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Importation of 2 winter-spring active dung beetles for southern Australia

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Abstract

Tunnelling and burial of dung by dung beetles improves water penetration, soil aeration, and movement of nutrients to the root zone, resulting in improved soil health and pasture growth. The lack of dung beetles in late winter and early spring represents an annual loss of 17-25% of that benefit in the main cattle grazing areas of temperate Australia. Two European dung beetle species, *Onthophagus vacca* and *Bubas bubalus,* were imported to fill that gap.

Refinements to rearing methods were investigated to eliminate causes of mortality and premature adult death, reduce the generation time to allow extra generations to be reared before release, and synchronise the beetles' life stage with the season at time of release. Temperature, photoperiod and other conditions were altered as more was learned about the success of different rearing methods.

Beetles were released in 2014 at five sites across southern Australia. Some were used by Dr Doube (Dung Beetle Solutions) to commence a field rearing program at three sites in SA. It is likely that beetles will be recovered within in 3-5 years and be present in sufficient numbers for collection and redistribution in a decade.

Executive Summary

Burial of dung by dung beetles improves water penetration, soil aeration and translocation of nutrients to the root zone, resulting in improved soil health and pasture growth. A recent analysis of the distribution of introduced dung beetles showed that there is a gap of dung beetle activity across southern Australia in late winter and early spring. The lack of dung beetles for 2-3 months of the year represents an annual loss of 17-25% of that benefit in the main cattle grazing areas of temperate Australia. This project introduced two species of dung beetles from southern Europe to fill that gap in seasonal activity in southern Australia.

The aims of the project were, by December 2014, to have:

- imported two new species of early spring-active dung beetles (*Bubas bubalus* and *Onthophagus vacca*), acclimatised the beetles while addressing quarantine requirements, and reared these species in sufficient numbers to enable releases at five sites across the expected home range of the beetles.
- provided starter colonies of both species to private companies and/or landowner groups.

To laboratory-rear sufficient beetles for multiple releases in spring 2014, a rearing method was required to maximise the reproductive potential of the species by eliminating causes of mortality and premature adult death, reduce the generation time to allow extra generations to be reared before release, and synchronise the beetles' life stage with the season at time of release. The first step was to start the lab colony with surface-sterilised eggs released from quarantine and reared in man-made brood balls. Subsequent generations were reared in natural brood masses. Temperature, photoperiod and other conditions were altered as more was learned about the success of different methods.

At the outset, it was known that both species were univoltine with an obligatory diapause. The strategy to reduce the generation time during laboratory rearing was to rear at a high enough temperature to shorten the time taken for the immature stages to develop and then provide an adequate but shortened period for diapause called vernalisation, all without sacrificing fitness.

This report goes into significant detail in the methods used and developed so that others may profit from our experience in future.

For *Onthophagus vacca*, many different options were tried to reduce the development time and the period of the vernalisation protocol to achieve two generations per year. Taking into account trade-offs such as larval survival, adult size, longevity and fecundity, the best outcome achieved was to rear *O. vacca* from egg to first egg in 27-32 wk by rearing the larvae at high temperature (25°C) and subjecting newly emerged adults to a vernalisation protocol consisting of 6 wk at 25°C 14:10 LD for feeding, 1 wk at 22°C 14:10 LD, 2 wk at 16°C 12:12 LD, 6 wk at 10°C 10:14 LD, 2 wk at 16°C 12:12 LD, 1 wk at 22°C 14:10 LD, 2 wk at 25°C 14:10 LD (in dry vermiculite) before setting up for oviposition at 25°C 14:10 LD. There was substantial variability in time for development from egg to adult that meant that each succeeding generation became less and less well-defined, until most stages were present at all times. From a mass-rearing perspective, this can be an advantage, as it evens out the workload associated with colony maintenance. However, if it is necessary to synchronise all the individuals of a colony, for example for field-

releases, the way to do it would be to lengthen the cold period, as attempts to further shorten the generation time failed.

The unsolved issue with rearing *O. vacca* remains the longevity and fecundity of adults. Dung beetles harbour a gut flora called a microbiome that is thought to assist with digestion of food. The mother transfers this material to her larva by lining the egg chamber with regurgitated and/or faecal material. Because the quarantine protocol for release of eggs strips the egg of all such material, it was hypothesised that that might explain the poor longevity and fecundity of adult *O. vacca.* Results of the preliminary genomics experiments suggest that the microbiome of European *O. vacca* is distinctly different to that of dung beetles in Australia. An experiment with *Onthophagus binodis*, itself an introduced but very successful species in Australia, was suggestive of a role of the microbiome and/or initial feed of small particles in increasing fitness. This finding has relevance to the process of introduction of other dung beetle species and further research is warranted on this topic.

Bubas bubalus has a much longer developmental time than *O. vacca*. The data showed that it has an inbuilt propensity for some individuals to complete development quickly and emerge as adults before winter, most to emerge as soon as temperatures begin to rise in spring and others to take very much longer, up to two years. The best result obtained with *B. bubalus* was by rearing larvae at 25°C for 23 wk, followed by a vernalisation protocol consisting of 1 wk at 22°C 12:12 L:D, 1 wk at 16°C 12:12, 6 wk at 10°C 10:14; 2 wk at 16°C 12:12, 2 wk at 22°C 12:12 and finally set up for oviposition at 22°C 14:10. The emergence period of the main part of the population followed from 1-20 wk with an average at 10 wk. The pre-oviposition period ranged from 6 to 7 wk so the overall time from egg to first egg was a minimum of 32 to a maximum of 61 wks (mean 42-43 wk) from egg to first egg. It appears impossible to rear tightly-defined generations and so the only way to rear is to accept that the colony will consist of all stages at all times and if a synchronised population is required, for example for field release, then it should be done by lengthening the cold period to slow down some of the population.

The plan was to release adult beetles that were sexually mature and physiologically synchronised with the local season and this was done with *O. vacca* at 5 sites (WA, NSW, and SA (3)). Unfortunately, the number of adults synchronised to the season was not great, but all of them were released. However, to complete the project, it was necessary to release *B. bubalus* and some *O. vacca* as immatures in their brood masses, but still moderately-well synchronised to the season. In the case of *O. vacca*, all immature beetles that would be expected to emerge in the summer of 2014-15 then feed and dig underground to overwinter were released in NSW and SA. *B. bubalus* immatures were buried at 6 sites (NSW (2), SA (3) and WA). The South Australian releases were made into field cages by Dr Bernard Doube (Dung Beetle Solutions) who is starting a field rearing program for both species (MLA Project ERM.0214).

Previous experience in releasing new dung beetles suggests that no beetles will be recovered for several years after release. The exception to this would be the field rearing being undertaken by Dr. Doube. Nevertheless, there is great interest in seeing when the beetles have established and the cooperating landowners and collaborators will be watching carefully. Once populations have reached a sufficient size, a program of cropping and redistribution would be useful to speed up the dispersal across the predicted range. This may take up to a decade; collection and redistribution of *Bubas bison,* the introduced species most similar to *B. bubalus*, started nine years after the original release. *O. vacca* is also univoltine, so it would be expected to take a similar time.

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Background

Although Australia has a rich dung fauna, most indigenous dung beetles are found only in bush and forest habitats and feed primarily on marsupial dung (Doube et al. 1991). As such, native beetles contribute little towards alleviating the problem of pasture fouling caused by cattle and other large exotic herbivores. To alleviate this problem, over 50 species of exotic dung beetle, mainly from Africa and Mediterranean Europe, have been imported and released in grazing areas throughout Australia. Of these, 23 species are now established (Edwards 2007) and are widely regarded as having a significant, on-going impact on the biological control of dung and dung-breeding flies (Nichols et al. 2008).

Although the analysis of the distribution of introduced dung beetles (Edwards 2007) has shown that Australia's tropical and sub-tropical cattle grazing areas are served by 7-13 species of dung burying beetles, temperate pastures have fewer than 4 or 5 species. Of these, most emerge in late spring and are most active during summer; only one is active during the autumn – winter period. The net effect is a 2-3 month gap in dung burial. Because tunnelling by dung beetles improves water penetration, soil aeration and nutrient translocation to the root zone, the effect is improved soil health and pasture growth. The lack of dung beetles for 2-3 months of the year represents an annual loss of 17-25% of that benefit. The purpose of this project is to introduce two species of dung beetles from Europe that will fill that gap in seasonal activity in southern Australia.

1.1 Biology of *Onthophagus vacca* (Linnaeus, 1767) (Coleoptera: Scarabaeidae)

Until recently, it was thought there was a wide range of colour morphs in *Onthophagus vacca*, from very light (or clear) elytra (Figure 1) to very dark ones. In 2010, Rössner, Schönfeld & Ahrens recognized that *Onthophagus medius* (the dark form) is indeed a separate species from *O. vacca*, on the basis of morphology as well as molecular phylogeny, although the morphological differences are subtle, especially for the smaller individuals. The distributions of the two species overlap widely, but *O. medius* is a more cold-tolerant species. It occurs in England, Belgium, northern Germany and northern Poland where *O. vacca* does not occur, but is absent in southern Spain, Morocco and Sardinia where *O. vacca* does occur. In the overlap zone, *O. vacca* is more likely to be found at low elevations and *O. medius* at high elevations (Rössner *et al.* 2010). The true *O. vacca* has a wider predicted distribution in Australia than *O. medius* (P. B. Edwards *pers. comm*.).

The papers referred to in following summary of the biology of *O. vacca* all pre-date 2010, and may be referring to *O. vacca* alone, *O. vacca* and *O. medius* mixed or *O. medius* alone, depending on where the study was undertaken or where the founders for laboratory colonies were collected. From the information in the papers, it is not possible to determine which species are involved, although it is reasonable to expect the information to be generally correct for the true *O. vacca*.

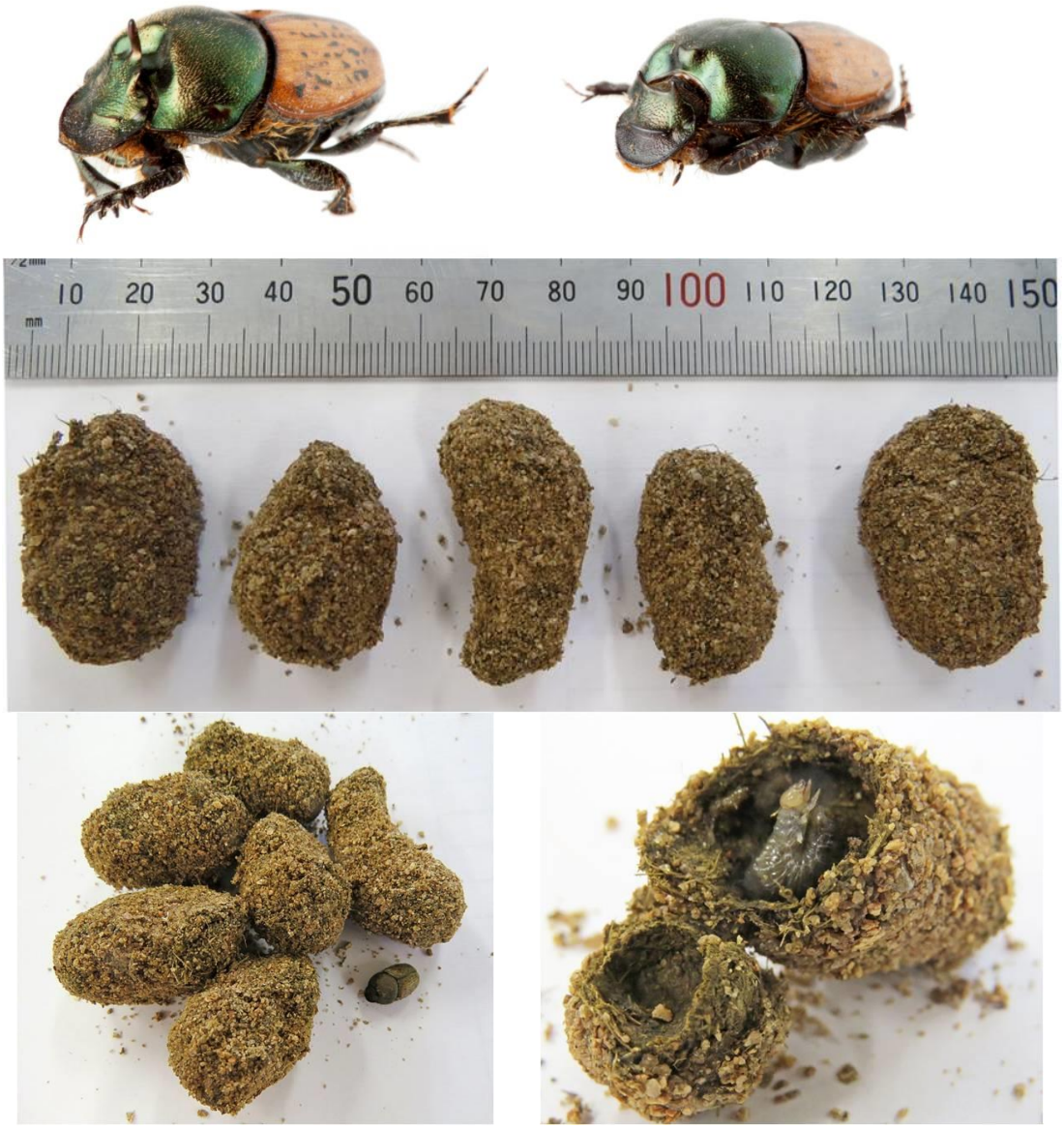


Figure 1. Life stages of *Onthophagus vacca*. Top left, adult male; Top right, adult female; Centre, natural brood masses; Bottom left, natural brood masses with adult female; Bottom right, natural brood mass opened to show larva.

