Chapter 6 – Measuring the distribution of parasites among saigas and livestock

6.1 Introduction

In measuring parasite abundance and distribution between host groups, it is important to consider how well parasite count data reflect underlying parameters of infection at the population level. This chapter begins with a critical appraisal of the field and laboratory methods used, and any errors and bias introduced by them. It then goes on to consider the interpretation of apparent differences in parasite abundance between groups of hosts, and the complications introduced both by non-random parasite distribution and observation uncertainty. The aim is to devise a way of comparing parasite burdens that deals rationally with both, and does not rely on unrealistic assumptions concerning data form and measurement accuracy. The results will be taken forward into chapter 7, and used to underpin the analysis of field data.

6.2 Sampling of parasite populations

6.2.1 Selection of saigas for sampling

As already discussed in chapter 4, it is impossible to be confident that either saigas or livestock in the present study were sampled in a truly random manner. In some cases, e.g. licensing restrictions on the age and sex of saigas hunted, bias is known and unavoidable. Another possible source of bias in saigas is the accidental selection of weaker animals, by shooting animals that flee less quickly.

Theoretically, if animals that are in poor body condition, or that have high parasite burdens, are less able to escape shooting, it should hold that they are over-represented in the sample. This representation may not be even, as small groups of saigas were often all killed, offering no opportunity for escape, while in larger groups only a small proportion of the group was shot, and the rest fled. The hypothesis that thinner or more heavily parasitised saigas were weaker and less likely to escape was tested by multiple bivariate correlation of group size at the time of shooting, abomasal

nematode burden and body condition score, using the Spearman rank correlation coefficient. Correlation was repeated using the Pearson correlation coefficient on $\log_{10}$-transformed counts, and *Marshallagia* spp. and *Nematodirus* spp. counts were analysed separately. In no case was a significant correlation found, suggesting no marked bias in either observed body condition or abomasal nematode count with the proportion of the group sampled.

6.2.2 Adult nematode counts

- *Incomplete sampling of gut contents*

Only a proportion of the gastrointestinal contents was examined in each animal sampled for adult nematodes. This was because:

(i) The entire gastrointestinal tract – and sufficient time to examine it - was not available for every individual.

(ii) Sieved gut washings often exceeded 20ml, the volume of the aliquot taken.

(iii) Where adult nematodes were abundant, and more than 40 specimens were picked out in the laboratory, counting was suspended to save time.

At least 10% of total abomasal contents was examined in all cases (normally more than 30%), and between 2 and 20% of small and large intestinal contents. Other workers have advocated that 1-10% of gut contents should be examined (Reinecke 1962; Eysker and Kooyman, 1993), or a maximum of 120 worms (Reinecke, 1984).

There is a risk that species of nematode that are actually present may not be observed in the sub-sample of gut contents examined. Artificially higher species richness might then be expected in hosts whose gut washings are examined more completely. No significant correlation was found, however, between the total proportion of digesta examined and the number of nematode species found, in either the abomasum of saigas (n=108, Spearman r_s=0, NS) or the small intestine of saigas and sheep (n=43, r_s=0.15, NS). There was a significant negative correlation between the proportion of digesta examined and the total calculated nematode burden (n=164, r_s=-0.37, $p<0.001$). This is because counting was suspended in samples found to contain many nematodes. The correlation became non-significant when burdens above 1,000

nematodes were excluded (n=95, r_s=-0.13, NS). The observed prevalence of infection was unaffected by the proportion of digesta examined (Table 6.1).

Table 6.1. The effect of examining only a proportion of digesta on the observed prevalence of gastrointestinal nematodosis. Samples from the abomasum and small intestine of saigas and sheep are included. n=152, Chi-square=4.14, 2df, NS.

Proportion examined, interval to	Nematodes present (n)	Nematodes absent (n)
0.1	49	11
0.3	41	4
more	44	3

- *Damaged saiga abomasa*

One adult and 4 sub-adult saigas were inadvertently shot through the abomasum. The volume of the contents of breached abomasa was significantly reduced relative to undamaged abomasa (median volume 10ml, and 30ml respectively, Mann-Whitney U=13.5, n=4 and 63, p=0.01). However, the calculated burden of abomasal nematodes was not lower in damaged abomasa (U=158, NS), and both *Marshallagia* spp. and *Nematodirus* spp. were found in washings from them. Samples from damaged abomasa were therefore included in subsequent analysis.

- *Frozen saiga abomasa*

The effect of freezing for several days on the number of nematodes recovered from the abomasum was assessed by comparing median burdens from abomasa that had and had not experienced freezing. There was no significant difference in either the medians of all nematode counts, or those of the separate counts of *Marshallagia* spp. and *Nematodirus* spp., in frozen and unfrozen abomasa (n=26 and 107, Mann-Whitney U=1237, 1291, 1315 respectively, NS).

- *Non-ligation of the gastrointestinal tract*

Failure to ligate the pylorus did not appear to allow significant movement of nematodes between the abomasum and small intestine, as *Marshallagia* spp. were recovered from the small intestine only very occasionally and in small numbers. *Nematodirus gazellae* was found in large numbers in both the abomasum and small intestine, but is known to infect both organs.

- *Extraction of nematodes from the small intestine*

Nematodes were recovered from the small intestine by extrusion, without subsequent washing (see chapter 4). This might lead to underestimation of small intestinal burdens, if some nematodes remain attached to the mucosa. Adult nematodes were found in all 5 sets of intestines opened and washed after extrusion (Table 6.2). Assuming that washing recovered all remaining adult nematodes, extrusion was successful in recovering on average 98.9%, and in no case fewer than 98%, of adult nematodes. No species were recovered by washing that were not already present in the extruded samples. Nematode burdens calculated from aliquots of extruded small intestinal contents are used without adjustment in subsequent analysis.

Table 6.2. Recovery of nematodes from the small intestine by extrusion and washing. Five sets of small intestines from sheep were subjected first to extrusion, then to a thorough mucosal wash. The number of nematodes recovered by washing was related to that first recovered by extrusion (n=5, Pearson r=0.96, p=0.011).

Mean burden calculated from aliquot of extruded contents	Range	Mean number of nematodes subsequently recovered by washing	Range
2224	200-6390	24	4-89

6.2.3 Immature parasitic stages

Of 18 saiga abomasa and 14 sheep abomasa steeped in water after washing, sedimentation recovered immature parasitic larvae from only 8, all sheep. Abomasal scrapes, however, were more successful in recovering late larval stages (Table 6.3). In sheep, both *Marshallagia* sp. and *Trichostrongylus axei* L5 were recovered from abomasal scrapes. *Marshallagia* were the most abundant, and were found in groups of 1 to 5 in the mucosal tissue, from which they were easily teased out. All larvae in this position were sub-adult: the males had well developed chitinised spicules, and the females had well defined ovejectors but were not gravid. They were most often found near the pylorus, but also in the fundus. Each cluster of immature nematodes was overlain by a small area of mucosal hyperaemia, which was visible only after removing the mucus layer. In saigas, 4 samples of inflamed abomasal tissue were taken and fixed in formalin: one of these was found to contain a nematode section on histological examination, but the species could not be identified.

Table 6.3. Immature parasitic stages found in the abomasa of sheep sampled in Chu in November 1999. 14 abomasa were examined by sedimentation in water, and 19 by inspection of the mucosal surface and scraping of hyperaemic areas. Ostertagine L3 and L4 are most likely to be *Marshallagia* sp., but larvae at this stage cannot be easily identified to genus.

Parasite species	Stage	Prevalence	Mean number	Median number (1st and 3rd quartiles)
		Sedimentation		
Ostertagine	L3	0.2	7	4 (3-9)
	L4	0.3	3	3 (2-3)
Marshallagia	L5	0.1	2	1 (1-2)
Trichostrongylus	L3	0.1	2	2
	L4	0.1	3	3 (2-4)
	L5	0.1	1	2
		Mucosal scrape		
Marshallagia	Immature adult	0.4	9	4 (2-10)
Trichostrongylus	Immature adult	0.2	4	5 (3-6)

Earlier immature parasite stages were found in both abomasal and small intestinal washings. This was not by design, as the sieves used were of broader aperture than those recommended for the recovery of trichostrongyloid larvae. Nevertheless, the presence of 3rd and 4th stage larvae in the gastrointestinal lumen does suggest ongoing infection: the implications are discussed in chapter 7.

6.2.4 Free-living stages

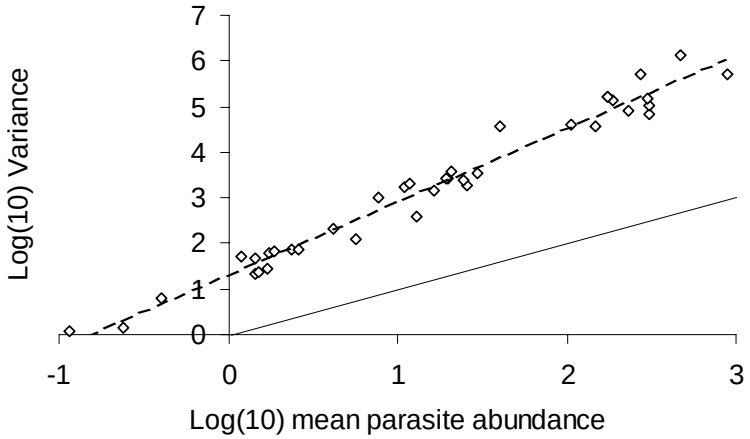
Free-living stages of parasitic nematodes were recovered from 4 of 19 herbage samples, all in Betpak-Dala in summer 1998. Results are discussed in chapter 7. None of the 8 samples taken in Ustiurt in spring 1998 contained parasite larvae, though adult free-living nematodes were extracted, suggesting that the method used was appropriate.

6.3 Distribution of adult nematodes between hosts

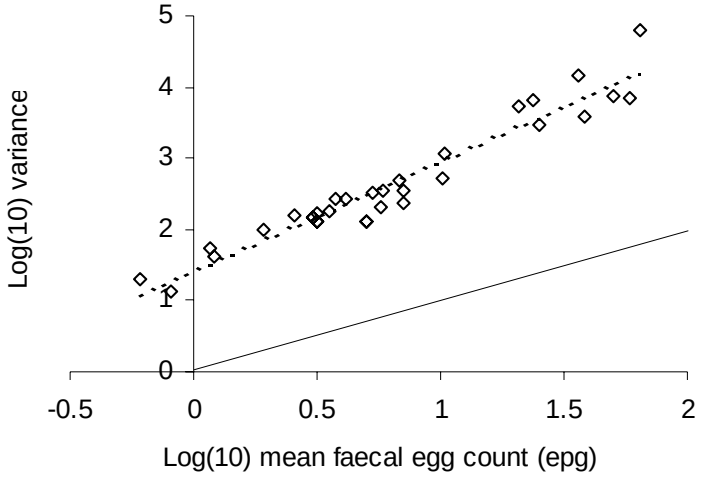
6.3.1 Overdispersion in parasite burdens

Overdispersion and aggregation, here used interchangeably, refer to right skew in the frequency distribution of animal counts in sampling units, such that the variance of the counts consistently exceeds their mean (Taylor, 1961; Taylor *et al*, 1978). This definition can be extended to detect overdisperion in parasite distribution between hosts (Shaw and Dobson, 1995). Fig. 6.1 summarises the relationship between the mean and variance of parasite and faecal egg counts from different host groups in

Kazakhstan. All points lie above the line of equality of mean and variance. Linear regression of Log_{10} (variance) on Log_{10} (mean) yields a slope significantly greater than unity, confirming that the distribution of parasites among the hosts sampled is overdispersed.



