Working paper on protocol for rearing and testing *Onthophagus taurus* and/or *Euonicellus fulvus* – for comment, discussion and revision

KG Wardhaugh, CSIRO Entomology
PO Box 1700, Canberra ACT 2601, Australia

Email: keith.wardhaugh@csiro.au

Introduction

Onthophagus taurus and *Euonicellus fulvus* are opportunistic, multivoltine species with a generation time of about 6 weeks at 26°C. When fed on good quality dung, females of both species attain sexual maturity in 1-2 weeks and will lay 1-2 eggs daily for four or more weeks. Egg to adult emergence will occupy about 4-6 weeks. Both species are therefore well suited for use in tests of excreted residues of veterinary parasiticides.

The following notes on their developmental biology and culturing have been compiled at the request of members of DOTTS (Dung Organism Toxicity Test Standardisation) during their inaugural meeting at Huntingdon Life Sciences, Huntingdon, UK on 26-27 February 2002. Similar procedures could also be used for rearing other species, such as *O. gazella* or *E. intermedius.*

Source colonies - laboratory versus field reared beetles

Both *Onthophagus taurus* and *Euonicellus fulvus* are very amenable to laboratory culturing. However, the maintenance of a laboratory colony is very demanding of both space and labour and requires continual access to large quantities of soil and fresh faeces. Moreover, because of selection, it is quite likely that the source colony will undergo qualitative change over time.

Accordingly, for the purpose of conducting bioassays of veterinary chemicals, it may be more practical to rely on the use of field-collected beetles. Field populations of *O. taurus* and *E. fulvus* are present in Europe and Australia and therefore both species could be available for sampling at any time of the year. Because *O. taurus* is also present in North America, it is the preferred species for ring testing.

Reliance on field-collected beetles has the acknowledged disadvantage of using insects whose immediate feeding history might well include exposure to toxic faeces, herbicides or other noxious compounds. This, of course, could adversely affect their subsequent survival and/or reproductive success. However, if collections are restricted to properties/localities with no recent history of parasiticide usage (> 8 weeks for topical or injectable formulations, or > 28 weeks for sustained release devices) and the collected insects are fed on 'clean' dung for say 10 days prior to use, then the potentially confounding effects of prior exposure are likely to be minimised. The limited laboratory data available suggests that beetles exposed to sub-lethal doses of MLs are not permanently debilitated by the experience and return to 'normal' within about 1-2 weeks – but there are no data for other compounds.

Minimising the impact of prior exposure will also be helped by an experimental design that contains adequate replication and allows for the random allocation of beetles between treatments.

However, the use of F_1 beetles might provide a more widely acceptable compromise between those in favour of using field-collected insects and those who prefer the use of laboratory-reared insects. If this is agreed then the protocol outlined below could also be used for maintaining the initial source colony, except that beetles would be held in 5 L containers at the rate of 5 pairs per container.

Rearing conditions

Insects used to assess the effects of veterinary parasiticides should be maintained under standard conditions. These may be summarised as follows:

***Temperature and RH*:** $26^{o}C \pm 2\,^{o}C$ and intermediate humidity (say about 60%). (In reality, I do not think that ambient humidity is especially relevant, since beetles are rarely found at the surface).

***Photoperiod*:** 16L:8D

***Soil*:** light, friable loam mixed with grade 1 vermiculite at the rate of 2 parts soil to 1 part vermiculite. Both soil and vermiculite should be passed through a 2mm sieve prior to mixing. The mixture should have a moisture content sufficient to allow a handful to retain its form after being lightly squeezed, yet dry enough to allow its easy passage through the sieve. It is possible that standard 'earthworm soil' may be acceptable, especially if mixed with vermiculite, which would allow it to cope with the additional moisture that drains out of the dung.

***Dung collection*:** Dung should be obtained from free-grazing animals that are allowed access to high quality (lucerne) hay for some 2 weeks prior to collection. This should ensure the production of dung with a high nitrogen content, which is important for dung beetle fecundity.

Where possible, dung should be collected directly from the animal. If collected from the ground it should be less than 12 hours old at the time of collection. This can be achieved by moving the source cattle to an area of fresh grazing. Collection should occur in the early morning to minimise colonisation by other dung-feeding insects.

Dung quantities and storage: A standardised dung beetle bioassay, as envisaged at DOTTS, will consist of an untreated control, a solvent treatment, a treatment involving a standard chemical at a specified concentration and five, 10-fold serial dilutions of the test chemical. Each treatment will be replicated 10 times, each replicate consisting of a single pair of beetles. Since each pair of beetles will be fed with 150 g of freshly thawed dung on 3 occasions over a 10 day period, the minimum quantity of dung required for a **single** bioassay will be about 40 L. If we allow for dung required for a 10 day period pre-maturation feeding, then an additional 10 L of dung will be required. This will mean that a herd of at least 10 cattle will be required to ensure an adequate collection of dung. After collection the dung will need to be thoroughly mixed to ensure uniform nitrogen and moisture content. With this

quantity of dung, it will be necessary for participating laboratories to have access to a large commercial mixer.