O. vacca is attracted to fresh cattle and sheep dung in open pasture and is active during spring and early summer (Lumaret and Kirk 1987) and regarded as having a major impact on the dispersal of cattle dung (Galante *et al*. 1995, Lumbreras *et al*. 1990). A study by Lumaret and Kirk (1987) showed that *O. vacca* has a very strong preference for open, non-shaded sites and over a season, only one of 454 beetles was taken at a site with more than 50% shade.

Adults emerge in early spring and feed on dung for several weeks until they are ready to reproduce. Adults fly only in the middle of the day when the ambient temperature exceeds 10°C (Mena *et al.* 1989). The male assists the female with the construction of the nest; females tending to construct the tunnels and males moving dung from the pad to the tunnel entrance. The nest structure of *O. vacca* is rather simple with a main vertical tunnel off which secondary tunnels lead to brood chambers (Sowig 1996a). Each is provisioned with a dung-mass of about 8 g (0.9 g dry weight) onto which one egg is laid (Figure 1). Presence of a co-operating male

increased the average number of brood chambers produced by a breeding female from 4 to 5 (Sowig 1996b). Brood chambers are more clustered and deeper in dry soil compared with moister soils. Breeding females stay an average of 115 h at a pad, with few staying longer than 144 h. Co-operating males tend to leave the pad somewhat earlier, averaging 87 h. Oviposition continues for 3-4 months. The larvae develop on the dung within the brood chambers and metamorphose into adults, which enter obligatory diapause until the following spring (Lumaret and Kirk 1991).

Three releases of *O. vacca* were made in the ACT in September-October 1980 and again near Toodyay in WA in October 1983 (Tyndale-Biscoe 1996). Release numbers at both sites were small and the species failed to establish (Edwards 2007). Based on voucher specimens in the Australian National Insect Collection, it is clear that *O. vacca* and O medius were imported together as the one species, which may explain some of the rearing difficulties experienced at that time.

Based on data on the distribution of the true *O. vacca* in Europe, the predicted range of *O. vacca* in Australia is as depicted in Figure 2 as modelled with BIOCLIM by Dr Penny Edwards (*pers. comm*.).

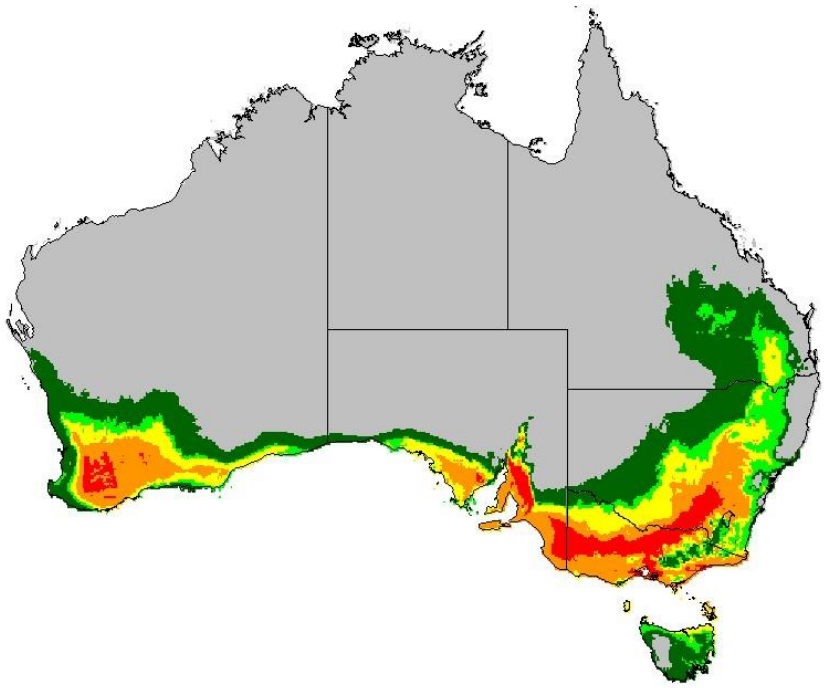


Figure 2. Predicted distribution of *Onthophagus vacca* in Australia. Red is the most favourable zone, followed by orange, then yellow, light green and dark green.

1.2 Biology of *Bubas bubalus* (Olivier, 1811) (Coleoptera: Scarabaeidae)

Bubas bubalus is a night flying, univoltine species adapted to the Mediterranean climate areas of southern Europe (Lumaret and Kirk 1987). It is typically a species of grassy pastures, but is also found in open woodland. It readily colonizes fresh dung and, in its native habitat, is one of the main species responsible for the degradation of cattle dung (Lumbreras *et al*. 1990). Although it exhibits no clear preference for particular types of faecal material (Kirk and Ridsdill-Smith1986), its need for large quantities of dung when egg-laying means that it is likely to become established in areas only where ample cattle dung is available.

Figure 3. *Bubas bubalus* life stages. Top left, adult male; Top right, adult female; Middle, natural brood mass with brood chamber dissected at each end revealing the egg; Bottom left, egg in brood chamber; note the black material lining the chamber; Bottom middle, late instar larva; Bottom right, faecal shell.

In Languedoc in southern France, *B. bubalus* emerges in late winter-early spring and begins oviposition after a brief period of intensive feeding (Lumaret and Kirk 1987). Oviposition continues through early summer, with a few adults emerging in the autumn and feeding before going underground to pass the winter with the other larvae, pupae or adults still in the faecal shells (Lumaret and Kirk 1991).

In the cooler areas of continental Spain (e.g. Salamanca province), the seasonal phenology of *B. bubalus* is rather different, with beetles being present in all seasons except summer (July-September). First emergence occurs in late autumn, but there is little activity or ovarian development until late winter-early spring, when oviposition increases sharply (Lumbreras *et al.* 1991). *B. bubalus* exhibits a similar pattern of seasonal activity in the dairying areas of northern Andalucia (Wardhaugh *pers. comm.*). Lumbreras *et al.* (1990) have concluded that autumn and spring peaks of abundance in northern Spain are, in part, a direct response to low winter temperatures, and in part the result of two discrete periods of emergence.

Eggs are laid into 10-15 cm sausage-shaped brood masses up to 30 cm below the surface, generally with one egg at each end (Figure 3). It appears that males play little role in the construction of the tunnels, as they never have the tibiae as worn as those of the females (Lumbreras et al. 1990). Population densities of 20 beetles per dung pad are common in Europe and such densities result in very rapid dung dispersal.

Previous work with *B. bubalus* in the quarantine laboratory of the Australian Animal Health Laboratory (AAHL) (Steinbauer and Wardhaugh 1992) showed that beetles collected in Spain in April (northern hemisphere spring), laid an average of 15 eggs per female, with the majority of the eggs being laid between mid July and mid September. The mean time from egg to adult was 110 d. Under laboratory conditions (18°C and 14:10 LD), there was no evidence of any interruption of juvenile development. Unfortunately, no beetles were released, mainly because numbers reaching the adult stage were too small (Steinbauer and Wardhaugh 1992).

B. bubalus is found mainly in the Mediterranean climate areas of southern Europe (Portugal, Spain, and France), where its distribution closely parallels that of B*ubas bison*.

B. bubalus is closely related to *B. bison,* which was first released in Australia at Dardanup, WA, in 1983 (Tyndale Biscoe 1996). Since then, *B. bison* has been successfully re-distributed to South Australia, Victoria, southern NSW and the ACT and its potential final distribution is shown in Figure 4 (P. Edwards *pers. comm.*) Once established, *B. bubalus* is expected to attain a similar distribution (Figure 5; P. Edwards *pers. comm.*), but because the two species occupy rather different seasonal niches, inter-specific competition is unlikely to affect the establishment of *B. bubalus* or the persistence of populations of *B. bison*. In Europe, *B. bison* begins oviposition soon after emergence in autumn. Oviposition continues through winter and ends in spring (Lumaret and Kirk 1987). In the case of *B. bubalus*, some individuals may emerge in autumn, but the start of oviposition is delayed until early spring and continues into summer (Lumbreras *et al*., 1991). Both species utilize a range of dung types but are most abundant in cattle dung.

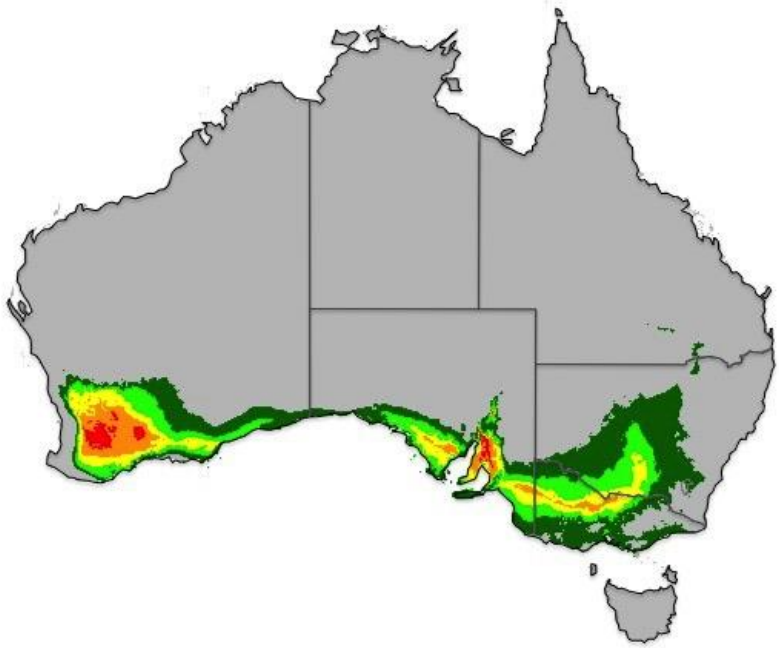


Figure 4: Predicted distribution of *Bubas bison* in Australia. Red is the most favourable zone, followed by orange, then yellow, light green and dark green.

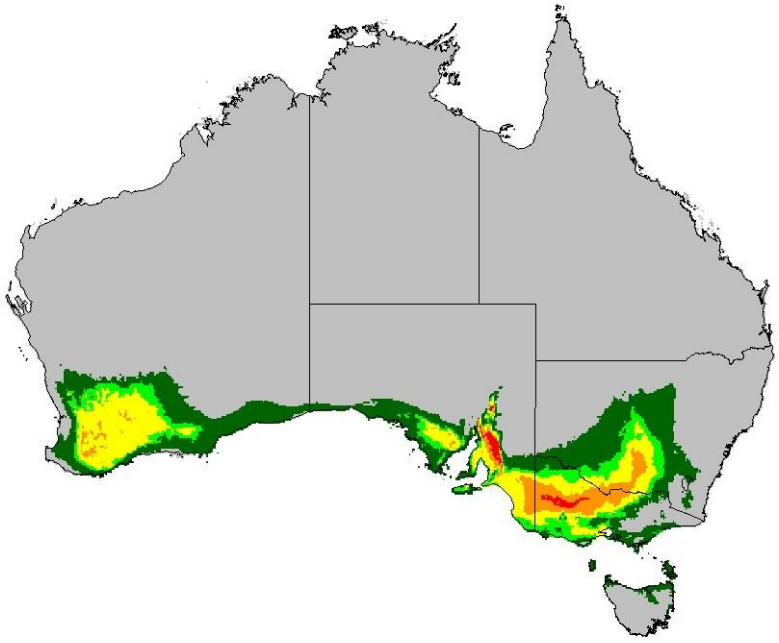


Figure 5: Predicted distribution of *Bubas bubalus* in Australia. Red is the most favourable zone, followed by orange, then yellow, light green and dark green.

Project Objectives

The objective of this project is to deliver the long term benefits of improved dung burial and pasture productivity in the main cattle grazing areas of temperate Australia by introducing two new species of spring-active dung beetle (*Bubas bubalus* and *Onthophagus vacca*)

By December 2014 have:

- imported two new species of early spring-active dung beetle (*Bubas bubalus* and *Onthophagus vacca*), acclimatized beetles while addressing quarantine requirements, and reared these species in sufficient numbers to enable releases at 5 sites across the expected geographical range of the beetles
- provided starter colonies of both species to private companies and/or landowner groups.

Methods and Results

Because the methods used for rearing each generation of beetles changed depending on the results from the previous generation, methods and results are combined for clarity. It is important to note that early in the project, the generations started to overlap, and eventually reached the point where it was unrealistic to maintain shipments and generations separate. There was little or no scientific value in keeping them separate, and efficiencies were gained by combining the generations.

3.1 Basic methods

3.1.1 Collection and freezing of dung supplies

Because both species are active in early spring when the dung is of high quality, sufficient dung was collected each spring and then frozen in packages to meet the project requirements for the following year (Figure 6). Dung was collected from farms where the cattle had not been treated with parasiticides. The dung was scraped off fresh pads with a trowel onto a dust pan, taking care not to collect any soil (Figure 6). It was then put into plastic garbage bins and returned to the laboratory for processing and storage. Once there, each bin was thoroughly mixed using an electric drill and paint stirrer and any insects and large pieces of grass were removed. For the first collections, the dung was put into 1 L rectangular plastic take-away containers for freezing and storage (Figure 6). Once the daily dung requirements were much larger, dung was scooped into plastic supermarket bags and placed into cardboard boxes (approximately 3 kg per bag; 4 bags per box). The boxes were sized (25 x 25 x 20 cm) to efficiently fit and stack into freezer facilities and be a suitable unit of size and weight for safe manual handling. Boxes were labeled with the collection date and source and then frozen.

