconclusive data set) from before and after anthelmintic drug treatment.

Figure 1


Density
0
0.002
0.004
0
200
400
600
800
True Egg Count (eggs per gramme)

285 Figure 2

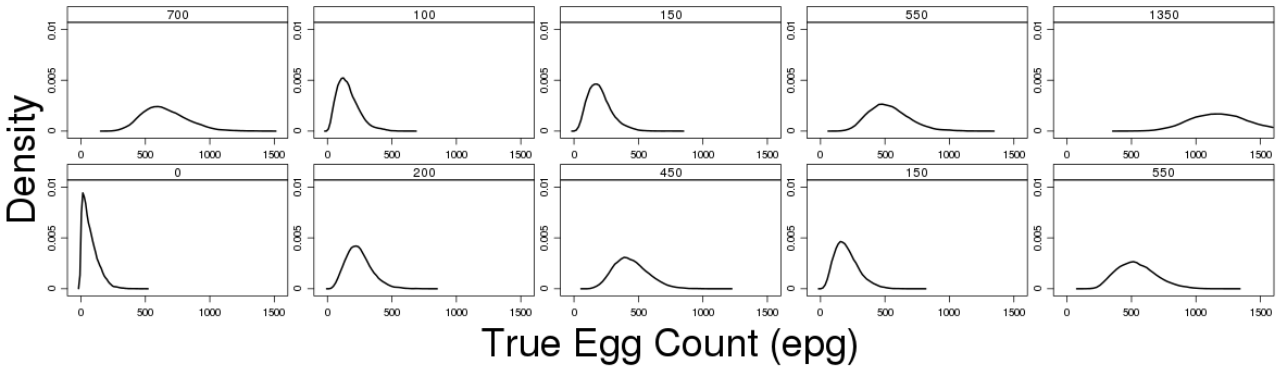

700
100
150
550
1350
0
200
450
150
550
Density
True Egg Count (epg)

