Ryan, U., Yang, R., Gordon, C. and Doube, B. (2011) *Effect of dung burial by the dung beetle Bubas bison on numbers and viability of Cryptosporidium oocysts in cattle dung.* Experimental Parasitology, 129 (1). pp. 1-4

http://researchrepository.murdoch.edu.au/5047/

Copyright: © 2011 Elsevier Inc.

It is posted here for your personal use. No further distribution is permitted.
Accepted Manuscript

Effect of dung burial by the dung beetle *Bubas bison* on numbers and viability of *Cryptosporidium* oocysts in cattle dung

Una Ryan, Rongchang Yang, Cameron Gordon, Bernard Doube

PII: S0014-4894(11)00196-2
DOI: 10.1016/j.exppara.2011.06.009
Reference: YEXPR 6250

To appear in: *Experimental Parasitology*

Received Date: 12 May 2011
Revised Date: 21 June 2011
Accepted Date: 24 June 2011

Please cite this article as: Ryan, U., Yang, R., Gordon, C., Doube, B., Effect of dung burial by the dung beetle *Bubas bison* on numbers and viability of *Cryptosporidium* oocysts in cattle dung, *Experimental Parasitology* (2011), doi: 10.1016/j.exppara.2011.06.009

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Effect of dung burial by the dung beetle *Bubas bison* on numbers and viability of *Cryptosporidium* oocysts in cattle dung.

Una Ryan*a,b, Rongchang Yang*a, Cameron Gordon*b, Bernard Doube*c.

*aDivision of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, Western Australia, 6150.
*bWater Corporation, Level 3, 629 Newcastle Street, Leederville, Western Australia 6007.
*cDung Beetle Solutions Australia, 37 Cave Avenue, Bridgewater, South Australia 5155.

*Corresponding author. Mailing address: Division of Health Sciences, School of Veterinary and Biomedical Science, Murdoch University, Murdoch, Western Australia, Australia 6150. Phone: 61 89360 2482. Fax: 61 89310 414. E-mail: Una.Ryan@murdoch.edu.au
ABSTRACT

*Cryptosporidium* oocysts were inoculated into fresh dung (~1.2 x 10⁴ oocysts per gram wet weight) and fed to dung beetles to assess the effect of dung burial by the dung beetle *Bubas bison* on the distribution of the oocysts in small cores of soil in the laboratory. The experiment consisted of five replicates of each of two treatments: controls (dung but no dung beetles) and the experimental treatment (inoculated dung and 7 pairs of dung beetles). After five days, when approximately 90% of the dung was buried, the surface and buried dung was recovered and subsampled. The oocysts in the subsamples were recovered and enumerated using qPCR. Oocyst viability was evaluated using an assay based on the exclusion or inclusion of two fluorogenic vital dyes, 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI). Results revealed that overall 13.7% of oocysts remained on the surface and 86.3% of oocysts were buried. The viability of oocysts in buried dung was only 10% compared to oocysts the surface dung (58%). Therefore, widespread dung burial by *B. bison* during the winter months could substantially reduce the numbers of *Cryptosporidium* oocysts available to be washed into waterways following winter rains.

Keywords: *Cryptosporidium*; dung beetle; *Bubas bison*; qPCR; reduction in oocysts.
1. Introduction

_Cryptosporidium_ spp. are protozoan parasites that infect a wide range of vertebrate hosts including humans (Xiao, 2010). The oocyst is the environmentally stable stage and is able to survive and penetrate routine wastewater treatment and is resistant to inactivation by commonly used drinking water disinfectants (Fayer et al., 2000). Currently cryptosporidiosis represents the major public health concern of water utilities in developed nations (Fayer et al., 2000) and has been responsible for >50% (165/325) of all water-associated outbreaks of parasitic protozoan disease reported in North American and Europe (Karanis et al., 2007). Five _Cryptosporidium_ species/genotypes are responsible for most human cryptosporidiosis cases; _C. hominis, C. parvum, C. meleagridis, C. felis, and C. canis_ (Xiao and Feng, 2008).

Over the past 20 years, cattle and particularly pre-weaned cattle have been identified as being one of the main reservoir host for the zoonotic _C. parvum_ (Xiao and Feng, 2008). On average, a 450 kg steer will excrete a total of 10 tonnes of wet dung per head per year and a 40 kg sheep about 0.6 tonnes (Anon, 2003). The environmental loading rate of _C. parvum_ in cattle has been estimated at between 3,900 to $1.7 \times 10^5$ oocysts cow$^{-1}$ day$^{-1}$ (Hoar et al., 2000; Atwill et al., 2003). Cattle can therefore potentially contribute significantly to contamination of drinking water catchments with _Cryptosporidium_.