Figure 6. Collecting and preparing dung for freezing. Left, Amar Singh showing the technique for collecting only the interior of the fresh pad; Right, packaging of mixed dung in 1 L containers for freezing.

About 2500 kg of dung were collected in 2012, of which about 1600 kg were used before the spring of 2013.

Based on the 2012/13 experience, each female *O. vacca* (and her accompanying males) required about 1 L of dung during her adult life. It took about the same amount of dung to produce man-made brood balls for a single female. For *B. bubalus*, the dung required per female was about 3 L, and an additional 3 L was needed for the manufacture of man-made brood balls.

To have sufficient dung for another importation in 2013 and to feed the laboratory colony, another 5000 kg were collected in 2013.

Because the existing 3 x 3 x 2.1 m walk-in freezer was too small to store the required amount of dung, a 6 m refrigerated shipping container was acquired in 2013.

3.1.2 Manufacture of man-made brood balls

The objective was to make man-made brood balls as similar to those of the female as possible, with the proviso that an excess of dung was used to compensate for any reduction of quality due to the manufacturing process.

The process of manufacture of man-made brood balls was refined over the life of the project. It basically involved pressing thawed dung to expel excess moisture, then mixing the dung well with a strong mechanical mixer and forming the balls by hand. Certain processes, such as autoclaving the dung, microwaving to reduce moisture content and use of a fungicide (Nipagen®) were found to be unnecessary. The final process was as follows:

- Thawed dung was pressed with a fruit press to reduce the moisture content to 75-85%.
- The dung was then mixed in a semi-industrial mixer for 2 minutes to prepare it for ball rolling.

- It was easier to roll the balls when the moisture content was about 85%, and to dry them slightly later.
- The balls were then frozen to ensure that any sciarid fly eggs that may have been laid during the preparation were killed.
- The balls were thawed at 35°C overnight in a constant temperature (CT) cabinet before use. If the balls were too wet, they were laid out onto trays and dried in the CT cabinet as required.
- A 8 mm diameter hole about 1.5 cm deep was made in each ball using the blunt end of a pencil, the egg or larva was then inserted into the hole and the hole closed over.

3.1.3 Media, containers and pests

As the rearing continued, changes were made to the media and containers used. In addition, procedures were developed to limit the effect of pests on the culture. These are discussed as appropriate in the sections under rearing for each year and generation. Figure 7 and Figure 8 show several of the standard rearing containers.

Some small-scale rearing and diapause experiments (primarily on *O. vacca*) were undertaken at the CSIRO European Laboratory in Montpellier, France and the results of those are referred to during the detailed discussion of both species.

Based on advice from previous dung beetle workers, the starting medium for rearing was sandy river loam from Bredbo, NSW. It was purchased from Lopilato Brothers Landscape Supplies, Hume, ACT. It was steam-sterilised (autoclaved) at 121°C for 1 hour and the moisture content adjusted to 20-30%.

Vermiculite at 60-70% moisture content was used to set brood balls into for rearing. The vermiculite was Grade 1 (particle diameter 0-1 mm) from Australian Perlite Pty Ltd.

Vermiculite had been used during the importation project in AAHL in 1990 to add to damp soil to reduce the moisture content *in situ*. In this project, it was used this way at first, but it was not sufficient to prevent the need to change the soil very frequently. Once its value as a medium for suppressing nematodes was appreciated (see below), it was used in a 1:1 sand/vermiculite mix to replace soil for oviposition containers in the laboratory.

The sand (plasterers' sand; 125–250 µm) was purchased from Paragalli Haulage, Queanbeyan, NSW and was determined to be the most similar to that used at the CSIRO European Laboratory in Montpellier, France. The sand was steam-sterilised (autoclaved) at 121°C for 1 hour, mixed with vermiculite (1:1) and the moisture content adjusted to 20-30%.

Sciarid flies are always a problem with mass-rearing of dung insects. Methods used to reduce the infestation by flies included yellow sticky traps in the lab and rearing rooms, ultra violet insect electrocutors, enclosure of brood boxes and rearing containers in fine net bags (Figure 7), and freezing of dung, brood balls and soil media.

Nematodes are naturally phoretic on beetles as a method of transportation from one food source (dung pad) to another. In culture, the numbers of nematodes can build up substantially to the point where they hinder the ability of the adult beetles to feed

and reproduce. It was found that the problem was substantially reduced by firstly, giving heavily infested beetles a few days in dry vermiculite, which had the effect of abrading the nematodes off the beetles, and ultimately by changing to the vermiculite and sand mix.

Mites are also phoretic on the beetles, and while there were some from time to time, the husbandry methods used for suppressing sciarid flies and nematodes were sufficient to keep these under control.

Figure 7. Standard rearing containers used in the project. Top left, tall 1.7 L containers used in quarantine for both species and for *Onthophagus vacca* in the laboratory. Top right, clear plastic boxes with vented lids used for various purposes including holding *Bubas bubalus* faecal shells and *Onthophagus vacca* brood masses until emergence; Middle, 5 L bucket with mesh lid without and with the gauze cover to exclude flies, used for both species in the laboratory; Bottom left, 20 L tote box with vented lid, and right, inside a gauze bag to exclude flies.

The tall 1.7 L containers (26 cm high x 10 cm diameter x 26 cm height) were the only ones used in quarantine. However, shifting to the 5 L containers (22 cm high x 18 cm diameter) for *O. vacca* and 20 L containers (41 cm high x 29 cm diameter) for *B. bubalus* (Figure 8) meant more efficient processing and collection of brood masses. The 20L black tote boxes (49 x 34 x 18 cm) were used for holding man-made brood balls and natural brood masses of *B. bubalus*.

Figure 8. Patrick Gleeson with brood masses of *Bubas bubalus* from a single 20L rearing bucket (at rear) in the tray of the mechanical sieve used in the laboratory.

3.1.4 Laboratory handling techniques

At regular intervals appropriate for the species, breeding containers were tipped out to collect the brood masses and adults. The adults were set up again with recycled or fresh media as appropriate.

The contents of the containers were sieved using mechanical sieves built specifically for the purpose (Figure 9). In quarantine, where space was at a premium, the machines were relatively small (overall: 1.16 x 0.55 x 0.84 m; sieve inserts 48.5 x 47.3 cm) compared to the laboratory machines (overall: 1.45 x 0.53 x 0.9 m, sieve inserts 61 x 46 cm) that were designed to handle up to 10 L of medium at a time. The sieve inserts for quarantine were made from perforated stainless steel sheet for ease of cleaning and decontamination while the sieve inserts for the laboratory machines were made from perforated galvanized steel sheet. The aperture size was selected to be as large as possible to facilitate rapid sieving but small enough to retain the smallest individuals of each species. After some experimentation, inserts were selected at an aperture size of 5 mm for *O. vacca* and 8 mm for *B. bubalus*. The sieves oscillated sideways.

Figure 9. Mechanical sieves constructed for use in the quarantine cubicles. The apertures of the sieve inserts were different sizes for the 2 species.

3.1.5 Egg surface-sterilisation protocol

As part of the preparation for the application to import the new species of dung beetles, an extensive review of the literature indicated that there are no known pathogens that are carried within a dung beetle egg, so all quarantine risks are to be found on the egg surface. Because bacteria-feeding (bacteriovore) nematodes are naturally phoretic on adult dung beetles as a way to move from one dung pad to the next, they will be found on field-collected beetles brought into quarantine. The quarantine risk is that some of these nematodes could be on brood ball material attached to the egg or transferred onto the egg surface during dissection from the brood ball.

The treatment protocol to eliminate the nematodes was based on the Australian import conditions for used agricultural machinery, that is, to wash the surface thoroughly and then treat with a disinfecting agent (Virkon®) (Australian Quarantine Treatment T9952). Virkon® is a patented solution of peroxygenic acid that is a broad-spectrum virucidal veterinary disinfectant. A 1% solution is recommended for disinfection of surfaces, laboratory equipment, body fluid spillages and laundry (www.virkons.com).

A series of experiments was undertaken to determine the natural load of nematodes on eggs dissected from brood balls, the capacity of either water- or Virkon®-immersion to kill bacteriovore nematodes, and the ability of Virkon® to clean nematodes off eggs.

The experiments showed that the number of nematodes was naturally extremely low on eggs dissected from brood masses and the rest of the experiments were done using eggs that had been placed for at least 10 minutes in a solution containing large numbers of nematodes. Neither washing in water or in 1% Virkon®) killed the nematodes (at least in time frames that were operationally practical). However, both processes had the effect of washing the nematodes off the surface of the eggs (Figure 10).

The following protocol (Table 1) was accepted by AQIS and is a combination of reducing the nematode load in brood balls and on eggs and an effective cleaning procedure of already very clean eggs.

Table 1. Protocol for surface-sterilisation of beetle eggs.

Objective	Method
Minimise the numbers of nematodes in brood ball material by suppressing the nematode populations in the culture containers	Use only pre-frozen dung in quarantine Replace the soil at regular intervals with steam-sterilised or autoclaved soil.
Minimise the nematode load on the eggs dissected from the brood balls	Discard eggs that have dung or beetle faecal material attached.
Surface-sterilise the eggs	Place eggs in histological cassettes which are secured in a mesh basket and lowered into a beaker of solution being agitated by a magnetic stirrer. Wash for 10 minutes in 1% Virkon® followed by five 1-minute rinses in water.

Figure 10. Surface sterilisation setup; Virkon solution at left (pink), followed by five plain water rinses.

3.2 Collection and importation of beetles

The two cubicles available in Black Mountain Containment Facility limited the numbers of beetles that could be managed at any one time. The tall 1.7 L containers were used maximise the numbers of containers that could be fit onto the benches, minimize the quantities of soil used yet provide sufficient depth for the beetles to tunnel and oviposit. About 200 containers was the maximum that could be managed per cubicle. In 2012 and 2013, one cubicle for used for each species. In 2014, both cubicles were filled with *B. bubalus*.

Given the subtle morphological differences between *O. vacca* and *O. medius*, care was taken to collect only in locations where *O. vacca* occurred alone.

3.2.1 Importations 2012

O. vacca and *B. bubalus* were collected in the Camargue region of France by Mr José Serin and other staff of the CSIRO European Laboratory at Montpellier, France with the assistance of Dr Jean-Pierre Lumaret of the University of Montpellier. Beetles were washed, picked over under the dissecting microscope to remove any mites and adhered dung (Figure 11). They were starved for 3 d and then packed in damp vermiculite and shipped to Australia by air freight (Figure 12). Numbers of beetles by sex are shown in Table 2.

Figure 11. Preparation of beetles in France for shipment to Australia. Left, José Serin washing beetles; Right, examining each beetle for phoretic mites.

Figure 12. Patrick Gleeson, Jane Wright and Keith Wardhaugh with the first shipment of beetles from France, on its way into the quarantine facility to be unpacked.

3.2.2 Importations 2013

The importation objective for 2013 was to introduce another population of each species from Spain to increase the genetic diversity of the introductions. The main collaborator with experience in Spain was unable to assist with collecting in 2013, so Mr Patrick Gleeson travelled to Europe to do the collecting with Mr Serin from the CSIRO European Laboratory at Montpellier. They were successful in collecting sufficient *B. bubalus* for a shipment from Spain, but were only able to collect a few *O. vacca* (for reasons unclear, although the very wet winter and cold spring might have been part of it). Therefore, Mr Gleeson returned to France, extended his stay by a week and collected *O. vacca* and more *B. bubalus* in the region surrounding Montpellier for a second shipment. Scarcity of *O. vacca* meant that they had to be collected from a property where cattle had been treated with ivermectin 3-4 wk earlier. Because adults are much less affected by ivermectin than the larvae and there is no known intergenerational effect (Lumaret *et. al.* 2012) and a reasonable time had passed, it was decided to go ahead with the shipment. However, once in quarantine, these beetles laid fewer eggs and died quickly – see section 3.3.1.

An opportunity arose to receive F_1 *O. vacca* adults from a laboratory culture (established from beetles collected in France) at the CSIRO European Laboratory. Accordingly, an additional beetle shipment was arranged in September 2013 to bolster the colony.

3.2.3 Importation 2014

A further importation of 785 *B. bubalus* was made in May 2014 (Table 2), to boost the numbers of this species in culture. The beetles were collected by Mr Serin in the region surrounding Montpellier, France. These fully occupied both available cubicles in quarantine.

Table 2. Summary of importations of *Onthophagus vacca* and *Bubas bubalus* from 2012 to 2014.

	Date	Shipment	Source	Male	Female	Total
Onthophagus vacca	25/05/2012	1	France	47	65	112
	16/06/2012	2	France	42	66	108
	18/05/2013	3	Spain	4	9	13
	20/06/2013	4	France	121	126	247
	27/09/2013	5	France	112	122	234
	Total			326	388	714
Bubas bubalus	25/05/2012	1	France	213	187	400
	18/05/2013	3	Spain	129	128	257
	18/05/2013	3	France	27	15	42
	20/06/2013	4	France	94	76	170
	1/05/2014	6	France	408	377	785
	Total			871	783	1654

3.3 Quarantine rearing and egg release

Beetles were reared in the CSIRO Black Mountain Containment Facility according to agreed DAFF quarantine protocols and surface-sterilised eggs were released for subsequent laboratory rearing (section 3.1.5).