(a) Counts from 21 adult worm populations in saigas, and 17 in livestock. Zero counts are included. Slope=1.61(95%CI 1.51-1.70), intercept=1.31 (95%CI 1.16-1.46). R^2=0.97, F=1195, p<0.001.



(b) Faecal egg counts in 2 groups of saigas, and 14 of livestock, differentiated by egg type. Slope=1.55(95%CI 1.39-1.71), intercept=1.40 (95%CI 1.24-1.55). R^2=0.93, F=387, p<0.001.

Figure 6.1. Log variance against log mean parasite count for the groups of saigas and livestock sampled in Kazakhstan. Using both direct and indirect measures of parasite burden, the variance was consistently greater than the mean, i.e. the slope of the fitted regression line was significantly greater than one. The dotted line is the linear regression fitted by least squares; the solid line indicates a 1:1 ratio between variance and mean.

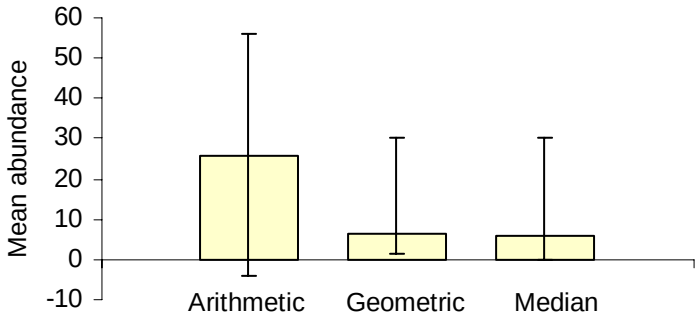
The distribution of parasites between hosts will affect the interpretation of apparent differences in parasite abundance between groups. Statistical tests that assume an underlying Normal distribution are not applicable if counts are markedly overdispersed. Non-parametric tests may be applied, but they always lack the power of their parametric counterparts (Siegel and Castellan, 1988), and often have restrictive assumptions of their own, that may not always be met. Transformation of parasite counts may normalise their distribution and allow the application of parametric tests. However, normality may not always be achieved, and the direct correspondence between the sample mean and parasite abundance is lost, confounding the interpretation of sample statistics (Fulford, 1994; Rozsa *et al*, 2000).

6.3.2 Describing parasite abundance and distribution

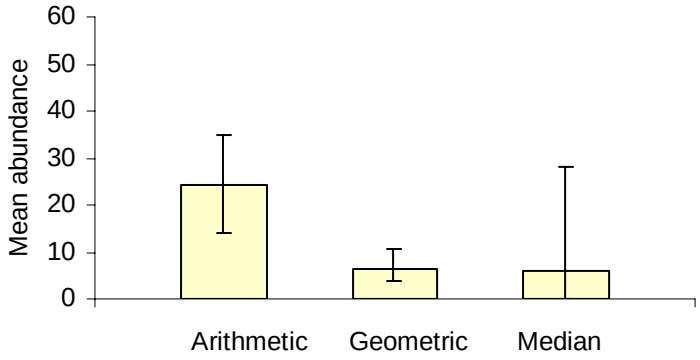
To illustrate the above difficulties, different sample statistics from populations of *Nematodirus gazellae* in saigas are compared in Fig. 6.2. This species is taken as an example because its distribution is highly aggregated in saigas (see section 6.4). The geometric mean was calculated by adding one to each count (to eliminate zeros), and taking logarithms. The mean log count and its 95% confidence intervals (CIs) were then back-transformed to give the geometric mean and CIs (Cox *et al*, 2000). The arithmetic mean is simply the average number of nematodes per host, and 95% CIs calculated as {1.96x SD/sqrt(n)}. Mean abundance reflects the total number of nematodes in the animals sampled, while mean intensity considers infected animals only, and can be used to calculate the overall parasite population density only if the prevalence is also given.

The distribution of *Nematodirus gazellae* in saigas was tested for overdispersion by multiplying the variance to mean ratio by the number of degrees of freedom, and comparing the result with the Chi-square distribution (Bliss and Fisher, 1953). Whether zero counts were included or not, the distributions differed significantly from the Poisson (χ^2=162, 280, 10164, 4875; df=10, 5, 86, 52 for abundance and intensity in Ustiurt and Betpak-Dala samples respectively, $p<0.001$ in all cases). The calculation of confidence intervals based on the Normal distribution is therefore fundamentally flawed. In the case of the Ustiurt sample, the lower 95% CI falls below

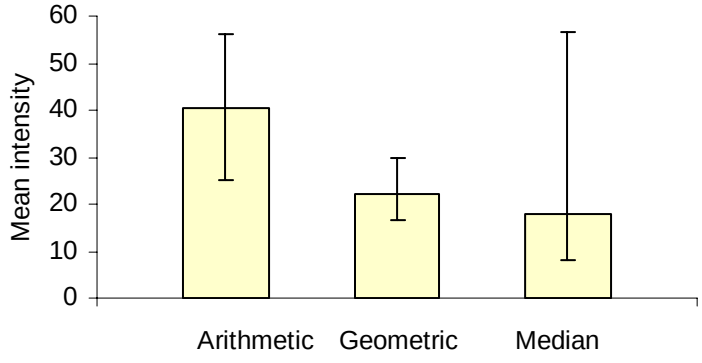
zero (Fig. 6.2*a*), which is absurd. The geometric mean has asymmetrical CIs that better reflect the overdispersed nature of the data, but it bears no simple relation to total parasite abundance in the sample (Fig. 6.2*b*). The median also poorly reflects parasite abundance, and is especially sensitive to the proportion of hosts observed to be infected.



(a) In the abomasa and small intestines of 11 adult saigas in Ustiurt.



(b) In the abomasa of 87 sub-adult saigas in Betpak-Dala, including zero counts.



(c) In the abomasa of 87 sub-adult saigas in Betpak-Dala, excluding zero counts.

Figure 6.2. Measures of central tendency of burdens of **Nematodirus gazellae** ***in saigas.*** Arithmetic and geometric means are shown with 95% confidence intervals, which are discussed in the text. Bars around the median mark the 1st and 3rd quartiles.

An alternative method of estimating variation in parasite counts is by bootstrapping (Efron and Tibshirani, 1993; Rozsa *et al*, 2000). One count is replaced with another from the same data set, and the mean re-calculated. Repeated replacement and resampling results in a frequency distribution of simulated means, from which confidence bounds (CBs) are drawn empirically. Bootstrap means and confidence bounds were calculated for *Nematodirus gazellae* abundance in the 2 groups of saigas, and are compared with the arithmetic means in Fig. 6.3. Bootstrap CBs cannot be negative, since they are drawn from the data. Increasing the number of iterations is expected to narrow the confidence bounds (Efron and Tibshirani, 1993). However, there was no appreciable difference in CBs around the means in Fig. 6.3 whether 200 or 10,000 iterations were used.

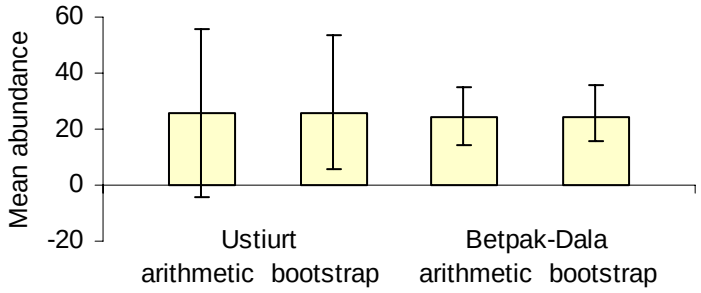


Figure 6.3. Mean abundance and 95% confidence intervals (CIs) of* Nematodirus gazellae *in 2 groups of saigas. CIs for the arithmetic mean were calculated from the Normal distribution in the usual manner. Bootstrap means and confidence bounds were calculated from 200 repeated samples, with replacement (see text), using the computer statistical programme Crystal Ball (Decisioneering Inc., Chicago).

6.3.3 Sensitivity of sample statistics to observation error

Measures of parasite abundance should take account of potential errors in collecting the data, and the robustness of sample statistics to them, as well as the underlying distribution of parasites between hosts. Counts of gastrointestinal nematodes may easily miss small numbers of parasites, while sampling small numbers of hosts increases the likelihood of missing the few largest parasite infrapopulations, especially if the parasite population is overdispersed.

Table 6.4 lists summary statistics for counts of *Nematodirus gazellae* in sub-adult saigas sampled in Betpak-Dala, and considers the effect of these potentially common sources of error on them. Poor detection of light infections by inefficient techniques will decrease the observed prevalence, and may paradoxically increase observed mean intensity, since only larger infrapopulations are counted. The missed parasites, however, contribute little to the total parasite population, and mean abundance is unaffected. Geometric mean abundance and intensity, however, are both substantially decreased. Median intensity increases, while median abundance decreases and must be zero when fewer than half the sampled hosts are observed to be infected. The addition of a high count, from a hypothetical heavily infected host missed in a small sample, increases both mean intensity and mean abundance. Such a disproportionate effect of a single observation on the mean may be statistically undesirable, but is biologically important as a reflection of parasite population abundance. The geometric mean and median are insensitive to the addition of this host. The bootstrap mean is faithful to the arithmetic mean across all samples. The addition of a heavily infected host results in a greater upward shift of the bootstrap CIs relative to the CIs of the arithmetic mean, which are constrained by the erroneous assumption of Normal variation about the mean.

The statistic that best describes the overall level of infection in the sample, and variation in parasite burdens between hosts, is therefore the bootstrap mean abundance and its empirical confidence bounds. There are circumstances in which prevalence and mean intensity are usefully considered separately (see chapter 7). However, mean abundance directly reflects in a single statistic the key biological parameter of parasite population density. It is robust to false negatives, and sensitive to large infrapopulations. Confidence bounds are wider above the mean than below it, reflecting overdispersion in parasite distribution.