A previous study examined the role that three species of European dung beetle (_Anoplotrupes stercorosus, Aphodius rufus, and Onthophagus fracticornis_) might play in the dissemination of _C. parvum_ oocysts (Mathison and Ditrich, 1999). In that study, beetles were fed cattle dung containing $5.9 \times 10^6$ oocysts of _C. parvum_ and examined after 24 hr of feeding. The authors reported that only small numbers of
oocysts were recovered from the beetles’ external surface, intestinal tract and faeces, suggesting that, although many oocysts were ingested, most were killed during passage through the gastrointestinal tract (Mathison and Ditrich, 1999).

In the present study, we assessed the effect of dung burial by another species of dung beetle, Bubas bison (Coleoptera: Scarabaeidae), also known as the scarab beetle, on the distribution and viability of C. parvum oocysts in fresh cattle dung in small cores of soil in the laboratory. Bubas bison is native to Europe and was released in the south of Western Australia (WA) in 1983 (Edwards, 2007) and is now established in a series of localities throughout its potential distribution across southern Australia (Edwards, 2007; Doube, 2008). Bubas bison breeds in cattle dung that it buries at 30 to 60 cm below the soil surface and, when abundant, can bury individual cattle pads within a few days (Doube, 2008 and unpublished). In such situations, the majority (>90%) of cattle dung produced from May to September each year is buried (Doube, 2008).

2. Materials and methods

2.1 Preparation of oocysts

The C. parvum cattle isolate (SC26), originally obtained from the Institute of Parasitology, University of Zurich was used in the present study. Oocysts were passaged in mice and were purified using ether extraction and a ficoll density gradient as previously described (Meloni and Thompson, 1996). Purified oocysts were counted using a haemocytometer, quantitated using qPCR as described below and stored in 1 x phosphate-buffered saline (1 x PBS) and antibiotics (100 IU/ mL penicillin G, 0.1 µg/
mL streptomycin and 2.5 μg/mL amphotericin B) at 4°C prior to use. Permission to use experimental mice was obtained from the Murdoch University Animal Ethics Committee (R2238/09).

2.2 Dung beetle experiment

The beetles were in the feeding phase of their development during which they feed and mate in shallow tunnels (5 to 10 cm deep) beneath the dung pad. Previous observations indicated that feeding/mating beetles commonly remain beneath a pad for 5 to 7 days before departing to find another pad (Doube, unpublished). Fresh cattle dung (538 gram) (80.8% moisture on a wet weight basis) from grass-fed cattle was inoculated with 6.1 x 10^6 oocysts ml^-1 and stirred continuously for 10 minutes to disperse the oocysts evenly through the dung. The dung was estimated to contain approx. 1.2 x 10^7 oocysts per gram wet weight and the beetles were allowed to bury the dung over a five-day period.

The experiment consisted of five replicates of each of two treatments; controls (dung but no dung beetles) and experimental treatments (dung and 7 pairs of dung beetles). Each soil core contained 0.5 kg of firmly packed damp plasterers sand (4.8% moisture on a dry weight basis) in a 16 cm long vertical plastic (PVC) core (7 cm internal diameter). There was a 5 cm space above the sand surface onto which was placed 50 g of oocyst-inoculated dung. Seven pairs of dung beetles were placed in each of five experimental cores. All 10 cores were covered with mesh and held at ambient temperature in a dry unheated laboratory (ambient 5 to 12 °C). The beetles used in the experiment were not fed for the 5 days preceding the experiment. They dug into the soil beneath the dung within 30 minutes after being introduced to the
cores and began to bury the dung.

After five days, when approximately 90% of the dung was buried, the cores were dissected and a portion (about 15–20 grams) was taken from the middle of each dung mass for oocyst enumeration and viability. The following samples were analysed: (1) the original dung/oocyst inoculum (three replicates); (2) beetle free control pads consisting of oocyst-containing moist dung which contained no sand (five replicates); (3) surface remains from the beetle treatment, which contained a mixture of surface sand and dung fragments (five replicates); (4) buried dung which also contained a mixture of sand and dung fragments (five replicates). The total wet weight of (2), (3) and (4) were determined for each core and a small portion (about 5 grams) was taken to determine the moisture levels of each sample.

2.3. Oocyst recovery and enumeration from dung samples

*Cryptosporidium* oocysts were purified from triplicate 1 gram sub-samples of the four sample types described above using a GC-Combo Dynabead immunomagnetic separation kit (Invitrogen, Victoria, Australia). DNA was extracted from all samples using a MoBio PowerSoil™ DNA isolation kit (MO BIO, Calsbad, California, USA). qPCR was conducted for oocysts detection and recovery using a previously described methodology (Yang et al., 2009).