After spiking (see below) the dung should be divided into 1.5 L aliquots (10 feeds), placed in sealed plastic bags and stored in leak-proof containers at -15^{o} C for 3 weeks prior to use. Freezing is intended to kill insects that may infest the dung at the time of collection Containers and the plastic bags of dung should be identified by colour-coded labels, which clearly identify treatment and/or drug concentration. However, the identity of each treatment should not be made available to those conducting the assays until all results have been processed, i.e. bioassays should be blind.

Samples of dung should be retained for moisture and nitrogen analysis.

Spiked dung: This protocol assumes that acetone would be a suitable solvent for both the test and standard chemical. In this working document I have assumed that ivermectin will be the standard chemical and that it will be used at a single concentration, tentatively suggested at 1 part per million (Table 1).

Table 1: Details of drug and acetone concentrations in spiked dung and control dung.

Treatment	Dung (kg)	Acetone (mL)	Test chemicals			
			parts per million	parts per billion	Active ingredient (g)	Technical grade @ 0.931 purity (g)
Dilution 1	5	90	1000	1000000	5.0	5.371
Dilution 2	5	90	100	100000	0.5	0.537
Dilution 3	5	90	10	10000	0.05	0.054
Dilution 4	5	90	1	1000	0.005	0.0054
Dilution 5	5	90	0.1	100	0.0005	0.0005
Acetone	5	90	-	-	-	-
ivermectin	5	90	1	1000	0.0005	0.0005
Control	5	-	-	-	-	-

The amount of solvent required will vary according to the solubility of the active ingredient, but the added volume must be constant for all treatments. In the worked example presented in Table 1, it has been assumed for convenience that both the test and standard chemical will have the same solubility and purity. The assumed solubility of 1 g of active ingredient to 18 mL of acetone is based on a recent study of a novel veterinary compound (RJ Mahon and KG Wardhaugh, pers.comm.) and is thus likely to be of the correct order of magnitude.

With a maximum concentration of 1000 mg/kg of dung (Table 1), the volume of acetone-drug solute to be added to 5 kg of fresh dung will be 90 mL. Since acetone is highly volatile, the addition of this quantity of solvent is unlikely to affect the ultimate consistency of the dung. However, problems may arise when the use of a less volatile solvent is necessary.

After adding the drug-acetone solute to the dung, the mixture should be vigorously stirred for at least 2 minutes to ensure an even distribution of active ingredient (for

chemicals that attach to particulate material it may be necessary for the period of stirring to be extended. The efficiency of the suggested mixing process needs to be tested before the program of ring testing is initiated.

Dung thawing: Dung should be thawed at room temperature over a period of 24 h prior to use. The bags of dung should be left in their leak-proof containers until the thawing process is complete. Quite frequently, the plastic bags deteriorate during freezing and thus leak during the thawing process. Their retention in leak-proof containers ensures minimal loss of dung fluid, which should be re-incorporated in the dung mass. Once thawed, surplus dung should not be re-frozen for later use.

Fig. 1. Dung, sealed in plastic bags and stored in leak-proof containers, left to thaw for 14 h at room temperature

Beetles: The larval stages of dung beetles appear to be more sensitive to drug residues than the parent insects, particularly when the latter are sexually mature. Initial assays should therefore concentrate on elucidating effects on the juvenile stages. Accordingly, beetles used for assay purposes should be fed for at least 10 days after emergence on dung voided by untreated livestock. This should be long enough to ensure that all beetles are sexually mature when first exposed to the test chemical. Female beetles can be considered sexually mature if their single ovary contains developing oocytes (Fig. 2). Some chemicals may act as both adulticides and larvicides, but this ought to be accommodated by an experimental design involving five serial dilutions.

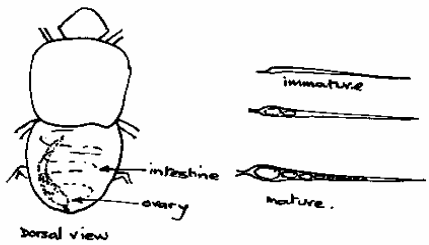
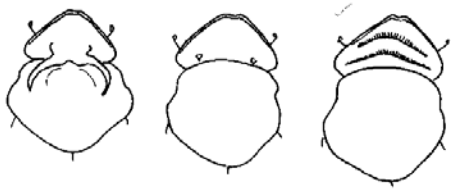


Fig. 2. Location of ovary and form of mature ovariole

Beetles should be divided into male : female pairs (Fig. 3) and allocated in batches of 20 to each of the various treatments. To ensure that this done randomly, each pair of beetles are assigned a randomly generated number. Random numbers are then sorted by ascending order into batches of 10 and allocated to successive treatments. The

eight treatments envisaged in Table 1 means that a single bioassay will require a minimum of 160 pairs of beetles (see below). The frequency of major males in each replicate should be recorded.



major male minor male female

Fig. 3. Head capsule and pronotum of males and females of *O. taurus*

Beetles should be held in secure, gauze-covered 2 L containers at the rate of 2 pairs per container. Containers should be filled to a depth of about 15 cm with a lightly compacted soil-vermiculite mix. Beetles should be fed with about 150 g (wet weight) of freshly-thawed cattle dung every third day for 9 days (say Tuesday, Friday, Monday, which avoids weekend work) and then checked for mortality and oviposition on day 10. At the time of each feed, old unused dung should be removed and checked for the presence of live and dead beetles. Gloves should be worn when feeding / handling beetles and should be changed between treatments to avoid cross-contamination. In treatments that employ serial dilutions of active ingredient, the order of feeding should be from the lowest to highest concentration. The control and acetone treatments should be fed first.