Figure 3

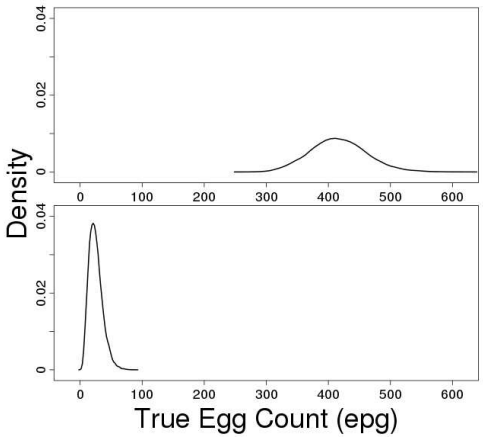
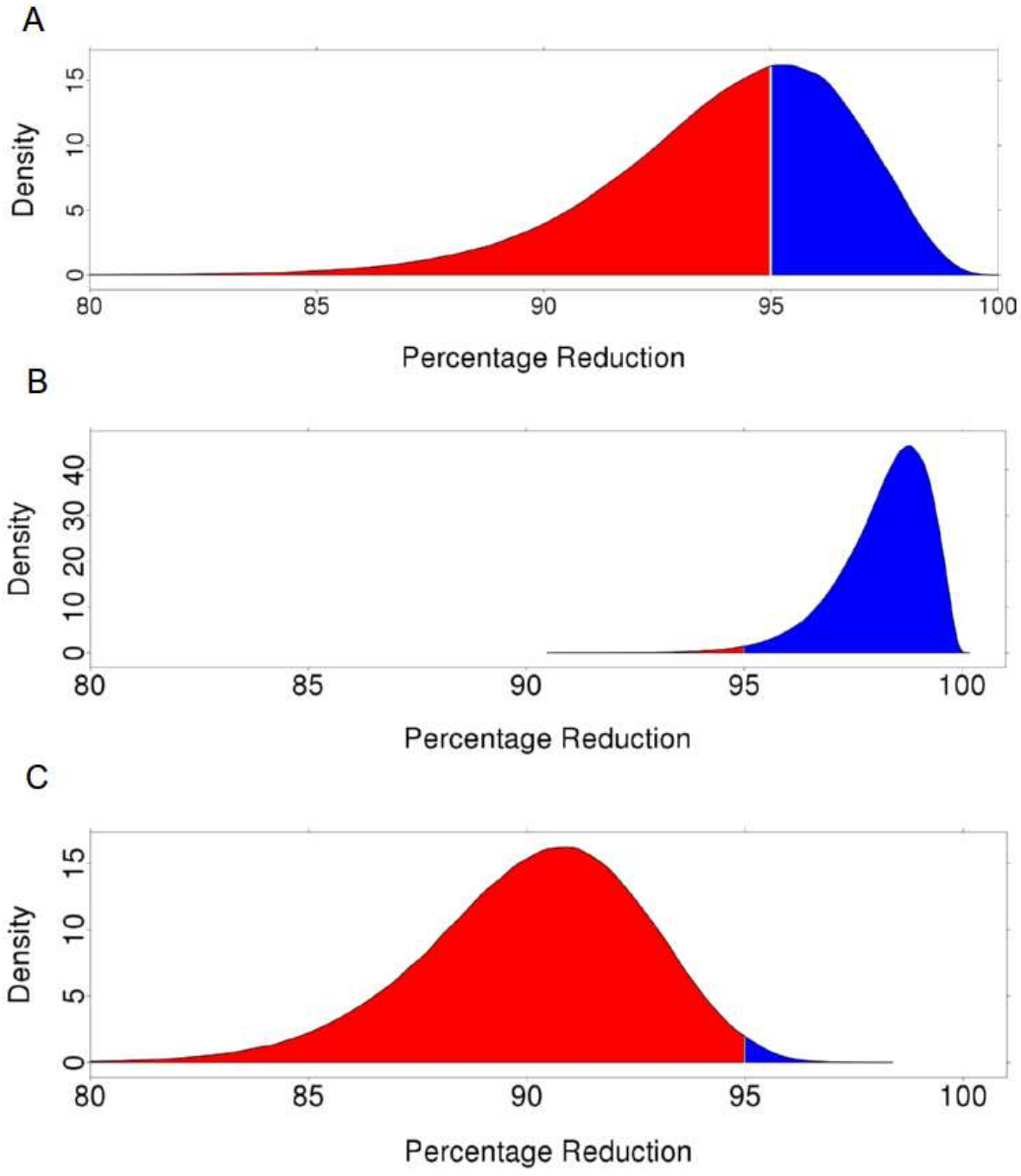


Figure 4


A
Density
0
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15
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90
95
100
Percentage Reduction
B
Density
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20
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40
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85
90
95
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Percentage Reduction
C
Density
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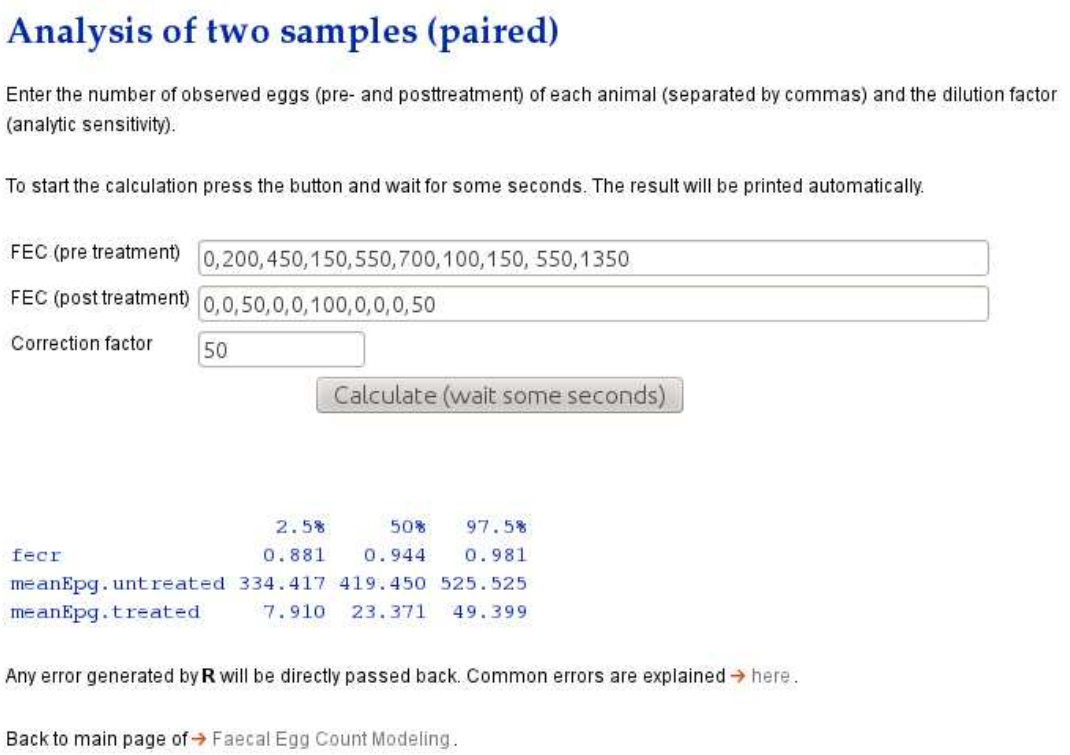
90
95
100
Percentage Reduction