The rearing containers were the tall 1.7 L plastic containers with a 3 cm stainless steel mesh vent in the lid (Figure 7). The height of the container allowed the beetles to have sufficient depth of soil to make tunnels and brood masses. The containers were filled to a depth of approximately 22 cm with sandy loam, which was then tamped down to approximately 20 cm depth (1.3 L) (Figure 13). A tennis-ball sized amount of dung (approximately 150 ml) was then placed on top of the soil and beetles added. One pair of *B. bubalus* and up to 3 pairs of *O. vacca* were put into each container. Extra dung was added if the beetles had depleted their supply before the scheduled feeding day.

The beetles were held at 22°C day 14°C night with photoperiod of 14:10 hr, light:dark. The contents of the containers were sieved every 7-14 d, as operationally appropriate, and the survival and number of brood masses recorded (Figure 13). The beetles were then reset with soil of an appropriate moisture content and fed.

Figure 13. Working on the beetles in the quarantine cubicle. Left, John Lester sieving beetles and collecting brood masses; Right, Freya Robinson and the large number of rearing containers fit into a cubicle.

The objective was to collect the eggs when they were only a day or two from hatching, so the brood masses were sometimes kept for a few days before dissection. The eggs were dissected from the brood masses and placed into histological cassettes. After the eggs were dissected out and the exact number of eggs was known, application was made to AQIS for permission to release the eggs. Once that permission was received, the eggs were surface-sterilised, the clean

cassettes and eggs were then transferred to a clean plastic box that had been wiped over with 1% Virkon® inside and outside, and then removed from quarantine.

While collecting eggs from imported *O. vacca* in quarantine, occasionally some eggs had already hatched or there were timing constraints due to holidays that meant that eggs could not be removed from quarantine. These were kept in their natural brood masses, buried in moist loam and left at 22°C and 14:10 L:D for development. Once adults emerged, they were set up in containers in the same cubicles, fed regularly and brood production recorded. There was no vernalisation treatment.

Figure 14. Preparation of eggs for release from quarantine. Top left, dissection of eggs from brood masses; Top right, eggs in cassette prior to being cleaned; Bottom left, cassettes secured in basket for washing treatment; Bottom right, washing beaker on magnetic stirrer.

Rearing in quarantine was challenging and labor-intensive, due to the cramped conditions (Figure 13) and the need to clean the cubicles very thoroughly after each session.

The beetles showed distinct preferences for the moisture content of media, with *O. vacca* preferring somewhat moister soil than *B. bubalus*. The soil had to have sufficient moisture to allow the beetles to dig self-supporting tunnels (*O. vacca* would not lay at all in dry soil) but the small size of the container meant that the medium would get too wet from the added dung within 2-3 wk of being brought in.

Addition of vermiculite to moist soil to dry it out was not successful on the long term although it did act as a moisture buffer and was routinely used from shortly after the start. This meant that hundreds of litres of medium needed to be changed over each fortnight. Because there was no way to dry out the soil in quarantine, damp media had to be double-bagged and autoclaved out of quarantine and taken to a glasshouse to be spread into trays to dry. The moisture content was then adjusted by addition of water in a cement mixer. Moistened re-used media could not be left to sit for any period of time for fear of infestation by sciarid flies. After the project acquired the refrigerated shipping container, 60 L bins of media were stored frozen.

Nematodes became an issue with rearing shipment 4 of *O. vacca* in 2013. When the containers were tipped out only 2 wk into rearing, the beetles were found to be covered with nematodes (especially around their mouthparts) and half the culture was dead. Overall feeding was low and the dung contained many nematodes. Whether the previous exposure to ivermectin had reduced the fitness of the beetles which allowed the nematodes to flourish or whether it was the nematodes alone is not clear. Nevertheless, this event led to important changes in rearing methods within quarantine and in the lab culture generally. Firstly, to prevent build up of nematode numbers the amount of dung given to each container was reduced and extra dung was added mid week only if necessary. Sand is used with success at the CSIRO European Laboratory and so plain sand and then sand and vermiculite mixed were tested as possible replacements for loam. They appeared to assist with suppression of the nematodes, probably due to their abrasive nature and absence of organic material. The standard medium used in the laboratory was then 1:1 plasterers' sand (125–250 µm) and vermiculite (sand was too heavy on its own for larger containers).

When nematode numbers began to build up, it was found that beetles could be washed with water to remove some nematodes and then and placed into boxes of dry vermiculite for a week to abrade off the majority of the rest.

Dissection of eggs from the brood masses was easier and more successful when the eggs were well developed (larger and harder) and less prone to physical damage. This meant that it was often helpful to delay dissection a few days after collection of the brood masses. By storing the brood masses at higher or lower temperatures within quarantine, egg development time could be managed to meet operational constraints.

Because *O. vacca* eggs developed quickly, eggs were dissected and released weekly. The longer development time of *B. bubalus* eggs gave extra flexibility. If very young *B. bubalus* eggs were dissected, they could be kept on moist filter paper in Petri dishes for a couple of days to swell up and harden before being surface-sterilised.

3.3.1 *Onthophagus vacca* quarantine rearing

Egg production by imported *O. vacca* in quarantine was very successful (Table 3) except for shipments 3 and 4 (2013) because of the nematode problem described above.

Table 3. Egg production by imported F_0 *Onthophagus vacca* in quarantine.

Year	Shipment	Source	♀ (n)	Egg (n)	Fecundity (eggs/♀)	Longevity ♀ (mean d)
2012	1 & 2	Field	128	1905	14.9	88
2013	3 & 4	Field	133	576	4.3	34
2013	5	Lab	121	1553	12.8	54

The small lab colony in quarantine produced up to 20 eggs per female (Table 4). The success of this very small culture was important to understanding the role of the microbiome in the ecology of the dung beetles (see section 3.4.3).

Table 4. Rearing results for *Onthophagus vacca* laboratory colony in quarantine. These insects were set up from eggs or newly-hatched larvae that could not be taken from quarantine for logistical reasons. Eggs produced by this culture were surface-sterilised and released from quarantine.

		Live emerged adults			Oviposition			
Shipment/ generation	# brood masses	♂	♀	% emerged	♀ set for ovip.	eggs	eggs/ ♀	Days (mean)
2012 F_1	25	13	11	96	7	63	9	88
2012 F_2 & 2013 (ship. 4) F_1	60	20	20	67	19	371	20	93
2012 F_3/F4 & 2013 (ship. 4&5) $F_1/F_2/F_3$	190	74	72	77	55	545	10	118
2012 & 2013 mixed generations	29	10	11	72	N/A*			

*Adults destroyed at end of project before oviposition could commence

3.3.2 *Bubas bubalus* quarantine rearing

In 2012, oviposition was low for the first 3 wk post arrival in quarantine. Egg production increased markedly once the beetles had been able to feed and build up fat body reserves sufficient to support egg production and maturation. Egg production was maintained for approximately 3 months thereafter. The production of 21.0 eggs per female (Table 5) compares favourably with an egg production rate of 15.2 eggs per female achieved at the AAHL by Steinbauer and Wardhaugh (1992).

In 2013 and 2014, imported beetles started to lay at 3 wk and 2 wk respectively after arrival and consistently produced large numbers of eggs (Table 5).

Table 5. Egg production by F_0 *Bubas bubalus* in quarantine

Year	Shipment	Source	♀ (n)	Egg (n)	Fecundity (eggs/♀)	Longevity♀♂ (mean d)
2012	1	France	186	3902	21.0	91
2013	3&4	Spain & France	210	2750	13.1	90
2014	6	France	377	5867	15.6	98

Because of the longer developmental time of the eggs, none hatched before dissection and so none were retained in quarantine.

3.4 Post quarantine rearing

Given that the project's objective was to lab-rear sufficient beetles for multiple releases in spring 2014, a rearing method was required to maximise the reproductive potential of the species by eliminating causes of mortality and premature adult death, reduce the generation time to allow extra generations to be reared before release, and to synchronise the beetles' life stage with the season at time of release.
The first step was to start the lab colony with surface-sterilised eggs released from quarantine and reared in man-made brood balls. Subsequent generations were reared in natural brood masses. Temperature, photoperiod and other conditions were altered as more was learned about the success of different methods.

At the outset, it was known that both species were univoltine with an obligatory diapause. *O. vacca* diapauses as an adult (Lumaret and Kirk 1991), whereas *B. bubalus* diapauses as an adult, pupa or larva (Lumbreras et al. 1990, Lumaret and Kirk 1991). Reduction of generation time required a vernalisation protocol to satisfy the diapause condition in a shorter period of time.

At the same time, it was most important that the vernalisation protocol not adversely affect the fitness of the beetles – that is, the life cycle should be shortened but not at the expense of survival and fecundity.

The issues of vernalisation, survival, longevity and fecundity were examined together as the project progressed, as it was not possible to examine one aspect without the others.

3.4.1 *Onthophagus vacca*

3.4.1.1.1 Vernalisation Protocol

The starting point for the vernalisation studies (Protocol 1, Table 6) was based on preliminary work done at the CSIRO European Laboratory, Montpellier, France plus information on the natural conditions that would be experienced in the Camargue region of France where the beetles were collected.

Figure 15 shows the temperatures, photoperiod and life history stages of *O. vacca* at Arles, France, close to the collection site. The strategy to reduce the generation time during laboratory rearing was to rear at a high enough temperature to shorten the

time taken for the immature stages to develop and then provide an adequate but shortened period of diapause during winter, all without sacrificing fitness.

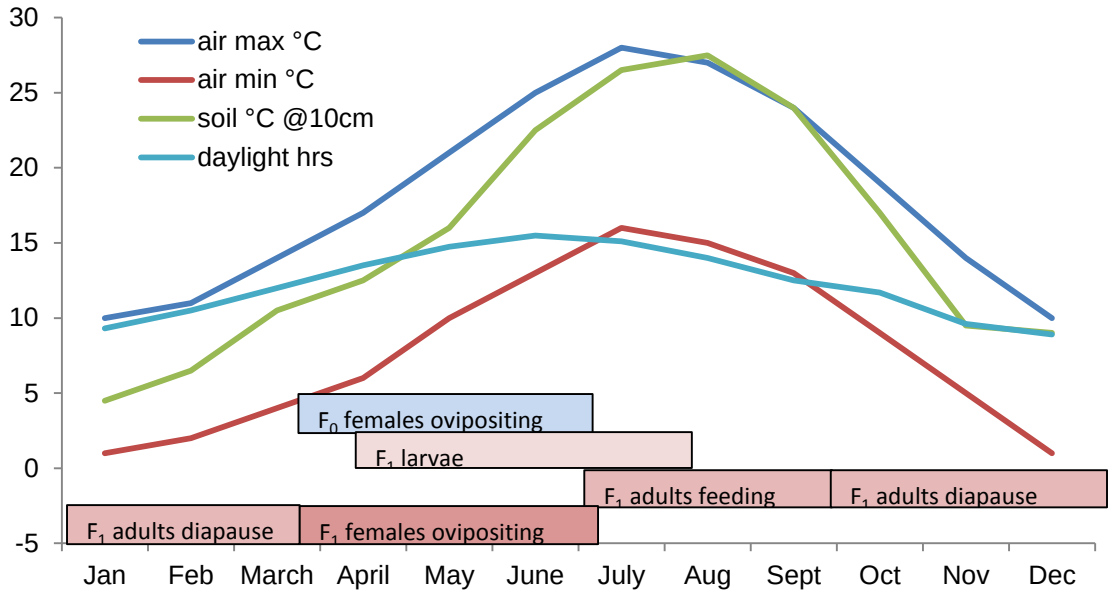


Figure 15. Temperatures and day length at Arles, France close to the collection site for *Onthophagus vacca* in 2012. Superimposed are the basic life stages of *Onthophagus vacca.*

The preliminary work on diapause manipulation undertaken in France achieved oviposition 18 wk after the start of a protocol that consisted of 7 wk at 26°C and naturally decreasing photoperiod, 4 wk at 12°C in the dark, then 26°C 14:10 L:D. It took 18 wk from adult emergence to first oviposition, in contrast to about 36 wk in nature. However, only about half of the females laid eggs and only 11 per female on average.

Given that the photoperiod had to be manipulated artificially in Canberra, it was decided to manipulate the temperature also, and step the temperature down in increments to acclimatise the beetles to colder temperatures, and likewise on the way back to warm temperatures. Therefore Protocol 1 involved 7 wk stepping down (3 at 20°C 12:12, 2 at 20°C 11:13, 2 @ 16°C 10:14), 4 wk provided at the coldest temperature (12°C 10:14), 4 wk stepping up (2 at 16°C 12:12, 2 at 20°C 14:10), to 25°C 14:10 for oviposition.

Table 6 lists all of the vernalisation protocols tested with *O. vacca* during the project, for ease of reference. The details are discussed sequentially in the following sections under the rearing for each year and generation.

Table 6. All of the vernalisation protocols used for *O. vacca* during the project. It begins from emergence of adults and ends at setup for oviposition.