Table 6.4. The effect of sampling and recovery efficiency on parasite count statistics. Raw data are counts of *Nematodirus gazellae* from the abomasa of 87 sub-adult saigas in Betpak-Dala. 'Low' refers to poor recovery of nematodes, simulated by deleting counts ≤10. 'High' supposes that a single extra, heavily infected, host is sampled, by the addition of a count twice that of the existing highest count. Bootstrap statistics were calculated from 200 repeated complete samples of the manipulated data set, with replacement.

	Preva-lence	Arithmetic		Geometric		Median		Bootstrap	
		Mean	95% CI	Mean	95% CI	Median	quartiles 1-3	Mean	95% CI
Intensity									
Raw data	0.61	41	(25-56)	22	(16-30)	18	(8-56)	41	(27-56)
Low	0.36	65	(42-88)	10	(5-20)	54	(25-68)	65	(43-95)
High	0.62	53	(24-83)	22	(17-30)	20	(8-58)	54	(32-89)
Abundance									
Raw data		24	(14-39)	7	(4-10)	6	(0-28)	25	(16-35)
Low		23	(12-33)	4	(2-8)	0	(0-28)	22	(13-32)
High		33	(14-52)	7	(4-11)	7	(0-33)	32	(17-54)

6.3.4 Comparing parasite burdens between hosts

Bootstrapping can be extended to a comparison of parasite abundance between samples. The mean abundance in each sample is first estimated by bootstrapping with replacement, and the two means compared. The process is then repeated many times, and the proportion of comparisons in which mean abundance in sample 1 exceeds that in sample 2 is noted. If the mean of sample 1 nearly always exceeds that of sample 2, this is unlikely to be due to chance, and sample 1 can be said to contain more parasites per host than sample 2.

Table 6.5. Different measures of statistical significance of differences between the abundance of* Nematodirus gazellae *in the abomasa of 46 adult and 87 sub-adult saigas culled in Betpak-Dala in November 1997. Bootstrap means were drawn from 200 repeated complete samples of each data set, with replacement, and compared over 1,000 iterations.

	Adult	Sub-adult	Test	Test statistic	p
Arithmetic mean (95% CIs)	19 (5-34)	24 (14-35)	t-test	t=0.594	0.554
Geometric mean (95% CIs)	2.9 (1-7)	6.5 (4-10)	t-test	t=2.562	0.012
Median (quartiles 1-3)	0 (0-8)	6 (0-28)	Mann-Whitney	U=1528	0.008
			Kolmogorov-Smirnov	Z=1.57	0.015
Bootstrap mean (95% CIs)	19 (8-33)	25 (16-35)	Comparison of bootstrap means	99.2% of sub-adult bootstrap means exceed those of adults	

Results of bootstrap comparison of means are compared with those of conventional parametric and non-parametric statistical tests in Table 6.5, again using *Nematodirus gazellae* in saigas as an example. The t-test on transformed data, and both non-parametric tests, suggest that parasite abundance is significantly higher in younger saigas, even though there is considerable overlap between parasite distributions in the two age groups. The transformed data, however, do not fulfil the assumption of normality made by the t-test (χ^2 =244, 86df, $p<0.01$, and χ^2 =67, 45df, $p<0.05$ for sub-adult and adult groups respectively), and the t-test is therefore not applicable. Neither non-parametric test, meanwhile, provides a true comparison of parasite abundance, since differences in the distribution of parasites in the samples being compared can affect the test statistics and lead to Type I errors (Sokal and Rohlf, 1995; Rozsa *et al*, 2000). Bootstrapping, meanwhile, suggests that there is only a 0.8% chance of the adult mean exceeding the sub-adult mean when the data are sampled with replacement.

More formal methods of comparing sample means using bootstrapping are possible (Rozsa *et al*, 2000). However, all assume that the data set is fully representative of the population sampled, and do not allow for observation uncertainty. In this respect, they cannot be used to make accurate probability statements. If, on the other hand, we have some knowledge of the underlying parasite distribution, bootstrap samples can be drawn from a more smoothly distributed set of values, refining our estimates of mean and variance (Hilborn and Mangel, 1997), and consequently our comparison of samples. Moreover, if the underlying mathematical distribution of the parasite population among hosts can be estimated, comparisons between samples may be based explicitly on this distribution, and deviation from it provides evidence that the sample is drawn from a parasite population that is significantly different in density and/or distribution. Investigation of the underlying distribution of the sampled parasite populations forms the basis of the next section.

6.3.5 Modelling parasite distribution

Distribution-based approaches to the analysis of parasite count data have most often used the Negative Binomial Distribution (NBD), since it provides a good empirical

description of parasite samples from a wide variety of host-parasite systems (Anderson and May, 1982; Barger, 1985; Shaw *et al*, 1998). The NBD has the further advantages of being relatively simple (it is described completely by two parameters, the mean, *m*, and the inverse degree of aggregation, *k*), and mathematically convenient (which allows it to be incorporated easily into models of parasite population dynamics).

The NBD has a variance to mean ratio between that of the Normal (or Poisson) and the Log normal distributions, and the slope of the regression between Log_{10} mean and Log_{10} variance is typically close to 1.5 (Shaw and Dobson, 1995). Parasite counts from saigas and livestock in the present study closely match this criterion (see Fig. 6.1), and the NBD might therefore be expected to provide a good empirical description of the parasite distribution.

This expectation was tested by fitting the NBD to the adult nematode counts using maximum likelihood estimation (MLE). The overdispersion parameter *k* was estimated using the iterative MLE method of Bliss and Fisher (1953). This was facilitated by the collection of counts into around 10 classes (Barger, 1985). Results were assessed by the Chi-square goodness of fit test, and a p-value of 0.01 taken as the criterion for rejection of the NBD, to avoid Type I errors when making multiple comparisons (Shaw *et al*, 1998). The method allows the estimation of confidence intervals for *k* by examining the slope of the likelihood function near its mimimum. A second MLE method, in which the negative log likelihood is minimised directly (Williams and Dye, 1994; Shaw *et al*, 1998), gave similar results. Where the number of infected animals in the sample was adequate, the analysis was repeated using positive counts only, to see whether a mixed prevalence-intensity model might better describe the data.

MLE used the following formulation of the NBD (Hilborn and Mangel, 1987):

$$\Pr(Z = s) = \frac{\Gamma(k+s)}{\Gamma(k)s!}\left(\frac{m}{k+m}\right)^{s}\left(1+\frac{m}{k}\right)^{-k} \qquad (6.1)$$

where *s* is the class number, and Γ represents the gamma function. The starting point for estimation of *k* was the corrected moment estimate (Smith and Guerrero, 1993):

$$k = \frac{\left(m^2 - \frac{v}{n}\right)}{v - m} \qquad (6.2)$$

where *v* is the variance, and *n* the sample size.

Results of MLE of NBD parameters for the most common abomasal nematode species in saigas are presented in Table 6.6, and fits to the NBD shown in Fig. 6.4. Adult and sub-adult saigas are treated separately because of obvious differences in the mean abundance of some parasites between them. Some parasite species were present in too few hosts to allow MLE and goodness of fit tests: summary statistics for these, including moment estimates for overdispersion parameters, are given in chapter 7.

The significance of the fit to the NBD is marginal for many of the parasite species considered, but in every case is better than the fit to the Poisson distribution. Omitting the zero class and testing parasite intensity against the NBD generally gave values for *k* similar to those for abundance (data not shown). However, the reduction in the sample size made it possible to test the fit for only 2 of the parasite populations, and in neither case was the fit improved. The case for describing parasite distributions in saigas using a combined prevalence-intensity model is therefore not pursued.

Table 6.6. The abundance of abomasal nematodes in sampled saigas, with maximum likelihood (ML) estimates of parameters of the negative binomial distribution. Goodness of fit was assessed using the χ^2 test: blank rows indicate that too few classes were filled to allow this test. None of the data sets fitted the Poisson distribution (χ^2 test on variance-mean ratio, $p<0.001$, see text). Graphical fits of NBD to the data are shown in Fig. 6.4.

Parasite	Age class	n	Proportional prevalence	Arithmetic mean abundance	95% bootstrap CIs	Median intensity	Moment k	ML k (95%CI)	χ^2 goodness of fit, df	p
Nematodirus	<1yr	87	0.61	25	16-36	8	0.25	0.34 (0.22-0.46)	11.97, 4	0.018
gazellae	Adult	46	0.33	19	7-34	14	0.15	0.11 (0.04-0.18)	2.084, 1	0.149
Marshallagia	<1yr	87	0.25	8	3-16	14	0.06	0.09 (0.04-0.13)	5.196, 1	0.023
marshalli	Adult	46	0.70	148	92-197	74	0.62	0.28 (0.16-0.39)	9.088, 3	0.028
Marshallagia	<1yr	87	0.28	3	1-5	15	0.10	0.05 (0.02-0.08)	-	-
mongolica	Adult	46	0.54	106	104-166	128	0.29	0.19 (0.10-0.28)	13.17, 1	<0.001
Marshallagia	<1yr	87	0.68	16	9-26	10	0.18	0.52 (0.34-0.70)	29.21, 3	<0.001
spp.	Adult	46	0.96	308	232-382	242	1.41	0.55 (0.36-0.74)	43.31, 1	<0.001