Figure 5



Analysis of two samples (paired)

Enter the number of observed eggs (pre- and posttreatment) of each animal (separated by commas) and the dilution factor (analytic sensitivity).

To start the calculation press the button and wait for some seconds. The result will be printed automatically.

FEC (pre treatment): 0,200,450,150,550,700,100,150, 550,1350

FEC (post treatment): 0,0,50,0,0,100,0,0,0,50

Correction factor: 50

Calculate (wait some seconds)

	2.5%	50%	97.5%
fecr	0.881	0.944	0.981
meanEpg.untreated	334.417	419.450	525.525
meanEpg.treated	7.910	23.371	49.399

Any error generated by **R** will be directly passed back. Common errors are explained → here.

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Supplementary Data S1.

Below is R code, with commentary after # for each line of code. This code analyses a faecal egg count reduction (FECR) for a paired study design of FEC before and after treatment, with a dilution factor of 50. Some options are given, which are not essential, which are the same defaults as the web interface. Also note that slightly different results (usually beyond the second significant figure) can occur with different defaults and runs on the same data, or with a different set.seed figure. This is because random number generators are used in the Markov Chain Monte Carlo (MCMC) . If the number of burnins is increased and nsamples is also increased, this small variability will be decreased at the cost of a longer running time.

R Code

```
library(eggCounts) #load eggCounts package into R
set.seed(12345) # this sets a seed for the random number generator (not essential)
preepg=c(0,200,450,150,550,700,100,150, 550,1350) #pretreatment epgs loaded to data vector "preepg"
postepg=c(0,0,50,0,0,100,0,0,0,50) #post treatment epgs loaded to data vector "postepg"
reduct1=fecr_mcmc(preepg,postepg, f.pre=50, f.post=50, model="paired",nburnin=5000, nsamples=10000, maxiter.pilot=30,thin=1) #Facecal egg count reduction executed and results stored in "reduct1".
reduct1 #this displays the results (below)
```

```
Model:  paired
Iterations = 5001:15000
Thinning interval = 1
Number of chains = 1
Sample size per chain = 10000

1. Empirical mean and standard deviation for each variable,
   plus standard error of the mean:

                    Mean       SD  Naive SE Time-series SE
fecr               0.9399  0.02692 0.0002692       0.003013
meanEpg.untreated 420.8912 47.05646 0.4705646       5.436109
```

meanEpg.treated 25.0693 10.99560 0.1099560 1.204413

2. Quantiles for each variable:

	2.5%	25%	50%	75%	97.5%
fecr	0.8814	0.9231	0.9444	0.9601	0.9806
meanEpg.untreated	334.4167	389.2149	419.4504	449.3533	525.5247
meanEpg.treated	7.9099	16.9213	23.3712	32.0040	49.3986

From the web interface, using the same data:

fecr	0.881	0.944	0.981
meanEpg.untreated	334.417	419.450	525.525
meanEpg.treated	7.910	23.371	49.399