		Number of weeks at each temperature													
		25°C	22°C	20°C		16°C		12°C	10°C	16°C	20°C		22°C	25°C	
Photoperiod (day hours : night hours)		14:10	14:10	12:12	11:13	12:12	10:14	10:14	10:14	12:12	12:12	14:10	14:10	14:10	
Feeding each week		2x	2x	2x	2x	1x	1x	1x	1x	1x	2x	2x	2x	2x	
Protocol	Importation year & generation														Total weeks
1	2012 F_1	-	-	3	2	-	2	4*	-	2	-	2	-	-	15
2	2012 F_2	-	5	2†	-	-	-	3	-	-	2^	-	2	-	14
3	2012 F_2	-	14	-	-	-	-	-	-	-	-	-	-	-	14
4	2012 F_3	4	13	-	-	-	-	-	-	-	-	-	-	-	17
5	2013 F_1	6	10	-	-	-	-	-	-	-	-	-	-	-	16
6	2013 F_1	6	4	-	-	4	-	-	3	2	-	-	2	-	21
7	2013 F_1	2	14	-	-	-	-	-	-	-	-	-	-	-	16
8	2013 F_1	2	16	-	-	-	-	-	-	-	-	-	-	6	24
9	2012/2013	6	1	-	-	2	-	-	6	2	-	-	1	2	20

*5 d at 12°C & 2 d at 20°C each week. †20°C day & 16°C night.

Protocol 1: Adults were given 2 d a week at 20°C to encourage feeding during their cold treatment.
Protocols 2 & 3: The first half of newly emerged adults were given a vernalisation treatment (protocol 2) and the second half were held at constant 22°C (protocol 3), as it was observed in quarantine that adults were ovipositing without vernalisation.
Protocol 4: Newly emerged adults were given 4 wk at 25°C 14:10 to feed heavily, as it was observed that feeding was lower at 22°C 14:10. Protocol length was also extended as it was observed in quarantine that adults were taking longer to sexually mature without a cold treatment. From previous protocols it was evident adults were being set up for oviposition too early and were consequently more susceptible to nematode infestation (from low dung removal combined with the moister rearing medium).
Protocols 5 & 6: These were used to investigate whether there was a difference in brood production between a cold treatment and continuous 22°C/25°C. This comparison showed higher brood production by adults undergoing a cold treatment.

Protocol 7: The time adults spent at 25°C was reduced after sufficiently high feeding was observed at 22°C in Protocol 5. It was proposed that a longer period at 22°C instead of 25°C may lengthen lifespan and prevent adults 'burning out' early on in the protocol.
Protocol 8: Adults from Protocol 7 did not feed highly during their 14 wk at 22°C as had been predicted, and had only 2 wk of sufficient feeding at 25°C. It was also observed that adults were taking longer to begin oviposition than the previous importation, therefore adults were given an extra 6 wk at 25°C at the end of their protocol to encourage high feeding to assist oviposition.
Protocol 9: Newly emerged adults were given 6 wk to feed highly at 25°C before starting on the vernalisation treatment. The period at the coldest temperature was extended to 6 wk because it resulted in a greater overall egg production and number of females laying. It was observed that feeding after cold treatment was slow to pick up, even at 22°C, and so adults were given 2 wk at 25°C at the end of the protocol to encourage feeding before being set up for oviposition. High dung shredding at 25°C after cold treatment was a good indicator that adults were ready to start ovipositing.

3.4.1.1.2 2012 Importation Shipments 1&2: Rearing of F_1 generation

Eggs released from quarantine were put into 30 ml man-made brood balls set into a layer of moist vermiculite in flat trays with a clear lid (Figure 16). The trays were placed in a constant temperature room at 20°C which was later raised to 25°C after the final tray was set up. The holes in the man-made brood balls were left open to observe egg hatch and subsequent larval development. Trays were checked several times a week and the mortality of eggs and larvae noted. Larvae were left to cover over the hole in the man-made brood ball with dung and faecal material.

Figure 16. Left, Eggs set up in artificial balls in flat rearing trays; Right, the boxes used for emergence from brood masse (2012).

It was observed for the first half of trays set up that many larvae were not covering themselves with dung but instead filling the hole beneath themselves with dung and excrement and eventually falling out of the top of the ball into the surrounding medium. Although they were replaced into a new hole in the ball, this certainly increased mortality.

There was substantial growth of fungus on the man-made brood balls and so after the larvae had covered themselves over the balls were covered with autoclaved moist soil. The soil was firmly packed around the balls to also contain larvae within their balls. This method, combined with making deeper holes in the balls, reduced the number of larvae exiting the balls and consequently lowered larval mortality from starvation. Initially the soil deterred fungal growth in the trays but did not completely solve the problem. Furthermore, the soil, being partially organic in origin, exacerbated the breeding of sciarid flies within the trays so was replaced with moist vermiculite.

Trays were checked daily for emerged adults and emergence holes. Once emergences were occurring brood masses separated from the medium and placed into a clear plastic box (26 x 19 x 6 cm) with three 3 cm holes in the base. The box was placed on top of another box of the same size, into which emerged adults fell and were easily collected daily (Figure 16).

Egg hatch overall was greater than 90%, although it fell towards the end of the females' lives. Larval mortality was high with 43% of deaths occurring during larval development. Larval survival varied by tray, probably due to differences in the moisture content of the man-made brood balls or changes in the moisture content of the vermiculite. Overall, 33% of the eggs set up in man-made brood balls emerged

as live adults (Table 9). Adult emergence occurred over a 3 month period with beetles taking an average of 89 d from egg set up to adult emergence.

As adult F_1 beetles emerged, they were grouped into cohorts to move through the vernalisation protocol 1. Cohort 1 was composed of adults that emerged between 13-28/8/2012, cohort 2 between 29/8/12 and 7/9/12 and so on. Thus cohort 1 corresponds generally, but not perfectly (due to differences in developmental time), to the first eggs laid by F_0 females in quarantine, and cohort 11, the last eggs laid in quarantine.

Adults were set up in autoclaved dry soil in the tall 1.7 L containers, with an average of 9 adults per container. Sexes were kept separate throughout the vernalisation protocol for ease of record keeping. Soil was changed every 1-2 months and adults were fed twice per week at the higher temperatures and once per week at the lower temperatures. At higher temperatures, the beetles fed voraciously on the dung, but once they were moved to 16°C, feeding dropped off dramatically.

A few of the first adults to go into the protocol were dissected at week 1, 4 and 7 to determine the state of the fat body and ovaries. At week 1 and 4, females had some fat body and ovaries without differentiated eggs. Females at the end of week seven had almost no fat body, indicating that they were starving. This corresponds to the observation that they had barely disturbed their dung supply. An observation from preliminary work in France was that beetles that had been kept for longer than two months at constant cold temperature laid very few eggs, which did not survive. Therefore it was decided to provide fresh dung and warm temperatures for 2 d per week while otherwise at 12°C or 16°C, to ensure that beetles remained healthy. In nature, temperatures do fluctuate and it was considered better to keep the beetles healthy than to stick rigidly to a fixed low temperature.

About 25% of adults died during the vernalisation treatment, with most dying at the beginning and end of the process (Table 7). The inference was that the beetles needed more feeding time after emergence and before the vernalisation protocol to build up a sufficient fat body to survive the cold period.

Table 7. Mortality of F_1 adult *Onthophagus vacca* during the vernalisation protocol.

		Percent Mortality					
	n	20°C 12:12	20°C 11:13	16°C 10:14	12°C 10:14	16°C 12:12	Overall
Female	319	5.0	4.1	3.8	5.3	4.4	26.3
Male	303	5.6	2.0	2.6	8.3	9.2	23.8
Total	622	5.3	3.1	3.2	6.8	6.8	25.1

Once the beetles had completed the vernalisation protocol, they were set up for oviposition in autoclaved moistened soil (2 L water: 50 L dry soil) in a new tall 1.7 L container, with an average of 3 females and 2 males per container, and fed twice weekly. The first containers of adults (cohort 1) were set up for oviposition on 22/11/12 as they were moved from 22 to 25°C. The first eggs were laid on 20/12/12. Oviposition quickly increased but the mortality of adults was very rapid, and so half the adults were moved back to 20°C on 16/1/13, and subsequent adults from the vernalisation protocol were kept at 20°C. This resulted in an increase in adult survival by a week or two but also the almost complete cessation of oviposition (which was distinctly different from the experience of the F_0 females in quarantine where they oviposited and survived for 2 months). The females (now quite old) kept at 25°C laid almost twice as many eggs (24) as their counterparts (13) at 20°C. As a

compromise condition, all the containers were moved back to 22°C on 12/02/12, to see if more eggs would be laid (live adults are of little value if they are not ovipositing).

Most of the eggs were produced by beetles of cohorts 1-3, which were those that spent most of their time at 25°C). Whether temperature or the quality of the adults was the major cause for the better rate of oviposition was not clear. Nevertheless, the best egg production by cohort 1 (8 eggs/female) was only half that achieved by their F_0 parents (15 eggs/female) so there was a clear fitness problem to be resolved.

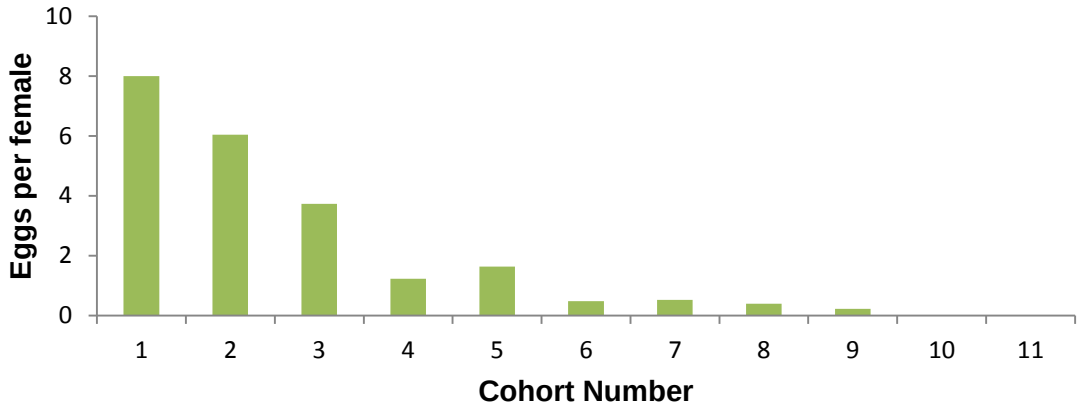


Figure 17. Eggs produced by *Onthophagus vacca* F_1 females. Cohort 1 is composed of the earliest adults, cohort 11 the latest adults to emerge.

It took 18 wk from adult emergence to first oviposition, in contrast to about 36 wk in nature. However, only about half of the females laid eggs and only 3.2 per female on average (Table 9).

3.4.1.1.3 Laboratory colony: Rearing of 2012 F_2 generation

The natural brood masses (F_2 generation) from the F_1 adults were sieved from the oviposition containers and put, without opening, into clear plastic boxes (26 x 19 x 10 cm) with a small mesh vent in the lid, buried in a mix of 2/3 autoclaved moist soil and 1/3 dry vermiculite for development. A 30 ml plastic cup was set in the corner of each tray to act as a pitfall trap to collect adults on emergence. The first few trays were set up at 25°C and the first adults emerged after 46 d but were smaller than the previous generation. Because size is usually correlated with fecundity it is important to rear large adults, so all succeeding trays were set up at 22°C so that the larvae would eat more over a longer developmental period.

The egg to adult survival rate for the F_2 natural brood masses improved significantly from that of the F_1 generation, with 66% of eggs set up developing into live adults (Table 9). It was expected that beetle-made brood masses would improve the adult emergence rate compared to man-made brood balls. Adult emergences occurred over a 5 month period with an average of 84 d from egg set up to emergence. Adults kept at 25°C for most of their larval development emerged an average of 54 d after egg set up, while those at 22°C took an average of 92 d. It was noted that this extra 5 wk for development was quite substantial and would make it more difficult to achieve rapid synchronisation.

As for the F_1 generation, the first cohorts of emerged adults were set up in autoclaved dry soil in the 1.7 L tall containers for their protocol with an average of 8 adults per container. Sexes were mixed this time to allow for possible copulation during the protocol. The soil was changed every 1-2 wk.

The vernalisation protocol was revised (Protocol 2, Table 6) with the major change being the addition of 5 wk at a warmer temperature (22°C) at the beginning of the protocol to encourage feeding. Constant 16°C was replaced with a 20°C day/16°C night to also encourage feeding into vernalisation. Part of the way through adult emergences in the laboratory colony it was observed that adults remaining in quarantine had begun ovipositing without a cold period and were producing a significant number of eggs (Section 3.3.1; Table 4). As the F_2 adults which had already undergone their vernalisation were not producing as many eggs as expected, it was decided not give newly emerged adults a vernalisation but instead keep them at a constant 22°C (Protocol 3, Table 6) to replicate the maximum day temperature in quarantine to see whether rearing without vernalisation was possible.

Adult survival through the resynchronisation protocols was lower than expected with only 33% of adults surviving to oviposit (Table 9). Most adult deaths occurred a month into the protocol at 22°C. Leaving the adults in soil at 22°C accounted for much of the mortality as it provided a favourable environment for nematodes. Beetles were washed with water and moved into clear plastic boxes with dry vermiculite to abrade off the nematodes. A decision was made to keep the adults in boxes of dry vermiculite throughout their protocol and set up any newly emerged adults this way.