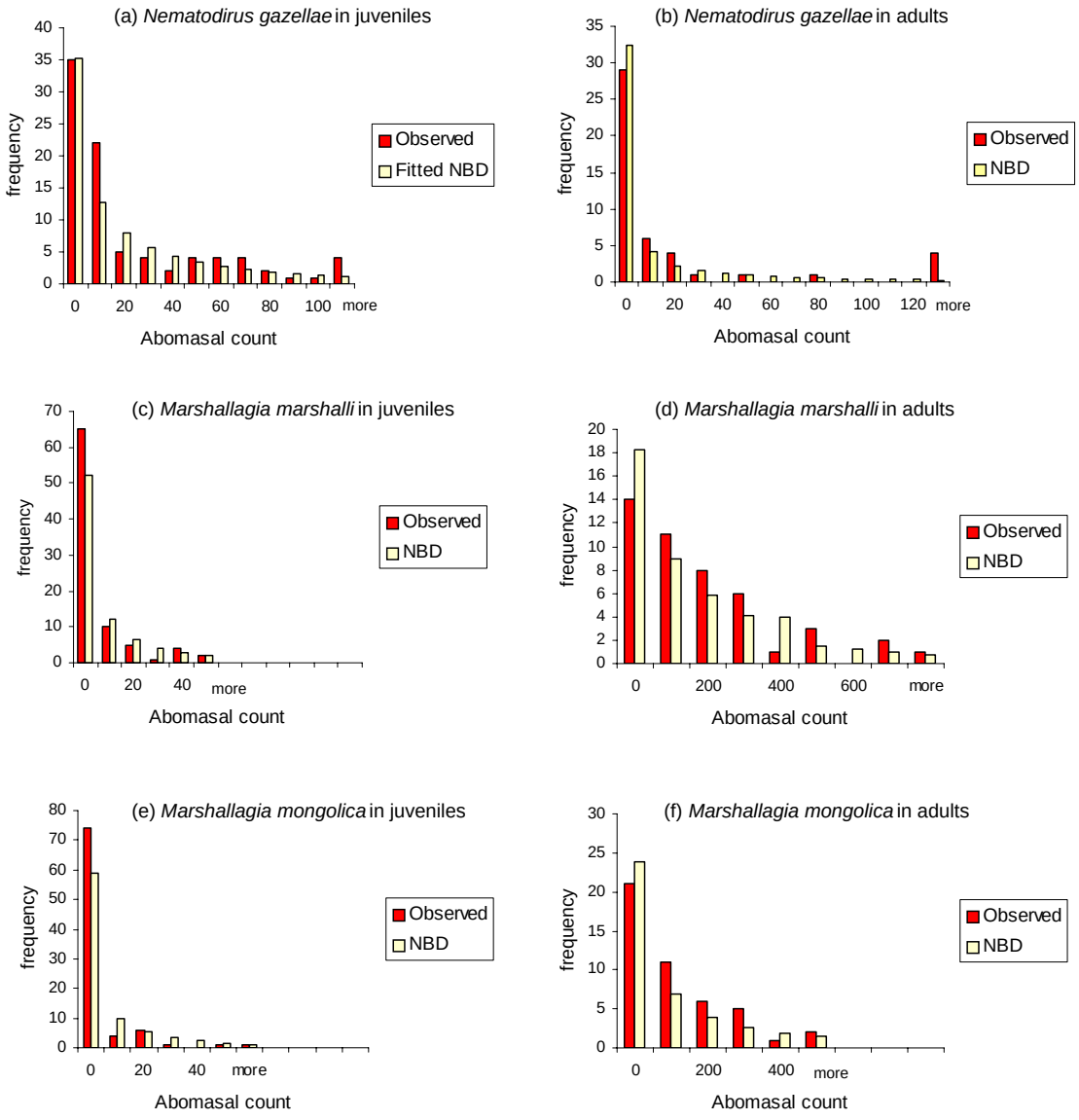


Figure 6.4. The observed distribution of abomasal nematodes among saigas of different ages in Betpak-Dala, with maximum likelihood fits to the negative binomial distribution (NBD). Juvenile refers to animals less than one year old. Note that the scale differs for *Marshallagia* spp. in adults, which were more abundant than other populations. Parameters and and goodness of fit of the NBD distributions are given in Table 6.6.

It should be noted that the goodness of fit test is biased to the fuller count classes (i.e. the 'hump' of the distribution), while MLE is biased to the 'tail', since the very small point likelihoods in this region disproportionately influence the likelihood function. It is possible, using the method of Bliss and Fisher (1953), to iteratively find the value of k that returns the minimum χ^2-value and therefore the closest overall fit. This was carried out for the same parasites, and results are presented in Table 6.7. As Fig. 6.5

shows, the fit is improved in all cases, mainly as a result of closer correspondence in observed and expected frequencies in the uninfected class. Values for *k* using this method were elevated for both *Marshallagia* species in adult saigas, but similar to MLE for other host-parasite combinations.

Table 6.7. Alternative fits to the Negative Binomial Distribution, obtained using the iterative method of Bliss and Fisher (1953), but minimising χ^2 directly to obtain the closest possible overall fit. Graphical fits to the data are shown in Fig. 6.5.

Parasite	Age class	k	χ^2 goodness of fit, df	p
Nematodirus gazellae	<1 yr	0.47	9.249, 6	0.160
	Adult	0.13	1.853, 1	0.173
Marshallagia marshalli	<1 yr	0.16	0.074, 1	0.786
	Adult	1.05	1.422, 2	0.491
Marshallagia mongolica	<1 yr	0.09	2.013, 1	0.156
	Adult	0.60	0.247, 1	0.619

Lumping together counts of both *Marshallagia* species reduced the fit to the NBD to non-significance (Table 6.6), and obscured the apparent differences in degree of aggregation of these parasites in saigas of different age classes. Analysis of *Marshallagia* burdens will continue to consider the 2 species as separate, despite the ambiguity of their morphological differences discussed in chapter 5.

The overall MLE fit of the NBD to the parasite counts tested was assessed by summing the χ^2-values and degrees of freedom (Hunter and Quenouille, 1952; Stear *et al*, 1995*b*). This demonstrated that the poor fits were not simply a consequence of the multiple comparisons (combined χ^2 =41.5, df=10; $p<0.01$). The good fit in some cases, however, and lack of a convincing alternative distribution, motivated the continued use of the NBD as a description of overdispersion in the data, and its exploration as the basis of comparison of parasite abundance between groups. The bootstrap comparison of mean *Nematodirus gazellae* abundance described in the previous section was repeated, this time by sampling directly from the fitted NBDs that best described each data set. The fitted NBDs were based on a class interval of 10 parasites, truncated at the class whose probability of occurring was less than 0.001, and scaled to a total probability of 1.

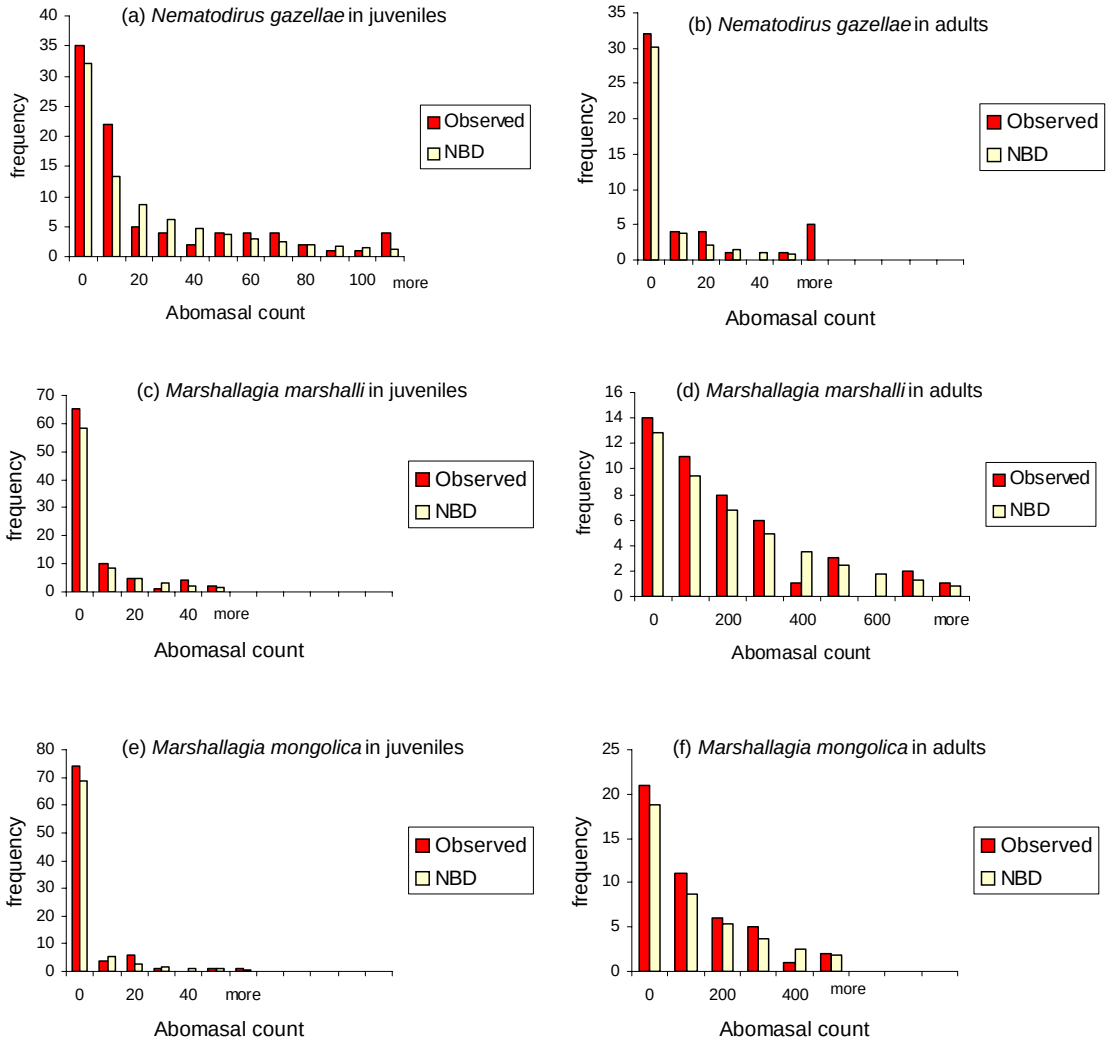


Figure 6.5. The distribution of abomasal nematode burdens in saigas in Betpak-Dala, and fits to the negative binomial distribution (NBD) by minimising χ^2. Juvenile refers to animals less than one year old. Note that the scale differs for *Marshallagia* spp. in adults, which were more abundant than other populations. Parameters and and goodness of fit of the NBD distributions are given in Table 6.7.

Bootstrap sampling from fitted distributions has the advantage that comparisons are independent of sample size. Sampling directly from the data is not, since larger samples are more likely to include heavily infected hosts. However, if the data are used to estimate the underlying parasite distribution, then fitted distributions may also be biased by the original sample size. To test for this, a bootstrap comparison was also made between the counts from adult saigas (n=46), and those from juvenile saigas truncated at the 46th sample collected. Bootstrap samples were drawn both from the data directly, and from the NBD estimated by MLE using these data. The mean and *k* were both lower for the truncated sub-adult sample than the full sample, and the NBD fitted less well (mean=21.5, MLE k=0.350, χ^2=9.07, 2df, p=0.011, c.f. Table 6.6).

Results from the bootstrap comparisons of means are given together in table 6.8. The probability that sub-adult saigas carry heavier abomasal burdens of *Nematodirus gazellae* than adults, given the assumption that the underlying parasite population is negatively binomially distributed, is high and agrees closely with the proportion of data-based bootstrap means that show a difference in this direction. Neutralising the effect of the larger sub-adult sample size results in a less consistent difference, whether bootstrap samples are drawn from the data or the fitted NBD. Mean abundance in sub-adult saigas is still mostly higher than in adults (the expected p-value given equality is 0.5), but confidence in the difference is weakened. The use of bootstrap comparisons of parasite abundance is discussed in section 6.5.