2.4 Assessment of oocysts viability

The viability of purified oocysts was determined based on morphology and the inclusion or exclusion of two flurogenic vital dyes, 4’,6-diamidino-2-phenylindole
(DAPI) and propidium iodide (PI) as previously described (Campbell et al., 1992; 1993).

2.5 Statistical analysis

Statistical analyses were carried out using SPSS Statistics 17.0 (Statistical Package for the Social Sciences) for Windows (SPSS Inc. Chicago, USA).

3. Results

3.1 Dung beetle processing of dung

From visual observation of the cores, it was estimated that ~90% of the dung was buried by day 5. The dung that remained on the surface of the sand in the core was present as dry fragments of the original dung pad mixed with a small quantity of dry sand (mean 52.6 grams, 4.8% moisture), while the dung recovered from within the core (ie buried dung) was present as fragments of the original dung pad mixed with a larger quantity of moist sand (mean 269 grams, 10.4% moisture).

3.2 qPCR analysis of dung beetle fractions

The mean oocyst recovery efficiency from the samples using immunomagnetic separation was 24% (based on qPCR analysis) and this was factored in to all calculations of oocyst numbers in the different fractions. Therefore, analysis of triplicate 1 gram sub-samples of the various samples revealed that the numbers of
oocysts in the different fractions ranged from 1,080 oocysts per gram of sample (g⁻¹) in the surface remains of the beetle treatment group to 11,698 g⁻¹ in the original dung inoculum (Table 1, Figure 1). Comparison of the numbers of oocysts in the control dung core (5,814 oocysts g⁻¹) with the numbers in the beetle-treated surface remains or unburied dung (1,080 oocysts g⁻¹), indicated that on a per gram basis, only ~19% of the oocysts g⁻¹ were still present in the surface remains.

The total number of oocysts in both the surface and buried dung can be estimated from the average mass of material recovered and the number of oocysts g⁻¹ of sample (Table 1). For the control cores, the average mass of dung remaining on day 5 was 21.4 g (69.0% moisture) and this contained 5,814 oocysts g⁻¹, therefore there were approximately 124,420 oocysts in the non beetle control. The average mass of dung mixed with sand on the surface in the +beetle group on day 5 was 52.6 g and this contained 1,080 oocysts g⁻¹, therefore there were approximately 56,808 oocysts on the soil surface. The average mass of buried dung and sand on day 5 was 269 g and this contained 1,329 oocysts g⁻¹, therefore there was an average of 357,501 oocysts in the buried dung. The combined total number of oocysts in the beetle-treated surface and buried dung and sand was 414,309/321.6 grams. This suggests that overall 13.7% of oocysts remained on the surface and that 86.3% of oocysts were buried. However, it was not possible to accurately compare the total numbers of oocysts in the surface remains with the control pad as only 24.1 grams of the original 50 grams of dung was recovered due to desiccation and both the surface and buried dung in the +beetle treatment, contained substantial amounts of sand resulting in much larger total weights being recovered.
3.3 Inactivation of C. parvum oocysts.

Analysis of triplicate 1 gram sub-samples of the various samples revealed that the initial viability of the oocysts in the original dung/oocyst inoculum was 92% (Table 2). The viability of the oocysts in the control core after five days was 62%. In the beetle treatments, the viability of oocysts in surface dung was 58%, whilst the viability of oocysts in the buried dung and sand was significantly lower at 10% (P<0.01).

4 Discussion

In the present study, burial of oocyst-inoculated dung by B. bison resulted in approximately 86.3% of oocysts being buried. Adult dung beetles feed by ingesting dung fluids, which they squeeze from the dung solids using specialised mouthparts (Edwards, 2007). Cryptosporidium oocysts are most likely to be suspended in the ingested dung fluids. It is therefore possible that the reduction in the numbers of oocysts is due to their digestion during passage through the intestine of the dung beetle. Those oocysts that persist underground are likely to be locked up in the solid dung matrix that subsequently provides food for the beetle larvae.

Viability testing using DAPI/PI staining indicated that there was a significant reduction in oocyst viability in beetle processed dung as oocysts in the buried dung had only 10% viability compared to 58% viability in the beetle processed surface dung and 62% viability in the control core. There was ~33-37% drop in viability in the oocysts in the control core and in beetle processed surface remains compared to the initial viability of 92% for oocysts inoculated into the dung. This is most likely
due to five days exposure to sunlight and ambient temperature, as these have been
shown to be important factors in oocyst inactivation (Reinoso and Bécares, 2008).
Beetle processing of the oocysts reduced the viability by a further 52%.