After completing the protocol, adults were set up for oviposition in moist soil in 5 L plastic buckets (22 x 18 cm diameter) instead of the previously used 1.7 L plastic containers. By this stage beetle numbers were increasing so rearing methods needed to become more time efficient. Switching to larger rearing buckets allowed a larger number of adults per container (average of 5 males and 7 females), and reduced labour time and increased flexibility in the timing of brood harvests, albeit with a reduction in detail of information collected. Brood masses were harvested and soil and dung were replaced weekly, however nematode infestation of adults still occurred regardless of the regular maintenance. Despite a lower number of F_2 adults set up for oviposition compared to the previous generation, female fecundity doubled with an average of 6.2 eggs per female (Table 9). The results showed also that it was possible to rear the beetles without vernalisation.

3.4.1.1.4 Laboratory colony: Rearing of 2012 Shipments 1&2: F_3 generation

Natural brood masses (F_3 generation) produced by the F_2 generation of adults were buried in moist vermiculite in the clear plastic boxes with vented lids and kept at 25°C for development so that development would be as rapid. As before, a 30 ml cup collected newly emerged adults. Adults took an average of 49 d from brood set up to emerge and, unlike the F_2 generation left to develop at 25°C, were mostly majors of a good size.

Emerged adults were grouped into cohorts and set up in dry vermiculite in the clear plastic boxes for the length of their resynchronisation protocol. Dry vermiculite prevented the accumulation of nematodes on the beetles and adult survival through the protocol was the highest yet, increasing by 50% from that of the F_2 generation (Table 9). A constant warm temperature protocol was used again (Protocol 4) with the protocol lengthened to allow 4 wk of intense feeding at 25°C immediately after adult emergence (Table 6). Feeding at 22°C as per the previous protocol (Protocol 3)

was observed to be lower than expected. The 2012 F_3 adults were combined with the 2013 F_1 adults for oviposition and their brood mass production is discussed under 3.4.1.1.6

3.4.1.1.5 2013 importation Shipments 3, 4 & 5: rearing of F_1 generation

The process was changed for this year to save time and also avoid some of the problems, albeit with the loss of some data on survival of eggs compared to larvae. Eggs released from quarantine were inserted into holes in 30 ml man-made brood balls with the hole closed up immediately and the brood balls were covered over with moist vermiculite in flat trays with clear lids and kept at 25°C.

F_1 egg to adult survival for shipments 3 and 4 improved from the F_1 2012 Importation (Table 9), likely due to lower larval mortality from closing and burying man-made brood balls immediately after egg set up. Egg to adult survival for shipment 3 was low compared to former F_1 generations with most mortality attributed to a poor egg hatch rate. Whether this was a result of the parents having come from a laboratory colony or of local factors is unknown. Shipment 3 and 4 adults took an average of 65 d and Shipment 5 adults an average of 61 d from egg set up to emerge.

Newly emerged adults were set up in dry vermiculite in the same plastic boxes used for the 2012 F_3 generation, with the vermiculite replaced every 1-2 months during the vernalisation protocols. Adult survival through the protocols was the highest yet with over 90% of emerged adults being set up for oviposition (Table 9).The initial non-vernalisation protocols used for the 2013 importations were devised around a 16 week time frame as it was found from the 2012 importation that adults began ovipositing at a minimum of 16 wk post-emergence without a vernalisation. The first few cohorts of emerged adults (all of shipment 3 and half of shipment 4) were kept at 25°C for 6 wk to feed before being moved down to 22°C for 10 wk (Protocol 5) (Table 6). Four cohorts were set aside for a vernalisation experiment which is discussed below. The remainder of shipment 4 adult emergences were kept at 25°C for only 2 wk and 22°C for 14 wk (Protocol 7) (Table 6). It had been planned for Shipment 5 adults to complete Protocol 7 however it was observed that feeding at 22°C was lower than expected so adults were given an additional 6 wk feeding at 25°C to assist oviposition (Protocol 8) (Table 6).

3.4.1.1.5.1 The effect of vernalisation and a fluctuating day/night temperature on fecundity

It was observed in quarantine from remaining 2012 offspring that a vernalisation was not required for *O. vacca* to begin to oviposit, raising the possibility that an easier rearing method could be developed. F_1 and F_2 adults left in quarantine were ovipositing without a constant 10°C cold period, and furthermore egg production was threefold (average 20 eggs per female) that of the equivalent generation of adults in the laboratory outside quarantine (average 6 eggs per female). In addition, adults in quarantine were ovipositing for a longer period (maximum of 93 d for F_2s) compared to those in the laboratory outside quarantine (maximum of 47 d for F_2s). The hypothesis was that the fluctuating temperature in quarantine from a warmer day (22°C maximum) to a cooler night (14°C minimum) increased adult longevity and therefore lengthened the period of oviposition.

The experiment was set up to assess: a) the need for vernalisation and b) the effect of a fluctuating day/night temperature during oviposition. Four cohorts of adults originating from shipment 3 and 4 (a cohort being adults with comparable emergence dates and ranges) were used. Each cohort was divided in two; half the adults were given a vernalisation treatment (Protocol 6) and the other half were not (Protocol 5) (Table 8). On protocol completion adults were set up for oviposition at either constant 25°C or given a 25°C day and 16°C night. Therefore each cohort underwent the four treatments as summarised in Table 8.

A vernalisation treatment improved the fecundity of the adults (overall 8.3 vs. 2.7 brood masses per female (Table 8). So although it was possible to rear *O. vacca* without a vernalisation period, better results were achieved with one. There was no obvious difference in fecundity between adults kept for oviposition at constant or daily fluctuating temperatures.

Table 8. Summary of the effects of vernalisation and fluctuating temperatures on oviposition of *Onthophagus vacca* in the laboratory. All oviposition under photoperiod 14:10 L:D

Vernalisation	Protocol	Temperature during oviposition	Females (n)	Brood masses (n)	Brood masses per female
No	5	Fluctuating	69	188	2.7
No	5	constant	28	70	2.5
Yes	6	fluctuating	57	493	8.6
Yes	6	constant	21	153	7.3

3.4.1.1.6 Mass Rearing Colony 2014

Natural brood masses produced by the 2012 F_3 and 2012 F_1 adults were set up at 25°C in clear plastic boxes (26 x 19 x 10 cm) with a small meshed vent in the lid and covered in moist vermiculite with a 30 ml cup buried in the corner to catch emerged adults. Approximately 30 newly emerged adults of mixed sex were set up in dry vermiculite in each clear plastic box to undergo Protocol 9 (Table 6).

Protocol 9 combines the positive components from previous protocols, as well as general observations and data analysis from previous generations. Newly emerged adults were given 6 wk at 25°C to encourage high feeding and build up of fat reserves to sustain them through vernalisation. Adults were gradually stepped down to 10°C to replicate a more realistic transition from summer to winter. Adults were kept at 10°C for longer than previous vernalisation protocols to ensure the majority of females had a sufficient number of cold days to fulfil the diapause requirement.

It had been noted that when adults were set up for oviposition immediately after completing the vernalisation protocol, their feeding was poor and they removed no dung for brood creation, indicating they were not ready to oviposit. The highest level of mortality in *O. vacca* adults occurred within these few weeks following oviposition set up and therefore lowered the potential egg production of the generation. Nematode numbers dramatically increased when beetles were not feeding strongly or removing dung and likely were a factor that contributed to adult mortality during this period. Therefore an additional step was inserted before setting up for oviposition. Protocol 9 provided 2 wk at 25°C for adults in containers with dry

vermiculite to feed and build up fat body prior to being set up for oviposition. The overall length of the protocol was increased from 16 wk to 20 wk, as experience from earlier generations showed that it took an average of 20 wk with a vernalisation from emergence to first oviposition. Adults set up after 20 wk began ovipositing within 1-2 wk of set-up.

The 2012 F_3 and 2013 F_1 adults were combined for oviposition with a total of 742 adults surviving through the protocols (Table 9). 590 of these adults were set up for oviposition within the lab and left to oviposit until death (most cohorts were not synchronised to begin ovipositing in spring 2014, so were left as breeding stock). These adults produced an average of 3.9 eggs per female. The remaining adults were field released immediately after the protocol or were set up for oviposition in the lab for several weeks and then field released. Allowing the adults to begin ovipositing in the lab was advantageous as it ensured the adults were ready to put down brood masses immediately on field release.

In 2014, adults of the various generations from the 2012 and 2013 generations overlapped significantly (Figure 18) and so these were combined into a single mass rearing colony (Table 9). The first cohort of eggs was set up in January 2014 and a total of 2519 brood masses were produced to the end of December 2014 (Table 9), of which the majority were field released. For the brood masses remaining in the lab, adult emergence continued and to the end of December 2014, egg to adult survival was 56% (Table 9).

All the mass-rearing colony adults were given a vernalisation treatment (Protocol 9 in Table 6) and good survival rate through the vernalisation was maintained with 76% of adults surviving to oviposition (Table 9). 347 adults were set up in the lab for oviposition and oviposited until death, producing an average of 4.4 eggs per female (Table 9). The remaining adults were either field released after completion of their protocol or were set up to oviposit for several weeks before being field released.

Table 9. Cross-generational comparisons of rearing of *Onthophagus vacca*, including comments on key lessons learned each generation.

Generation	Dates (first egg to last adult)	Eggs	Survival egg - adult	Live emerged adults	Survival adult emergence - oviposition	Adults set for oviposition	Eggs per female	Comments and Key Lessons
2012 Importation (Shipments 1 & 2: May, June)								
F_0	Adults set up 25/5/12, destroyed 3/9/12				na	220	15	Oviposition for 3 months
F_1	31/5/12 - 11/4/13	1905	33%	622	75%	465	3.2	Man-made brood balls, not buried. High larval mortality. 25% of adults died during vernalisation. 25°C too hot for good oviposition.
F_2	20/12/12 - 5/12/13	788	66%	523	33%	171	6.2	Natural brood masses, buried. Survival of immatures doubled compared to previous generation. Adults in vermiculite without vernalisation successfully oviposited, *suggesting vernalisation not required.*
F_3	4/7/13 - 8/8/14	516	46%	200	84%	161	na	Adults from this generation were combined with the 2013 F_1 adults for mass-rearing. Their output is presented under Mass Rearing Colony
2013 Importations (Shipments 3 & 4: May, June)								
F_0	Adults set up 18/5/13, destroyed 12/9/13				na	252	4.3	These adults had shorter survival and laid fewer eggs than those of 2012; whether due to greater age at collection, the effect of the ivermectin or nematode infestation is not clear.
F_1	7/6/13 – 28/3/2014	576	52%	299	96%	277	na	Man-made brood balls, buried. Survival of immatures poor (but better than 2012). Survival during vernalisation was excellent. Adults from this generation were combined with the 2012 F_3 adults for mass-rearing. Their output is presented under Mass Rearing Colony.

Generation	Dates (1st egg to last adult)	Eggs	Survival egg - adult	Live emerged adults	Survival adult emergence - oviposition	Adults set for oviposition	Eggs per female	Comments and Key Lessons
2013 Importation (Shipment 5: September)								
F_0	Adults set up 28/9/13, destroyed 25/6/14					234	12.6	Adults shipped from French laboratory colony after emergence from the vernalisation protocol.
F_1	4/10/13 - 26/11/2014	**1553**	**21%***†	**323***	92%	298	na	Eggs set up in man-made brood balls at 25°C 14:10
Mass-rearing Colony								
2012 F_3 & 2013 F_1	4/7/13 - 19/11/14					590	3.9	742 adults survived through to oviposition. The ones synchronised to the season were field released when ready for oviposition; some were set up for oviposition for some weeks prior to field release and their egg output is included below. The remaining 590 that were not adequately synchronised were set up for oviposition and produced eggs until death (eggs/♀ is for this group only).
2012 F_4 & 2013 F_2	6/1/14 - 23/12/2014	2426	56%*†	1036	81%	269*	4.4*	844 adults survived through to oviposition; 269 of these were set up for oviposition and produced eggs until death (2426); eggs per female are for this lot only and their egg count is included in the figure). Remaining adults were field released when ready for oviposition; some were set up for oviposition for several weeks prior to field release and their egg output is included in the 2426 figure. Some brood masses from the 2426 were also field released.

Generation	Dates (1st egg to last adult)	Eggs	Survival egg - adult	Live emerged adults	Survival adult emergence - oviposition	Adults set for oviposition	Eggs per female	Comments and Key Lessons
Quarantine Colony: Eggs released to laboratory								
F_2 & F_1	5/4/12 - field release 2/10/14	384	27%	84	76%	64	na	Most eggs set up in surrogate brood masses or with maternal gift/particles in man-made brood balls. Most adults field released when ready for oviposition.
F_2,F_3,F_4 & F_1,F_2,F_3	14/8/14 - field release 18/10/14	216	21%	46	na	na	na	Most eggs set up in surrogate brood masses or with maternal gift/particles in man-made brood balls. Most adults field released when ready for oviposition.
F_2,F_3,F_4 & F_1,F_2,F_3	14/8/14 - field release 18/10/14	224	na					Eggs set up in surrogate *O. binodis* brood masses. All brood masses field released.