Table 6.8. Bootstrap techniques for comparing the mean abundance of* Nematodirus gazellae *in adult and sub-adult saigas in Betpak-Dala. The data sets from which bootstrap samples were drawn are: 1. Raw counts; 2. The first 46 animals sampled in each age class, making sample sizes equal; 3. The underlying negative binomial distribution (NBD) predicted by MLE (see text, section 6.3.5); 4. NBD from MLE, on the first 46 animals sampled in each age class. P-values in each case are based on the proportion of adult bootstrap means that exceeded sub-adult bootstrap means. 1,000 comparisons were made, each based on 1,000 bootstrap samples drawn with replacement from the observed or fitted parasite distribution.

Bootstrap drawn from:	Mean abundance (CI)		Mean difference in bootstrap means (95% CI)	p-value
	Adults	Sub-adults		
1. Data	19 (8-33)	25 (16-35)	5.4 (0.8-9.9)	0.008
2. Data (equal n)	19 (8-33)	21 (9-39)	2.2 (-2.6-6.8)	0.174
3. NBD	19	25	14.5 (11-18)	0.006
4. NBD (equal n)	19	22	9.1 (-7 to 21)	0.181

6.4 Faecal egg counts

Indirect measurement of parasite burdens has many potential advantages (see chapter 4), especially as a means of obtaining a large sample size without having to kill the animals sampled. Its utility depends on the following questions:

- Does the indirect measure (in this case faecal egg count, FEC) provide a true reflection of adult parasite prevalence and burden?
- Are the methods used reliable and repeatable, allowing comparison of results within and between studies?
- Do the statistical properties of the counts allow meaningful comparison between host groups?

These questions are considered with respect to FEC in saigas and livestock below.

6.4.1 Relationship between FEC and prevalence of infection

The efficiency of FEC in detecting gastrointestinal nematode infection is considered separately for the 3 recognisable egg types: *Nematodirus* spp., *Marshallagia* spp., and other trichostrongylids (principally *Ostertagia*, *Trichostrongylus* and *Haemonchus*).

Table 6.9. Comparison of the prevalence of gastrointestinal nematodosis in saigas and sheep using the McMaster's faecal egg count (FEC) technique, and post mortem nematode extraction. Saigas from the Betpak-Dala population are included: J=juvenile (<1 year old); A=adult. χ^2 values refer to a comparison of positive and negative frequencies using each measure of infection, in a 2x2 contingency table, and are not given where expected frequencies include values <5. Asterisks mark significant differences in median intensity of infection (*Mann-Whitney U=8, 1df, p=0.039; **U=13, 1df, p<0.001). A more concentrated suspension of faeces was used for the McMaster's test on samples from saigas, compared with those from livestock, and FEC from saigas and livestock are therefore not directly comparable.

Parasite genus	Host	n	Mean abundance	Median intensity	n positive, nematode extraction	n positive (FEC)	χ^2 (1df)	p
Marshallagia	All	72	175	50	62	19	52.2	<0.001
	Saiga J	34	13	9**	25	3	29.4	<0.001
	Saiga A	14	448	409**	14	11	-	
Nematodirus (abomasum)	All	72	28	23	32	45	-	
Nematodirus (total)	All	37	28	203	27	7	21.8	<0.001
	Saiga J	6	48	1108*	6	6	-	
	Saiga A	8	23	210*	7	1	-	
Other tricho-strongylids	Sheep	13	463	433	12	8	-	
	Saigas	144	2	5	8	0	-	

Direct and indirect measurements of the extent of infection are compared in Table 6.9. The overall prevalence of all nematode types was higher when measured by *post mortem* worm counts than by McMasters FEC. Furthermore, the efficiency of FEC in detecting nematode infection varied between parasite genera and host groups. Apparent false positive FEC were obtained for abomasal nematodirosis, and are likely to be caused by nematodes in the small intestine. When only complete gastrointestinal washes were considered, FEC detected about a third of *Nematodirus* infections, a proportion comparable to that for *Marshallagia* in the abomasum. FEC, however, were more successful in detecting nematodirosis in juvenile than in adult saigas, and *vice versa* for marshallagiosis. This may be a consequence of higher *Nematodirus* burdens, and lower *Marshallagia* burdens, in the younger age class, compared with

adults (see Table 6.9). Trichostrongylid species other than *Marshallagia* were found in the faeces of sheep but not saigas. False negatives in sheep were again associated with lower nematode burdens (n=8 and 4 for true and false negatives, median intensity 592 and 171, Mann-Whitney U=4, p=0.042).

These results suggest that:

(i) McMasters FEC were quite inefficient in detecting nematode infection in the animals sampled, in spite of modification to allow for low faecal egg density (see chapter 4).

(ii) The success of detecting nematode infection by FEC is a function of the intensity of infection.

Both points have implications for the interpretation of data in the present study, and are given further consideration below.

6.4.2 Detection of small gastrointestinal nematode burdens

Coverslip flotation was used in an attempt to detect faecal egg density below the threshold of the McMasters test (see chapter 4). More nematode infections were detected by coverslip flotation than by McMasters FEC across all host-parasite combinations (Table 6.10). Overall, 50% of *Marshallagia* and *Nematodirus* infections found in saigas and sheep were detected by coverslip flotation, significantly higher than the 33% detected by the McMasters technique (n=121, χ^2 =8.123, 1df, p<0.05).

Table 6.10. A comparison of McMaster's and coverslip flotation techniques for detecting nematode eggs in the faeces of saigas and sheep. McM=number positive using McMaster's technique; CS=number positive using coverslip flotation.

		Marshallagia		*Nematodirus*		*Trichostrongylus*		All nematodes	
	n	McM	CS	McM	CS	McM	CS	McM	CS
Saigas	49	7	18	20	34	2	3	29	55
Sheep	279	8	27	26	79	13	27	47	133
All livestock	516							129	260

The relative sensitivities of the McMasters, coverslip and Soviet loop methods were compared using a single sample of horse faeces known to be infected with strongyles. Strongyle eggs are similar in size and appearance to those of the trichostrongylids. Their density in the original sample was assessed by McMasters examination of 1g of faeces: the well-mixed sample was then diluted to simulate samples containing eggs at different densities, and all 3 methods used to search for eggs at each dilution. Results are shown in Fig. 6.6. The coverslip technique was the most sensitive, detecting strongyle eggs down to an egg density equivalent to 5epg, while the loop method did not detect eggs below a dilution equivalent to 165epg. The McMasters technique became unreliable below 42epg. An attempt was made to repeat the analysis on the same, refrigerated, sample 4 days later, but egg recovery was lower and results were not comparable.

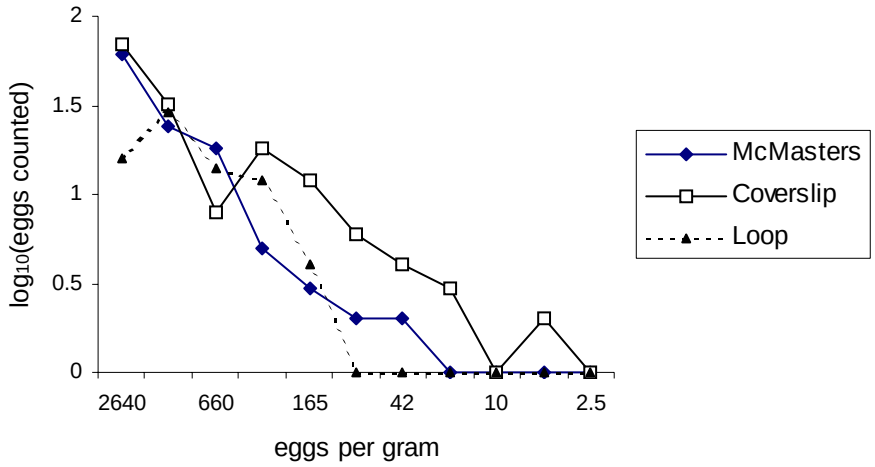


Figure 6.6. The relative success of 3 different flotation techniques in detecting strongyle eggs present at a known density in equine faeces. The reference egg density per gram was taken from serial McMasters examination of one gram of undiluted faeces.

Results therefore agree that coverslip flotation is a more sensitive test than the McMaster's technique for small burdens of the nematodes considered.

6.4.3 Quantifying nematode egg density in faeces

The McMasters test is designed to quantify faecal egg density in ruminant faeces. Extrapolation of the eggs counted to give a FEC (eggs per gram, epg) assumes that eggs are randomly distributed both through the sample after mixing, and across the

counting chambers of the McMaster slide (Peters and Leiper, 1940). Sufficient faecal material should be examined to obtain repeatable measurements of faecal egg density. To test these assumptions, and determine the amount of faecal material needed for repeatable results, duplicate slides were examined from a subset of samples. Test samples from the foal infected with strongyles were included.

FEC from 129 samples were verified by examining a second McMaster slide from the same, mixed, sample. There was a highly significant correlation between the numbers of eggs found on the two slides (n=129, Pearson r=0.943, $p<0.001$). In 62 cases, a total of 5 slides were examined. Deviation from the null (random) distribution of eggs among slides was assessed by computing Fisher's formula (Hunter and Quenouille, 1952) below:

$$\chi^2 = \frac{\sum (x - \bar{x})^2}{\bar{x}} \qquad (6.3)$$

where x is the count on each slide, and $\bar{x}$ the mean of each series of 5 slides.

χ^2 was then summed across all 62 series, and compared with the χ^2 distribution for (62 x 4) degrees of freedom. The resulting value of χ^2 =348 (248 df) is not significant at the 5% level, and the distribution of eggs among slides does not therefore differ significantly from Poisson. The distribution of eggs and coccidial oocysts among the 12 marked rows of 36 McMaster counting slides was similarly compared with the Poisson, and again found not to deviate significantly from it (χ^2=21,514; 396 df, $p>0.05$).

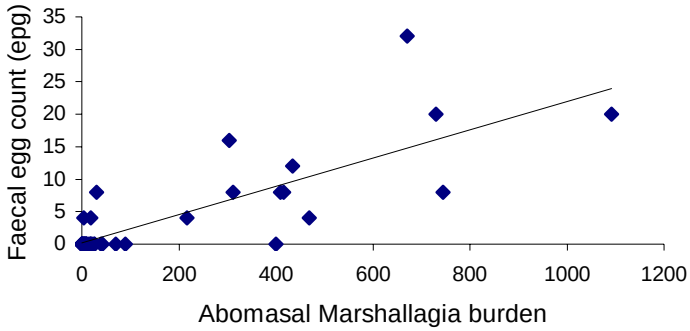
The assumptions of random mixing of eggs between and within slides, and the repeatability of the modified McMaster method used, are therefore satisfied.