The B. bison beetle has an annual or biennial breeding pattern (depending
upon temperature), with adults emerging in autumn and breeding during winter and
spring. It has a Mediterranean distribution pattern, and is thus likely to be suited to
similar climate zones in Australia and overseas (Edwards, 2007). Since their original
release, B. bison has been recovered in the south of WA, South Australia, Victoria,
Southern New South Wales and Canberra and Tasmania (Doube, unpublished),
although the numbers of beetle populations remains unknown. The beetles bury dung
during winter months (Doube, 2008) and therefore widespread dung burial by B.
bison during the winter months could substantially reduce the numbers of
Cryptosporidium oocysts available to be washed into waterways following winter
rains.

Currently, apart from the data reported here, there is no information on the fate
of oocysts carried underground with buried dung. It appears unlikely that these
oocysts could escape the dung matrix and be washed into the ground water and find
their way into the waterways. However, if this can occur, it is important to determine
whether the soil will act as a biological filter and prevent oocysts reaching the
waterways. McGechan, (2002) analysed soil structure and suggested that Giardia and
Cryptosporidium (oo)cysts are the only colloidal contaminants with a sufficiently
large diameter to have their movement restricted by physical sieving though soil
pores, but this effect is likely to vary with soil type and compaction. A study by
Atwill et al., (2002) on the filtration efficiency of soils in limiting the transport of C.
parvum reported that vegetated buffer strips of similar soils with bulk densities
between 0.6 to 1.7 g/cm³, \( \leq 20\% \) slope, and widths of at least 3 m should generally function to remove 99.9\% of *C. parvum* oocysts from overland flow and shallow subsurface flow generated during events involving low to moderate precipitation (\( \leq 4 \) cm/hr). These data suggest that the soil matrix will act as a filter and inhibit movement of oocysts, but this needs to be assessed under a variety of field conditions.

It is also possible that dung beetles might act as vectors for the dispersal of *Cryptosporidium* oocysts from infected dung to uninfected dung. Oocysts could be carried on the beetle surface or in the gut and excreted once a new pad is colonised. However, the previous study by Mathison and Ditrich (1999), reported that only small numbers of oocysts were recovered from the beetles’ external surface, intestinal tract and faeces, suggesting that large numbers of oocysts would not be transferred between dung pads.

In conclusion, data from this preliminary study indicates that widespread dung burial by *B. bison* during the winter months could substantially reduce the numbers of *Cryptosporidium* oocysts available to be washed into waterways following winter rains and that *B. bison* may be an ideal candidate for a biological control agent for *Cryptosporidium*. More extensive field studies are required to confirm this.

**Acknowledgements**

This study was funded by Water Corporation, 629 Newcastle Street, Leederville, Western Australia. We thank Loene Doube for editing the manuscript.
References


Parasitology 124, 80-9.

Figure 1. Oocyst numbers based on a 24% efficiency of recovery of oocysts from triplicate 1 gram sub-samples of the original dung-oocyst inoculum, control cores, surface material from the +beetle treatment and a sand/dung mixture from the subsoil.
Table 1. Oocyst numbers per gram in sub-samples and total numbers of oocysts from the original dung-oocyst inoculum, control core and surface and buried dung from beetle treatments. Numbers are corrected for a 24% oocyst recovery efficiency.

<table>
<thead>
<tr>
<th></th>
<th>Dung-oocyst inoculum</th>
<th>Control dung</th>
<th>Beetle treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Surface remains</td>
</tr>
<tr>
<td>Mean oocyst numbers per gram</td>
<td>11,698 g⁻¹ ± 2,278</td>
<td>5,814 g⁻¹ ± 1,635</td>
<td>1,080 g⁻¹ ± 305</td>
</tr>
<tr>
<td>Total number of oocysts*</td>
<td>6.1 x 10⁶ oocysts/538 g</td>
<td>124,420 oocysts/21.4 g</td>
<td>56,808 oocysts/52.6 g</td>
</tr>
</tbody>
</table>

*Note: dung burial in the treatment groups resulted in mixing of the plasterers sand in the cores with the dung and therefore the weights listed consisted of a mixture of dung and sand.
Table 2. Viability (%) of oocysts per gram as determined by DAPI/PI viability staining in sub-samples from the original dung-oocyst inoculum, control core and surface and buried dung from beetle treatments. * = statistically significant differences.

<table>
<thead>
<tr>
<th></th>
<th>Dung-oocyst inoculum</th>
<th>Control core</th>
<th>Beetle treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Surface remains</td>
</tr>
<tr>
<td>Mean</td>
<td>92% ± 5%</td>
<td>62% ± 6%</td>
<td>58% ± 7%*</td>
</tr>
</tbody>
</table>
Research Highlights: Effect of dung burial by the dung beetle *Bubas bison* on numbers and viability of *Cryptosporidium* oocysts in cattle dung.

- First study to access fate of oocysts buried by dung beetles
- Significant reduction in oocyst numbers and viability
- Potential biological control agent for *Cryptosporidium*