* = preliminary values; na = not applicable; † = survival egg-adult for completed emergence trays; ‡ Most of these adults were released into the field just as they were ready to oviposit or shortly after starting to oviposit. Note: Figures do not include 2012 & 2013 cultures that remained in quarantine

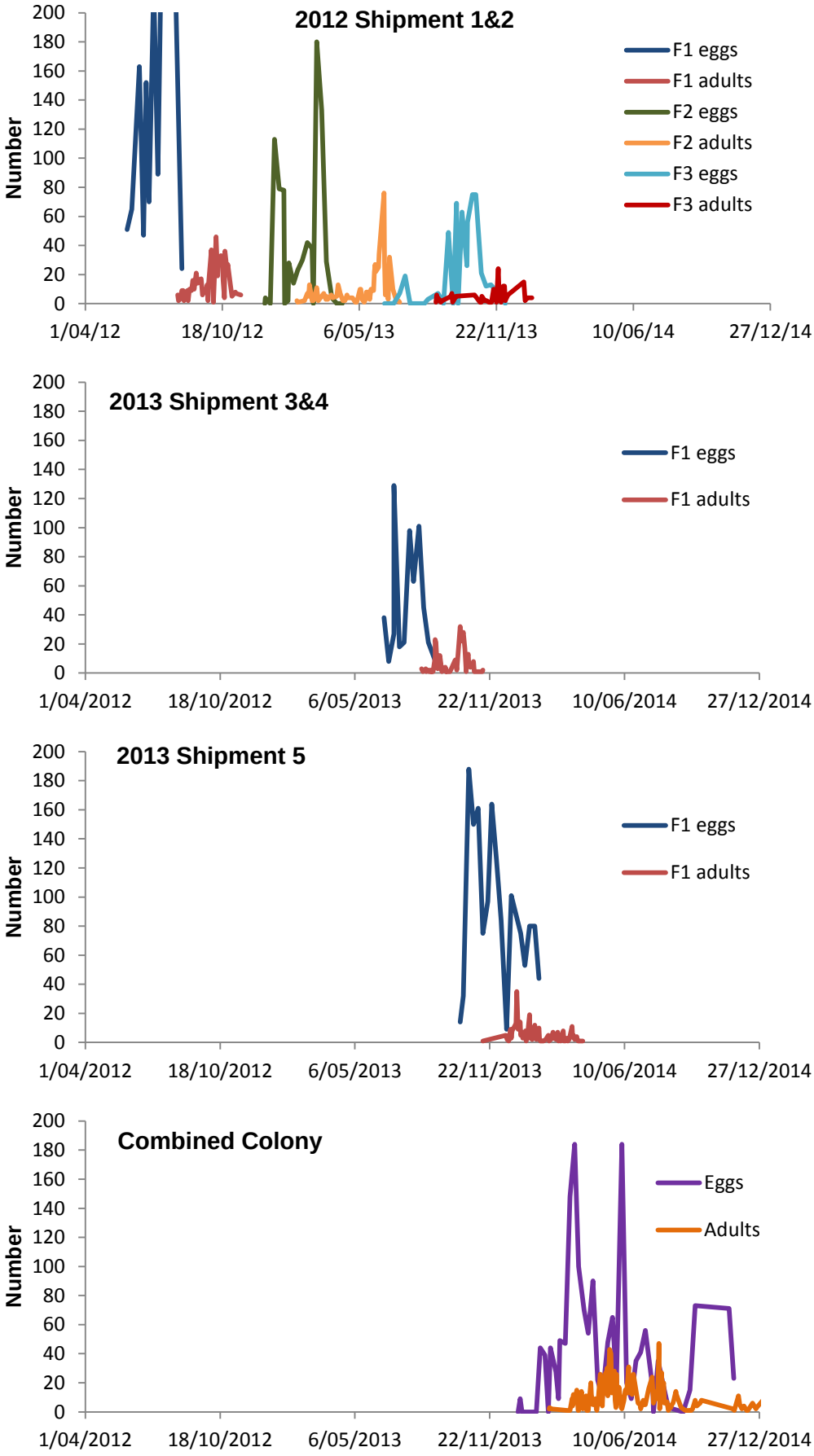


Figure 18. Plots of eggs and adults of each generation of *O. vacca*.

3.4.1.1.7 Generation Time for *Onthophagus vacca*

The development time for *O. vacca* from egg to adult varied dramatically with temperature. In early 2013, development from egg to adult of 2012 F_2s was an average of 92 d (13 wk) at 22°C but only 54 d (7 wk) at 25°C. But the development was variable, and over all of the rearing at 25°C in this project, it took 6-10 wk for all adults to emerge. The number of weeks from egg to egg for *O. vacca* reared at 25°C followed by vernalisation Protocol 9 ranged from 27 to 32 wk (Table 10).

Table 10. Time for a generation of *Onthophagus vacca* reared at 25°C followed by vernalisation Protocol 9.

	Weeks
Development at 25°C (egg-adult)	6-10
Vernalisation protocol 9	20
Pre-oviposition period (to first egg)	1-2
Total	27-32

3.4.1.1.8 2014 *Onthophagus vacca* eggs from quarantine colony: microbiome rearing

It was observed that the 2012 F_1 adult emergence rate from beetle-made brood masses left in quarantine was significantly higher at 95% (Table 4) than the 33% adult emergence rate of 2012 F_1 eggs released from quarantine and set up in man-made brood balls (Table 9). Likewise, those 2012 F_1 adults left in quarantine produced significantly more eggs per female (Table 4) than the 2012 F_1 adults outside quarantine (Table 9). The likely importance of the maternal gift (small particles + secretions + microbiome) (see section 3.4.3) in supporting egg to adult development and adult fecundity was therefore acknowledged. While the European maternal gift could not be provided for quarantine reasons, a maternal gift from locally available dung beetle species was possible.

In February 2014 the adults of the colony in quarantine started to oviposit. A trial was set up to establish whether it was possible to rear *O. vacca* eggs released from quarantine through to adults, using surrogate brood masses from other dung beetle species. Two locally available species, *Onthophagus binodis* and *Onthophagus granulatus* were collected and set up as laboratory colonies for the purpose. The egg chamber of the surrogate brood mass was carefully opened and the *O.binodis* or *O. granulatus* egg was removed and replaced with an *O. vacca* egg. A total of 63 eggs (2 releases a week apart) were surface sterilised as usual and released from quarantine. Each egg was placed in the chamber of a surrogate brood mass, then the chamber was carefully sealed back up and the brood mass buried in a plastic tray of moist vermiculite and kept at 25°C and 14:10 LD photoperiod. This trial proved it was possible to rear *O. vacca* from egg to adult using surrogate brood masses, resulting in a 45% adult emergence rate.

From August to December 2014 the next generation of adults of the quarantine colony oviposited. A total of 384 eggs were surface sterilised and released from quarantine to be set up in surrogate brood masses (Table 9). *O.binodis* brood masses were used as surrogates as they are a similar size (albeit slightly larger) to *O. vacca* brood masses. The brood masses of *O. binodis* were harvested in

synchrony with harvest of *O. vacca* eggs. As before, the egg chamber of the brood mass was opened and the *O. binodis* egg was replaced with an *O. vacca* egg. These surrogate brood masses were then buried in moist vermiculite in small, vented plastic boxes and held in a constant temperature room at 25°C and 14:10 LD photoperiod.

The impact of using the maternal gift in surrogate brood masses to improve egg to adult development and adult fecundity in *O. vacca* was not evaluated as most of the surrogate brood masses were buried in the field before adult emergence was possible. The egg to adult development of the 168 remaining surrogate brood masses was low with a 27% adult emergence rate. The low emergence rate is attributed to poor hatch rate with 48% of eggs not hatching. These eggs were laid by adults in the final two months of oviposition in quarantine, so it is possible that the eggs were less viable as the laying females were getting old. At the end of December 2014, the emerged adults had not reached sexual maturity and therefore no data on adult fecundity was collected.

3.4.2 Bubas bubalus

3.4.2.1.1 Vernalisation Protocol

Little detail is known about the diapause behaviour of *B. bubalus*. In Spain, Wardhaugh (*pers. comm.*) found small numbers of *B. bubalus* adults during autumn and winter, but the vast majority in spring. The winter beetles were newly emerged (little or no tibial wear, small fat body) and sexually immature. Lumbreras et al (1990), working in northern Spain, suggested that those adults emerging in autumn derived from eggs laid in the early spring, those that emerged in spring from eggs laid in summer (July). Lumaret and Kirk (1991) concluded that *B. bubalus* overwinters as larvae, pupae and as indicated in Figure 19.

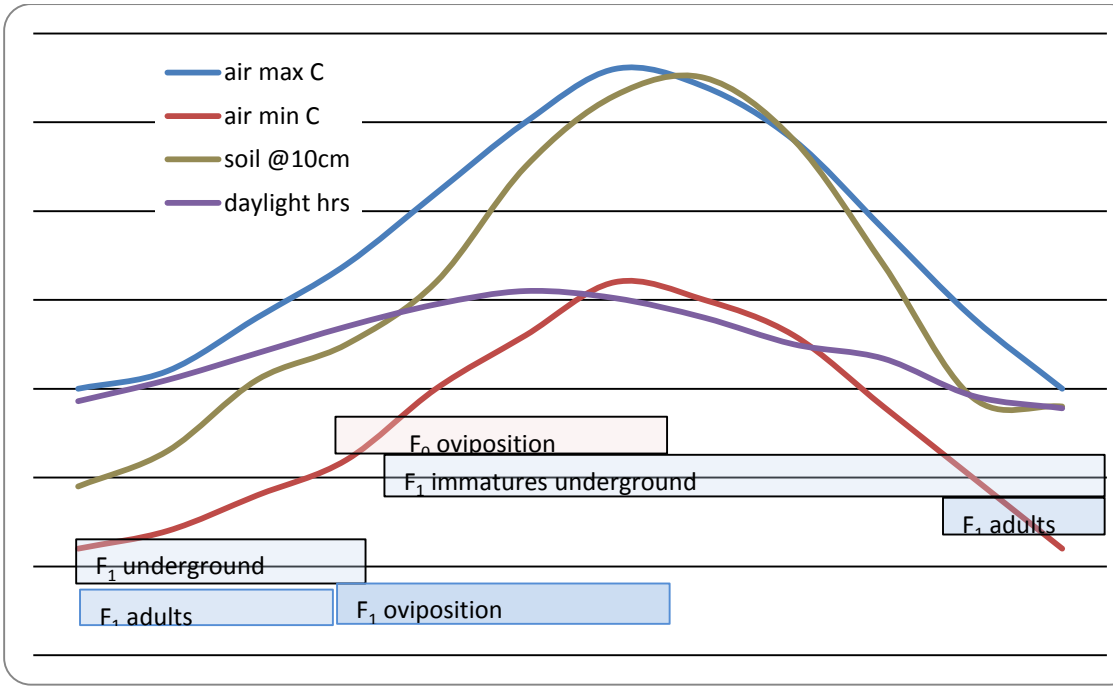


Figure 19. Temperatures and day length at Arles, France close to the collection site for *Bubas bubalus* in 2012. Superimposed are the basic life stages of *Bubas bubalus*

In nature, the brood balls are 10-30 cm below the soil surface, and experience very warm temperatures in summer and cold temperatures in winter (Figure 19). What is most interesting is that adults emerge and begin oviposition while the soil and air temperatures are still very cool.

Steinbauer and Wardhaugh (1992) reared *B. bubalus* at about 23°C in the quarantine laboratory at AAHL; egg to adult took 64-41 d (mean 120 d). Unfortunately, it is not known whether these adults were physiologically ready to oviposit, or whether they were in some kind of reproductive diapause. Only 50 of an original 210 eggs emerged successfully. From a mass-rearing point of view, to be able to force uninterrupted development and oviposition would be ideal.

3.4.2.1.2 2012 Importation: rearing of F_1 generation

Eggs that had been released from quarantine were put into man-made brood balls (60 ml) set into a layer of moist vermiculite in flat trays with a clear lid in the manner described for *O. vacca*. The same issues of fungal growth and larvae leaving the balls were experienced and so after a time, all brood balls were covered with medium.

Prior to the developing beetles entering their vernalisation protocol a sciarid fly infestation became evident. This was probably to be expected as CSIRO records show that this was an inherent problem when beetles were mass-reared. Fly larvae infest the dung balls and ultimately cause the death of beetle larvae and pupae. As *B. bubalus* spends more time in the brood ball than *O. vacca*, this species is more subject to damage of this type. Various non-chemical control methods were implemented (see 3.1.3) and the *B. bubalus* rearing was segregated from the *O. vacca* rearing.

Egg hatch overall was good at an average of 87% (Table 17.), although it was poorer at the very beginning and end of the oviposition period.

The F_1 larvae were reared at 20°C, and after 146 d, the oldest larvae had yet to pupate. Therefore, it appeared likely that to get uninterrupted development (as was the case with Steinbauer and Wardhaugh (1992)) the larvae would need to be reared at a higher temperature. As a result, the rearing temperature was increased to 23°C on 28/9/12 and then to 25°C on 15/10/12. Trays of beetles were then divided into 2 groups, so that there were approximately equal numbers of larvae that had been set up on a particular day, in each group. For example, cohort 1 trays were those set up from eggs on 1-8/06/12, cohort 2 from 19-20/6/12, cohort 3 from 25/6/12, and so on until cohort 14 on 2/10/12. Group A contained 1665 larvae Group B contained 2201.