6.4.4 Faecal egg density as a predictor of adult nematode burden

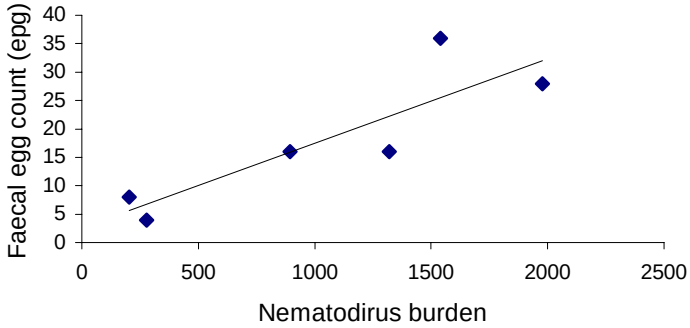
Prediction of adult nematode burdens using FEC relies on the existence of a linear relationship between nematode burden and faecal egg count. This is often not true, for example because of density-dependent reduction in fecundity (see chapter 4).

The relationship between nematode burden and FEC using the McMasters technique was examined for *Marshallagia* and *Nematodirus* infections of saigas. There was a significant correlation between abomasal *Marshallagia* spp. burden and the density of *Marshallagia* type eggs in saiga faeces, irrespective of host age (Fig. 6.7*a*). The correlation was not strengthened by logarithmic transformation of either or both axes, but was still significant after log-log transformation. Linear regression was attempted in order to obtain a first estimate of *Marshallagia* egg production in saigas. Counts were transformed for regression in an effort to normalise the variance. Estimated regression coefficients are given in the legend of Fig. 6.7*a*. The low burdens of *Marshallagia* in juvenile saigas, coupled with the low sensitivity of McMasters FEC, probably results in underestimation of egg production in the most lightly infected hosts. Linear regression using counts in adult saigas only, however, gave an estimated slope very close to that for all ages combined.

Only total *Nematodirus* burdens were considered, giving a smaller sample size. Just 5 of the FEC from adult saigas were positive, and none exceeded 4 *Nematodirus* eggs per gram. Correlation between burden and FEC was not significant (r_s=0.42, p=0.31). Among juvenile saigas, the 6 paired observations were significantly correlated (Fig. 6.7*b*). Regression was again attempted in spite of the limited data, as there are no existing published estimates of egg production by *Nematodirus* in saigas.



(a) *Marshallagia* in 48 saigas of all ages. Pearson's r=0.82, p<0.001. Linear regression coefficents on log-log transformed data: slope=$2.17x10^{-2}$(SE 0.002), intercept=0.21 (SE=0.65), ANOVA F=91.3, p<0.001. (Adult saigas only: slope=$2.16x10^{-2}$ (SE 0.07), intercept=0.31 (SE 3.7), ANOVA F=9.68, p=0.009.)



(b) *Nematodirus* (abomasum and small intestine) in 6 saigas less than one year of age. Pearson r=0.87, p=0.024. Linear regression on log-log transformed data: slope=$4.17x10^{-4}$ (SE<0.001), intercept=0.77 (SE 0.12), ANOVA F=16.39, p=0.015).

Figure 6.7. The relationship between gastrointestinal nematode burden and faecal egg count in saigas.

6.4.5 Predicting nematode burden in lightly infected hosts

Given the low sensitivity of the McMaster test in detecting light nematode burdens, the potential of the coverslip technique as a quantitative measure of faecal egg density was investigated. The test could, in theory, be extended to measurement of egg density because a known quantity of faeces is placed in the test tube used for flotation. The number of nematode eggs found on McMaster FEC was compared with that found on the coverslip in a series of samples of saiga and sheep faeces. The two measures were significantly correlated (n=63, r_s=0.78, p<0.001). However, coverslip egg counts were poorly correlated with adult burdens of both *Marshallagia* (n=63, r_s=

-0.20, p=0.34) and *Nematodirus* (n=39, r=0.34, p=0.11) found on *post mortem* examination.

The number of eggs recovered by McMasters FEC and by coverslip flotation were both compared with the number of eggs subsequently extracted from a subset of 12 samples using Direct Centrifugal Flotation (DCF; MAFF, 1986). DCF was repeated 3 times on each sample, and the total number of eggs recovered taken to represent the best estimate of the actual number of eggs in the sample. Of the 3 flotation techniques used, the number of eggs found by DCF correlated most strongly with that found by the McMasters technique (r=0.94, p<0.001). The DCF count was also closely correlated with coverslip flotation (r=0.93, p<0.001), while the loop technique poorly predicted the number of eggs found on DCF (r=0.53, p=0.08). Coverslip flotation recovered fewer eggs than DCF, and repeated sampling of the flotation tube revealed that many eggs remained in suspension after the coverslip was lifted (Table 6.11). DCF is therefore more efficient at recovering eggs than flotation without centrifugation, and is advantageous where resources allow. The observed prevalence of strongyle eggs in equine faeces at a range of dilutions, however, did not differ significantly using DCF and coverslip flotation (Table 6.12).

Table 6.11. The number of strongyle type eggs recovered by successive attempts at Direct Centrifugal Flotation (DCF) and coverslip flotation without centrifugation (CS) on the same sample of faeces, suspended in 0.9% NaCl solution. The mean (and standard deviation in brackets) is given for 10 samples of infected equine faeces at different dilutions. The efficiency is taken as the number of eggs recovered on the 1st repetition of each technique, divided by the total number recovered by 3 successive DCFs.

Repetition	CS		DCF	
1st	26	(20)	148	(212)
2nd	8	(7)	25	(18)
3rd	5	(3)	9	(8)
Efficiency (%), range	14	(7-52)	81	(54-93)

Table 6.12. The effect of centrifugation on the observed prevalence of strongyle type eggs in equine faecal samples. DCF=Direct Centrifugal Flotation; CS=coverslip flotation without centrifugation. Each technique was applied twice to 11 samples diluted to expected egg densities of 3epg to 3,000epg. There was no significant difference in the observed prevalence (χ^2 test for association = 0.524, 1df, NS).

	CS	DCF
Positive	14	16
Negative	8	6

In summary, the number of eggs recovered by coverslip flotation appears to bear a strong relation to the McMasters faecal egg count. However, added variation makes it a less reliable predictor of adult nematode burdens. Coverslip flotation may still be useful in detecting infection below the threshold of the McMaster's test.

6.4.6 The distribution of FEC between hosts

The use of faecal egg counts to estimate absolute nematode abundance relies on a convincing linear relationship between the two, and proper calibration of FEC to adult nematode burden. In the absence of such detailed data, however, FEC can still give an indication of relative nematode abundance in different host groups, provided there is reason to believe that the two are correlated. In this case, distributional assumptions will affect the interpretation of comparisons between FEC in the same way as for direct counts (see section 6.3 above). Additional sources of error are likely to arise from the extrapolation of overall egg density from the small aliquots of faeces examined.

The relative abundance of nematode eggs in the faeces of different groups of saigas and livestock in Kazakhstan is summarised in Table 6.13. Positive samples are grouped into classes depending on the dilution used in the McMasters FEC. Members of positive classes were generally too few to allow the fit to the NBD to be assessed. However, coverslip flotation established that eggs were actually present in many of the samples negative on McMasters FEC. The zero epg class can therefore be split into genuinely negative and positive but sub-threshold components, the threshold being 4 or 50 epg, depending on the McMasters dilution used. Linear regression of the transformed egg counts using each technique (Fig. 6.8) was used to predict egg abundance in samples negative on McMasters FEC, but positive on coverslip flotation. In this way, mean egg density in the <50epg class was estimated for each host group.

Table 6.13. Faecal egg prevalence and density in groups of saigas and livestock sampled in Kazakhstan. Mean faecal egg density in positive samples is expressed as eggs per gram (epg). Easily discernible nematode egg types were counted separately: 'Other trichostrongylid' refers to typical trichostrongylid eggs that are neither *Marshallagia* nor *Nematodirus* spp. The McMasters dilutions differed for samples from saigas and livestock, and results are not comparable. BD=Betpak-Dala.

			Marshallagia epg in positive samples			*Nematodirus* epg in positive samples			Other trichostrongylid epg in positive samples		
Host	Location	n sampled	n positive	Mean	sd	n positive	Mean	sd	n positive	Mean	sd
Saigas	Ustiurt	55	5	21	9	16	48	49	0	-	-
	BD	45	16	12	9	32	20	19	0	-	-
Cattle	Ustiurt	9	2	50	0	2	125	106	2	50	0
	BD	78	0	-	-	1	50	-	15	55	16
Sheep	Ustiurt	50	16	50	0	12	50	0	17	136	153
	BD	207	11	55	16	15	57	18	6	71	27
	Chu	107	12	61	22	67	112	77	26	124	182
Goats	All	47	0	-	-	3	50	0	1	50	-
Camels	All	9	0	-	-	1	50	-	1	100	-

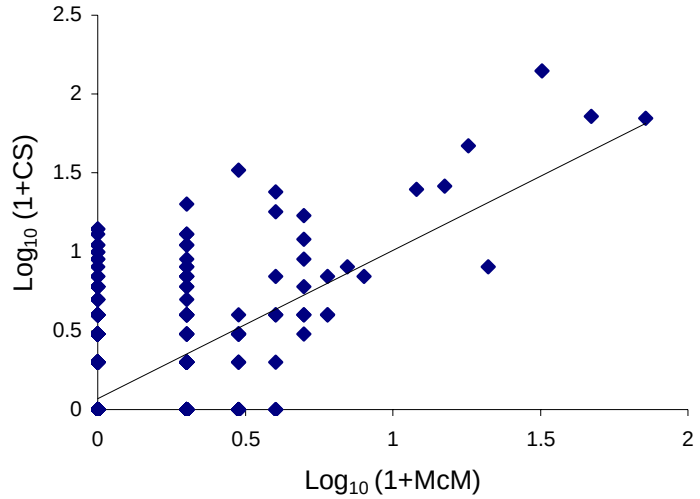


Figure 6.8. Regression of the number of nematode eggs found on coverslip flotation (CS) on the number found on McMasters count of the same sample (McM). n=985, r=0.61, ANOVA F=571, 984df, p<0.001. Slope=0.390 (SE 0.016), intercept=6.5×10^{-3} (SE 5×10^{-3}).

Class means (in eggs per gram) are an artifice of the dilution used for FEC. In reality, each class incorporates a range of counts, whose true distribution within and beyond the class boundaries is hidden by this rounding effect. To account for this, and derive a more realistic distribution, the 50epg classes for livestock counts were divided along 10epg intervals, and counts in each class assumed to be Poisson distributed among these intervals, either side of the class mean (Table 6.14). Members of each 10epg

class so derived were then summed to give the final smoothed distribution. The process is illustrated in Fig. 6.9 for *Marshallagia* eggs in the faeces of sheep in Betpak-Dala. Counts from the <50epg class were included, truncated at 10epg so that they did not artificially inflate the zero class. This adjustment successfully reflected overdispersion in the observed distribution of McMasters-negative, coverslip positive counts (Table 6.14).