Brood and beetle counts: Checking for broods (balls of dung containing a single egg) and the presence of live and dead beetles should occur on day 10. This should be done by removing unused surface dung and then passing the remaining contents of each container through a 2 mm sieve. The unused dung should be first checked for the presence of live and dead beetles. Commonly, broods are formed as separate entities and are exposed during sieving. However, on occasions broods will be attached to the base and/or sides of the container and can be damaged during removal, even when this is done with the utmost of care. Thus, it is suggested that such broods are left untouched, covered with 15 cm of moist vermiculite and allowed to incubate *in situ* (see below).

A further problem at this stage of the process is that some beetles construct feeding balls which are of similar appearance to broods; male and/or female beetles may reside in these feeding balls for 2-3 days and can sometimes be missed when checking for broods. These beetles normally return to the surface within 2-3 days so the brood containers should be sieved and checked for parent beetles on, or about, day 14.

Brood emergence: At 26^{o}C, the period of egg to adult emergence is normally around 4-5 weeks. Since egg-laying may have occurred at any time over a 10 day period, it will be necessary to begin checking for the emergence of F_1 beetles from day 30 onwards (i.e. 20 days after the broods were sieved out). Thereafter, beetles should be checked 3 times weekly until emergence is complete, i.e. when successive checks indicate 7 days without any emergence. If at any stage during the period of

emergence, the surrounding vermiculite shows signs of drying (i.e. does not adhere when squeezed), it should be re-moistened.

Newly-emerged beetles should be checked for obvious deformities and a random sample of 10 males and 10 females from each treatment should be killed and individually weighed. The frequency of major males in each replicate should be recorded.

Brood survival: Because of the presence of feeding balls and/or the propensity of beetles to attach broods to the sides or base of the oviposition container, the number of broods (B) produced by any pair of beetles can be determined only after emergence is complete. This is taken as:

$$B = L + D - P; \text{ where}$$

L = number of live beetles
D = number of non-viable broods
P = number of parent beetles recovered on day 14.

The number of non-viable broods is determined by the destructive sampling of brood balls that do not have an emergence hole (Fig. 4). On opening non-viable broods, it is often possible to estimate the stage at which death occurred on the basis of the size of the egg-chamber. If death occurred at or soon after hatching, then the size of the internal chamber is usually only 1-2 mm in diameter. With late-instar larvae the chamber will have grown to a diameter of 5-7 mm. When development has progressed to the pupal stage, the insect is encased in a smooth, spherical shell (faecal shell stage), which is formed by the expulsion of gut contents prior to pupation.

Fig. 4. Broods with holes formed by recently emerged beetles.

Juvenile development time: At 26^{o}C beetle emergence is normally spread over 15 or more days. To calculate development time, dates of egg laying and emergence are taken respectively as the day on which broods were collected (d) and the day on which newly emerged beetles were recovered (e). Development time (t) for each beetle is given by the expression t = b – e. Mean time from egg to adult emergence (devt) is calculated as:

$$devt = \sum n_1t_1+n_2t_2 \ldots\ldots+n_it_i \,/\, \sum n_1+n_2\ldots.+n_i$$

where n is the number of beetles emerging each day.

Statistical analysis

The effect of treatment will be assessed on the following parameters: percentage adult survival (male, female), number of brood balls, percentage F_1 egg to adult survival, F_1 developmental time (male, female), and the weight of the F_1 offspring (male, female). For each parameter, ANOVAs will be performed using the parameter as the dependent variable with 'treatment' as the independent variable. If no differences are detected between male and female values for percentage adult survival and the weight of F_1 offspring, values for male and females will be combined into a single dataset for reanalyses to increase statistical rigour. Data are expected to be non-homogeneous among treatments (e.g., F_1 survival may range from 0 to near 100% across treatments). Hence, raw data will be rank-transformed prior to analyses (see Conover, W.J. & Iman, R.L. (1984) Rank transformations as a bridge between parametric and nonparametric statistics. The American Statistician, 35, 124-129.).

To ensure that sample sizes used in bioassays are sufficient to detect a 50% effect 90% of the time, power analyses will be performed (e.g., Zar 1984, p. 171. IN Biostatistical Analyses, 2nd edition. Prentice-Hall, NJ). If results of the power anlyses indicate that sample sizes were insufficient to meet this standard, the bioassay will be repeated, with results combined across bioassays for analyses.

Dated: 16 May 2002