Table 6.14. The distribution of faecal egg counts within the classes derived from extrapolation of McMasters egg counts. In 70 samples of saiga and sheep faeces, the distribution of coverslip flotation (CS) counts in each McMaster's class was assessed against the Poisson using the one-sample Kolmogorov-Smirnov test. The test statistic, Z, and its p-value are shown. One egg found on McMaster's flotation represents 50 eggs per gram of faeces.

No. eggs found on McMasters	n	Mean CS count	Kolmogorov-Smirnov Z	p
0	29	5	1.819	0.003
1	10	6	0.904	0.39
2	9	9	1.320	0.06
3	4	15	0.596	0.87

Estimation of egg density in the <50epg class, and correcting for rounding error, allowed egg distributions in the larger livestock samples to be compared with the NBD (e.g. Fig. 6.10). Results for sheep in Betpak-Dala are shown in Table 6.15. The good fits of the FEC to the NBD formed the basis for comparisons of faecal egg abundance between host groups using bootstrapping. Results are in chapter 7.

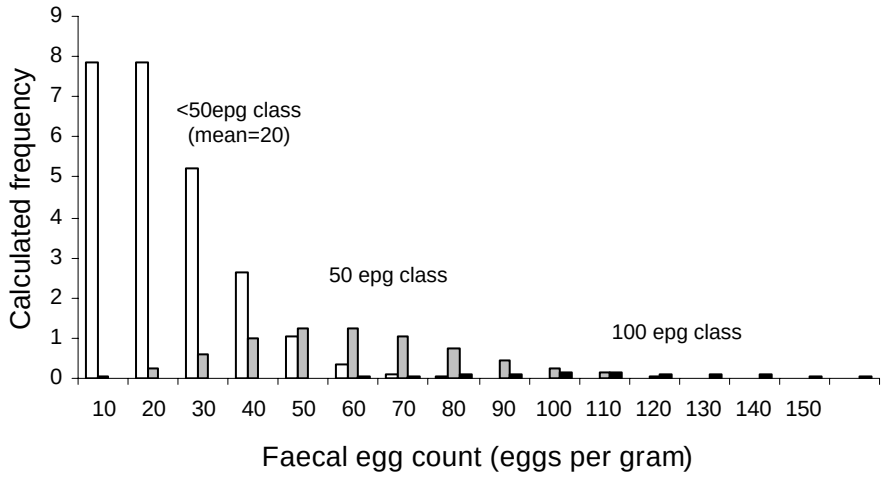


Figure 6.9. Correction of rounding error in counts of Marshallagia spp. eggs in sheep faeces. Faecal egg counts are assumed to be Poisson distributed around class means. Summing the resulting Poisson distributions results in a smoothed distribution (see Fig. 6.10).

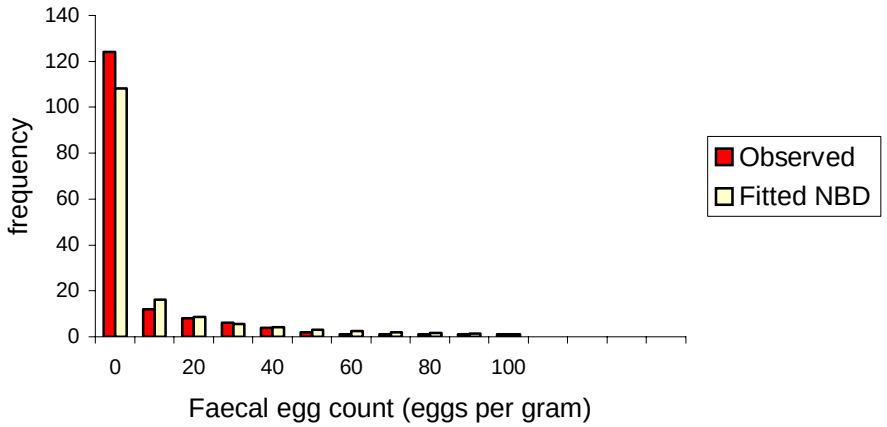


Figure 6.10. Smoothed distribution of faecal counts of Marshallagia sp. eggs in adult sheep in Betpak-Dala, and the maximum likelihood fit to the negative binomial distribution (NBD).

Table. 6.15. Nematode faecal egg abundance in sheep in Betpak-Dala, assuming an underlying NBD, and Poisson distribution about each class. Zero counts and false negative FEC (the <50epg class) are included (see text). 95% CIs about the mean were calculated by bootstrapping from the fitted distributions. Sample sizes are given in Table 6.13. χ^2 refers to the goodness of fit test to the NBD.

	Lambs (<1 yr old)					Sheep (>1 yr old)				
	Mean (epg)	95% bootstrap CI	k	χ2, df	p	Mean (epg)	95% bootstrap CI	k	χ2, df	p
Marshallagia	2	(0-5)	0.022	-	-	7	(5-10)	0.164	4.672, 6	0.587
Nematodirus	10	(4-16)	0.219	1.385, 1	0.239	5	(3-7)	0.122	6.348, 5	0.274
Other trichostrongylid	3	(0-9)	0.023	-	-	5	(3-7)	0.109	4.373, 1	0.037

6.5 Discussion

This section discusses the following questions:

- Do the methods used in this study provide reliable measures of parasite population abundance and distribution?
- Would they be useful in other studies?
- What are the key difficulties in analysing the data collected, and have they been satisfactorily overcome?

6.5.1 Sampling methods

Many inaccuracies in parasite counts can be introduced at the time of collection, and some workers have advocated more painstaking sampling methods as a way of saving time and error in the laboratory (Eysker and Kooyman, 1993). However, the remote field locations, adverse environmental conditions, and lack of time and resources in this study ruled many of these out. Instead, the field methods used for collecting parasites were intended to reduce the time and resources needed, minimise the volume of the material collected, and defer detailed examination to the laboratory.

In respect of avoiding obvious sources of bias and error, the collection methods used for adult gastrointestinal nematodes were successful. Non-ligation of the gut, extrusion from the small intestine, damage and freezing of saiga abomasa, and the taking of aliquots of sieved gut washings did not appear to greatly affect the species or number of parasites recovered. These modifications of standard techniques could be usefully employed in other surveys of gastrointestinal nematodes of wildlife, especially where water or containers are scarce, rapid sampling is necessary, or there is a limit to the material that can be carried back from the field. Freezing of abomasa, inevitable in this study, could be used as a means of preserving organs until extraction is possible, without fear of affecting the success of subsequent recovery of adult nematodes.

In the laboratory, the practice of examining aliquots of sampled material until a representative number of nematodes have been picked out for identification is widely

used in the enumeration of trichostrongylid burdens (Reinecke, 1984; MAFF, 1986; Eysker and Kooymans, 1993). In this study, the proportion of the sample examined did not seem to affect the number of species recovered. Examining aliquots rather than the whole sample carries an inherent risk of missing rare species. This is acceptable in the present study, which focuses on patterns of abundance in the more common species.

Recovery of immature stages was less successful. Temperatures in the field could not be maintained at a suitable level for long enough to allow larval migration out of the abomasal mucosa. Pepsin digestion would have encountered the same problem. It is worth noting that, unlike related nematodes such as *Ostertagia* and *Teladorsagia*, clusters of immature *Marshallagia* sp. found in sheep in south Kazakhstan were not associated with raised nodules, but only small areas of mucosal hyperaemia. Gross examination of abomasa for these stages should therefore be appropriately detailed. These tissue stages of *Marshallagia* sp. could be hypobiotic larvae (Michel, 1974; Giangaspero *et al*, 1992; El-Azazy, 1995). Irgashev (1973) described well-developed secondary sexual features in *Marshallagia marshalli* as early as 10 days after infection, at which time most larvae were in the mucosa. He called this the "pre-imaginal stage", and considered it a normal part of development, though in Uzbekistan a greater proportion of worms were at this stage in adult sheep in the winter than at other times of year. It would be interesting to know whether *Marshallagia marshalli* and *Marshallagia mongolica* differ in their propensity to arrest, and at what stage and season they do so. The implications of hypobiosis for the epidemiology of marshallagiosis are discussed in chapter 9.

The poor success of herbage larval counts in recovering free-living trichostrongyloid stages was disappointing in light of their adaptation for low larval density (chapter 4). The amount of herbage collected (100-1,000g dry mass) may still have been too small. Alternatively, the parts of the plants sampled (mainly green leaves) may not be the principal sources of infection, or the time of sampling may not have coincided with peak larval availability. Herbage samples were mostly taken in mid-morning: larvae may migrate earlier in the day, when there is dew, or later, when temperatures rise. Migration and larval availability may only follow rain, or larvae may be too sluggish once on the herbage to migrate through the Baermann apparatus. Controlled

studies of larval extraction from herbage under different conditions are needed to refine methods of extraction: to date, few results have been published from either temperate or non-temperate areas (see chapter 8).

6.5.2 Comparing burdens between host groups

Exploratory analysis of nematode burdens in saigas showed that standard parametric tests on transformed data, and non-parametric tests, are both unreliable in detecting differences in mean parasite abundance between host groups. This conclusion was also reached by, among others, Fulford (1994), Wilson *et al* (1996), and Wilson and Grenfell (1997). Alternative statistical methods for comparing means, however, have been harder to identify. Bootstrapping and maximum likelihood techniques were advocated by Pacala and Dobson (1988), Williams and Dye (1994), Wilson *et al* (1996) and Guyatt *et al* (1999), but their use in complex models is restricted, and generalised linear modelling techniques have often been preferred (Wilson and Grenfell, 1997; Shaw *et al*, 1998; Elston *et al*, 2001). The present study seeks to make a relatively small number of direct comparisons of parasite abundance between host groups, and the advantages of bootstrapping can be usefully harnessed.

Bootstrapping from a fitted distribution is generally preferable to sampling the data directly, as this reduces the influence of individual counts on the calculated statistics (Hilborn and Mangel, 1997). The flexibility of the NBD, and its demonstrated fit to a wide variety of parasite sample data, makes it an obvious choice for such a distribution (Shaw *et al*, 1998). In all cases, the NBD provided a better fit to the nematode counts in saigas than the Poisson distribution. The fits to the NBD were, however, less close than those previously reported for trichostrongylids of domestic ruminants (e.g. Barger, 1985). This was largely the result of a poor fit to the uninfected and lightly infected host classes (the ‘hump’ of the distribution). This is a consequence of the maximum-likelihood fitting methods, in which the small point likelihoods of high burdens have a large influence on the likelihood function. This was demonstrated by the improved fit when the goodness-of-fit statistic was minimised directly, giving the fuller classes disproportionate influence over the choice of k. Estimated values for k using this method were nevertheless similar to those using MLE for most host-parasite combinations.

Unequal sample sizes may compromise comparisons between sample means, as larger samples taken from an overdispersed parasite population are more likely to contain high burdens. Fitted distributions share the problem, since they are biased by the data used to estimate the distribution parameters. This resulted in both Type I and Type II errors when comparing parasite burdens between groups of saigas. The problem of comparing samples of unequal sizes can be solved by discarding the excess counts from the larger group of hosts, but this is wasteful of data. Nevertheless, this procedure is recommended as a check where differences in parasite abundance may be related to relative sample size. Simulated resampling could equally be used.

The NBD is inherently problematic when searching for sub-structure in parasite abundance and distribution within host groups. Parasites distributed as a NBD within a host group are only distributed as NBD in sub-groups if the mean, *m*, and *k* of those NBD are equal (Grafen and Woolhouse, 1993). Finding differences in estimated *m* and *k* in the sub-groups invalidates the assumption that the parasites are distributed as NBD in the group as a whole. Since such heterogeneities can be pursued down to the level of the individual host, it would seem unlikely that the underlying distribution of any natural population of parasites truly follows the NBD. Such hidden heterogeneities may in fact cause poor fits to the NBD. Thus, the NBD did not fit combined counts of both *Marshallagia* species. Ignoring the subtle and uncorroborated morphological differences in the *Marshallagia* specimens examined would dramatically affect their described distribution between hosts, underestimating aggregation of the parasite population and concealing differences in aggregation between host groups. This lends further weight to the arguments for careful examination of genetic variation within parasite populations outlined in the previous chapter.

Alternatives to the NBD have been used to describe parasite populations, but all have shortcomings. The Poisson-lognormal distribution has been suggested (Elston *et al*, 2001), not because it is a better description as such, but for analytical convenience. Taylor's power law (Boag *et al*, 2001) solves some of the issues of unequal sample size, but requires multiple data sets from the same parasite population. Poulin's index of discrepancy (Poulin, 1993), meanwhile, cannot be compared across unequal sample sizes, and says nothing about supra-Poisson variation in parasite distribution between

hosts. The poor fit of the NBD to the uninfected class of hosts may be exacerbated by false negative counts, especially if faecal egg density is used as the measure of infection, and distributions that fit this class separately from the infected hosts may have advantages. The mixed Bernoulli-Poisson and Bernoulli-NBD models developed by Welsh *et al* (1996) to describe the distribution of rare free-living mammals may be useful in this regard. Fitting distributions to the burdens of infected hosts only, however, only exacerbates the problems of small sample size, and was not possible for the saiga parasites counted. Prevalence may provide a simple and useful reflection of parasite abundance (Guyatt *et al*, 1999). However, the relationship between prevalence and intensity is not always reliable (Shaw and Dobson, 1995), and prevalence shows no sensitivity to the tail of the distribution (Fulford, 1994).

The use of the NBD as a description of parasite distribution in natural populations therefore has drawbacks, both theoretical and practical. The lack of appropriate alternative distributions hinders progress in the statistical modelling of parasite counts, but a natural distribution that is appropriate in all circumstances may not exist. In practice, selection of statistical models for comparisons of parasite abundance involves a trade-off between accuracy and analytical convenience, which should be evaluated on a case-by-case basis (Grafen and Woolhouse, 1994). This process has been a primary purpose of this chapter. A logical extension of the bootstrapping and maximum likelihood techniques used here would be a Bayesian statistical framework for parasite distribution. The posterior distribution would not have to be defined mathematically, but would incorporate information from prior assumptions concerning overdispersion, modified by the available data, however limited. Such a framework could also explicitly incorporate observation error in a way similar to, but more elegant than, that described above for faecal egg counts.

The NBD is therefore adopted as an imperfect but workable description of parasite distribution among saigas, and of faecal egg counts among the livestock sampled in the present study. Bootstrapping from the fitted NBD provides a more reliable and robust comparison of mean parasite abundance between host groups than other currently available techniques, provided differences in sample sizes are taken into account. In the next chapter, these methods will be applied to the analysis of field data with respect to the hypotheses already put forward.

6.5.3 Faecal egg counts (FEC)

The general advantages and problems of FEC as a measure of parasite population abundance have already been discussed in chapter 4. In this chapter, approaches to some of the more serious problems are described. These include non-random distribution of eggs within samples, false negatives, and rounding error. Egg identification and density-dependent reduction of egg production may also confound the interpretation of FEC.

FEC may not accurately reflect adult nematode burdens if it is difficult to differentiate the eggs of different species, the more so if species differ in their rates of egg production. This is unlikely to be of great importance in the present study, since the eggs of *Nematodirus* spp. and *Marshallagia* spp. can be reliably distinguished from those of the other trichostrongyloids (Thienpont *et al*, 1979). Further differentiation to species level would require more advanced techniques, such as the detection of specific antigens (Ellis *et al*, 1993) or DNA (Zarlenga *et al*, 2001).

The question of whether density-dependent reduction in egg production renders FEC meaningless as an index of adult worm burden was addressed in two ways. Firstly, plots of FEC against worm burden were assessed for non-linearity (see Fig. 6.6) and none was found, though data were few and variation large. Secondly, the relationship was assessed separately in young and adult hosts, in case previous exposure and acquired immunity depress egg production. No difference between age classes was found for *Marshallagia* spp., but eggs of *Nematodirus* spp. were fewer in adult saigas in proportion to the number of adult worms present. Again, data were few, since this species occurs in both the abomasum and small intestine, and there was time to sample only a small number of intestines. Preliminary evidence, therefore, suggests that FEC can be used as a reliable index of adult nematode burden for *Marshallagia* spp. in saigas of all ages, and *Nematodirus* spp. in young saigas only.

The distribution of eggs within samples was assessed by comparing egg counts within and between McMasters slides with a theoretical random distribution. In all cases, fit to the Poisson was satisfied. Other workers (e.g. Peters and Leiper, 1940) have used this criterion as an indication of consistency in the counting technique.

Irrespective of egg distribution within samples, dilution-flotation techniques carry an inherent risk of overlooking small numbers of eggs, as only a small aliquot of faecal material is effectively examined. The lower threshold of the McMasters test in its standard form is, in theory, 50epg (Burrows, 1980), though Rossanigo and Gruner (1991) noted a great increase in the coefficient of variation in egg counts below 100 epg. The sensitivity of the test can be increased either by examining more material sequentially, or by concentrating the existing material (Hunter and Quenouille, 1952). The extent to which samples can be concentrated is limited by obstruction of the field of view by excessive faecal debris, while examination of supplementary material is theoretically without limit. The diminishing returns from such prolonged searches, however, were noted early on by Stoll (1930), and Hunter and Quenouille (1952) subsequently calculated that FEC would be most efficient when the mean number of eggs detected was at least 4. Even by concentrating saiga faeces, this was not achieved. Detection of very low counts was therefore only possible using a concentration-flotation technique modelled on Direct Centrifugal Flotation (DCF) (Stoll, 1930). The centrifugation step was omitted due to lack of facilities in the field.

DCF has been found to recover between 10 and 88% of helminth eggs in faeces (Farr and Luttermoser (1941), compared with an average of 16.5% using McMasters flotation (Rossanigo and Gruner, 1991), and it is generally considered the more sensitive test for trichostrongylid eggs in ruminant faeces (MAFF, 1986). Alcaino and Baker (1974) compared DCF with and without centrifugation, and found that centrifugation increased the number of nematode eggs recovered from a wide range of host faeces, but did not significantly increase observed prevalence. This was also found in the present study. As a quantitative method of calculating FEC, DCF variously matched (Wilson, 1957) or undercounted (Levine *et al*, 1960) the number of trichostrongylid eggs in sheep and cattle faeces relative to the McMasters technique, but in each case was better able to detect low densities of eggs. Coverslip flotation in the present study was a useful adjunct to McMaster counts, and was able to detect patent infections missed by McMasters. The detection of low counts had little effect on the calculated mean faecal egg abundance, but did affect observed prevalence, and allowed more precise characterisation of faecal egg distribution. It is impossible to estimate the absolute sensitivity of either method without independent accurate knowledge of the egg content of the samples (Moriya, 1954; Thrusfield, 1995).

The distribution of faecal egg counts among hosts, when the detected false negative counts were included, was well described by the negative binomial (NBD). The NBD has been successfully fitted to faecal egg counts in various species by Hunter and Quenouille (1952), Northam and Rocha (1958), Roberts and Swan (1982), Sreter *et al* (1994) and Stear *et al* (1995*b*). All either combined counts into classes, or used the number of eggs observed directly. The present study, by allowing for variation about the observed count, refines the estimated distribution of eggs among hosts. This process improved the fit to the NBD, and is a reasonable way of dealing with rounding error. The assumption of Poisson distribution about the class mean appears to be satisfactory given the limited data available. The advantage of describing the distribution of FEC in such detail lies in the meaningful comparison of parasite abundance between host groups, which is discussed with respect to adult nematode counts in the previous section.

Refinements to the laboratory techniques used in the present study are possible and well described in the literature, but are not justified when either the conditions favour basic methods (Dangjin, 1996), or when an approximate measure of parasite abundance at the population level is more valuable than accurate enumeration of eggs in a few hosts (Reinecke *et al*, 1962; Rossanigo and Gruner, 1991). The inadequacy of standard FEC techniques in detecting low densities of eggs has been previously noted, especially in relation to bovine faeces (Levine *et al*, 1960; Michel, 1968). Where the detection of light infections is important, modifications such as those used in the present study are likely to be useful. The concurrent requirements of detecting light infections and of examining large numbers of hosts cause difficulties when operator time is limited, and will pose a dilemma in many wildlife studies as well as those of extensively grazed livestock. In this respect, new techniques that automate the enumeration of nematode eggs in relatively large quantities of faeces (e.g. Mes *et al*, 2001) are likely to be extremely helpful.

The large degree of variation observed in FEC, so problematic when ascribing significance to them, is a function of both the underlying parasite distribution and the vagaries of egg production by the nematodes and faecal production by the host. The faecal egg sampling and counting methods used in this study are effective in reducing observation error to a minimum, and deal with irreducible variation in a rational way.

Comparison with adult nematode burdens allows FEC to be used as a measure of both prevalence and intensity of infection in the animals under study, and the description of count distributions provides a basis for comparing these statistics between host groups. This forms the basis for the analysis of field data in the next chapter.