Group A was started on the vernalisation protocol on 16/11/2012 and Group B stayed at 25°C for another 2 months until commencing the vernalisation protocol on 16/1/13 (Table 11).

Table 11. Days that beetles were exposed to different temperatures and photoperiods. The initial 20°C period varied from very short to long, due to the sequential nature of the trays being set up as the eggs were released from quarantine. Cold period of the vernalisation protocol shaded. Post-vernalisation adults were set up at 22°C and later 25°C for oviposition.

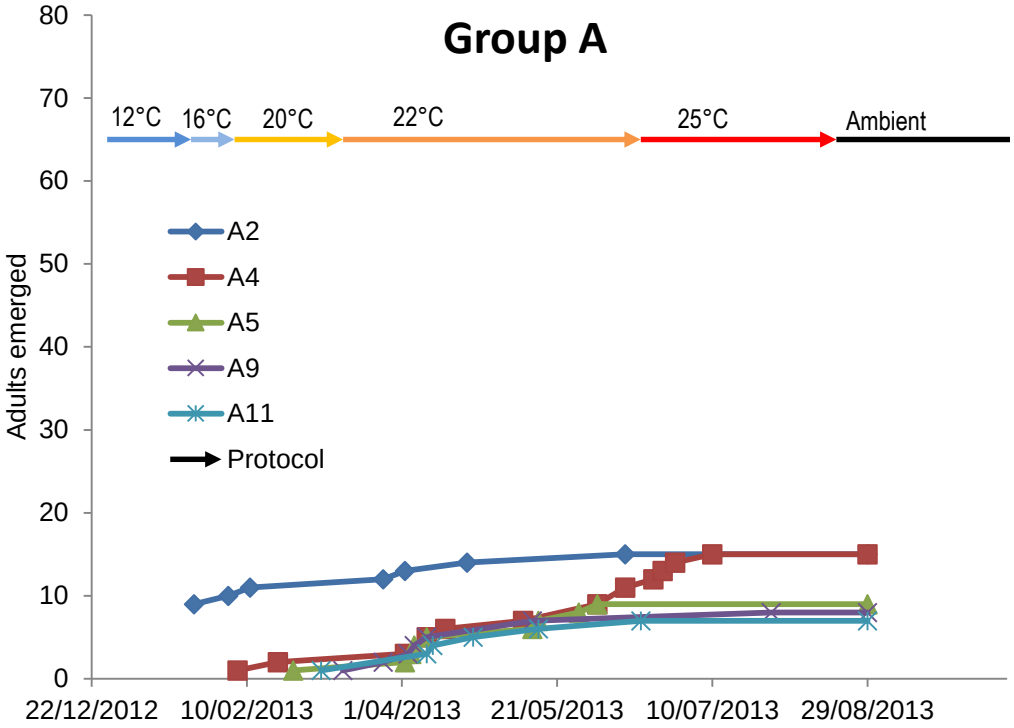
		Group	
Temperature °C	Photoperiod light:dark hr	A	B*
20	0:24	8 - 101	0 - 119
23	0:24	17	13 - 17
25	0:24	32	93
20	0:24	33	7*
16	0:24	8	7*
12	10:14	27	28
16	12:12	14	14
20	14:10	35	7
22	14:10	96	89
25	14:10	63	63

*The ramping down periods at 20°C and 16°C were reduced to 1 wk in an attempt to shorten the protocol to make up for some of the extra time at 25°C.

Emergence trays containing *B. bubalus* broods were checked every few days for adult emergences. Beetles generally tunnelled to the surface of the trays and were found walking around the perimeter of the tray. Over the period of beetle emergence, newly emerged adults (up to 3 pairs) were set up directly into 5 L buckets, containing moist soil and supplied with dung weekly.

The beetles that emerged from their faecal shells of their own accord were termed natural emergences. If a faecal shell containing an adult was damaged irreparably while being checked over, the adult was deemed to have had an artificial emergence and was set up for oviposition, sometimes with adults that had naturally emerged. However, from early containers where natural and artificially emerged beetles were separate, it was clear that the artificially emerged beetles were not yet ready to feed and commence oviposition and therefore beetles were left to emerge naturally wherever possible.

Some Group B adults emerged either at 25°C, during vernalisation as the temperature was decreasing or at 12°C. The earliest that a Group A beetle emerged was a day after being moved during vernalisation from 12°C to 16°C. However, most adults emerged after vernalisation upon being moved to 22°C, with the patterns of Groups A and B not noticeably different, with natural emergence tapering off in early July 2013 (Figure 20).



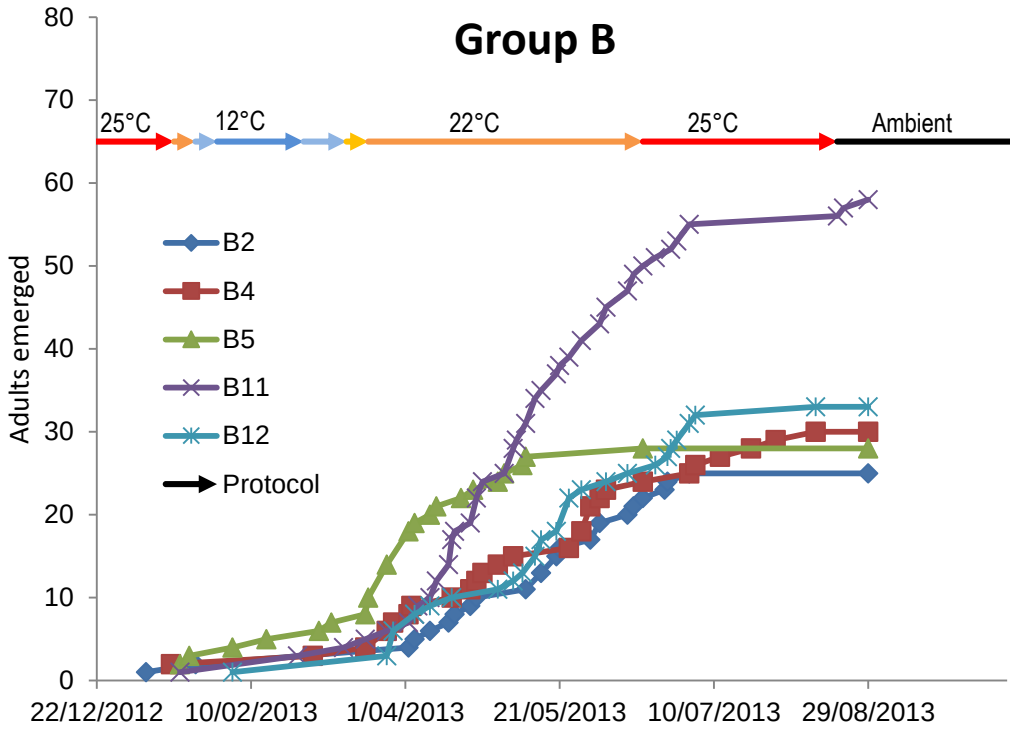


Figure 20. Emergence of adult *Bubas bubalus* after vernalisation treatment for some cohorts with substantial emergence. Cohorts for each group are shown with more than 7 emergences in Group A, or 25 emergences in Group B.

After 96 and 89 d at 22°C (Treatments A and B respectively), followed by 63 d at 25°C, natural emergence had effectively ceased in both groups, and so trays were

moved to ambient conditions in a glasshouse on 19/8/2013 in an attempt to trigger final emergences under more natural (late winter/early spring) conditions. This treatment had no substantive effect (Figure 20) and it was decided to finalise emergence artificially by breaking down the trays to search for non-emerged adults and assess what other development stages might be present. Apart from about 150 adults considered artificially emerged (Figure 21), there were a few callow adults and pupae, as well as some unhealthy diapausing late-instar larvae. All the larvae were destroyed but the pupae & callow adults were kept. A few of the more advanced ones survived to adult.

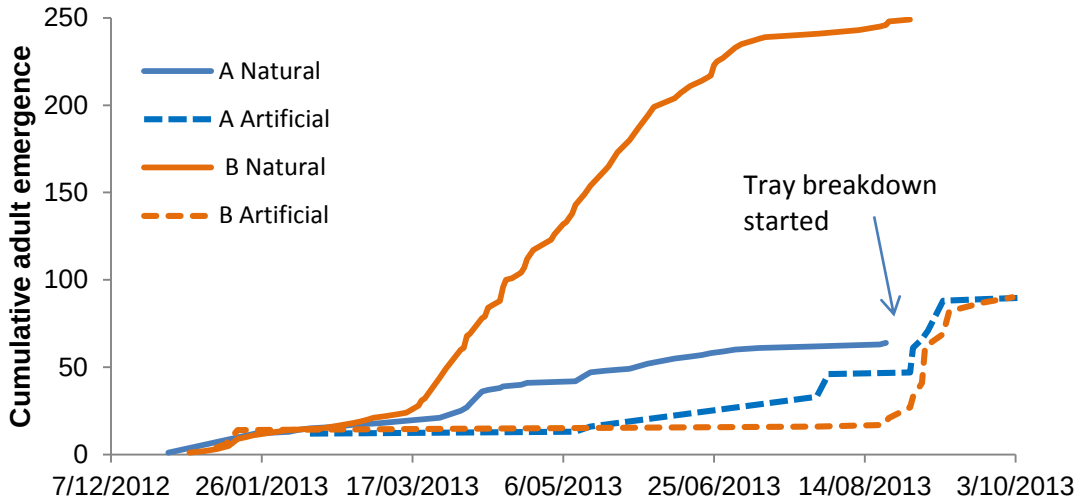


Figure 21. Cumulative adult emergence across cohorts for Group A and B treatments, divided into natural and artificial emergences.

Neither vernalisation treatment resulted in large numbers of adult beetles of either sex emerging from each cohort of eggs (Table 12). Survival to adult in Group B was about 15% overall compared to 9% for Group A. The most striking difference was the much higher percentage of natural emergences for Group B compared to Group A. These results suggested that a longer period at 25°C, to maximise larval feeding and development before the onset of the cooler temperatures, was advantageous to survival and to natural emergence of adults that are ready to breed.

Table 12. Comparison between Group A and B for numbers of adults produced, survival to adults and proportion of natural emergence.

		Adult emergence (n)			Survival to Adult			Proportion of adults	
Group	n	Natural	Artificial	Total	Natural	Artificial	Total	Natural	Artificial
A	1665	64	90	154	0.04	0.05	0.09	0.42	0.58
B	2201	249	90	339	0.11	0.04	0.15	0.73	0.27

Average development time from egg to adult for F_1 *B. bubalus* was 304 ± 3 d. The average development time for beetles under vernalisation Group A and Group B was 317 ± 5 d and 298 ± 4 d respectively. Despite the vernalisation treatment the juvenile beetles were put through, no clear differences in development time between Group A or B can be discerned (Figure 22). The first adult emergence occurred after 149 d of development and the last after 456 d. Beetle emergence across all egg cohorts and each vernalisation treatment was variable. The data suggests that the earlier egg

cohorts developed faster than the later cohorts. This corresponds to the fact that the earlier cohorts (cohorts 1-6) spent longer at 20°C - 23°C (between 88 and 118 d) than the later cohorts (cohorts 7-15) (between 13 and 81 d) and had developed further, prior to being moved to the 25°C A and B Groups and the cold phase of the vernalisation protocol.

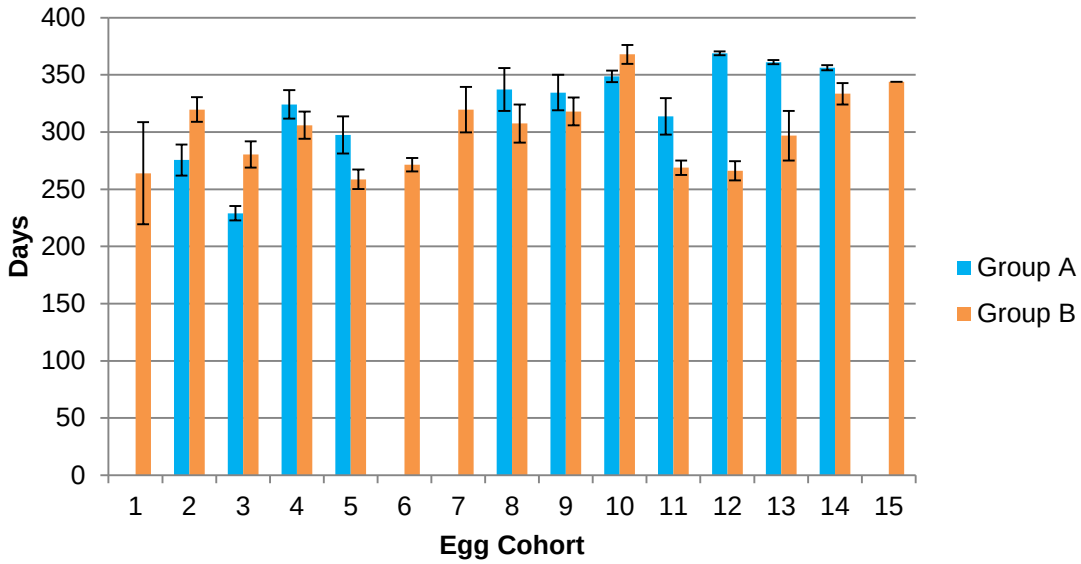


Figure 22. Average number of days ± standard error for F_1 *Bubas bubalus* development from eggs to adult emergence per vernalisation treatment. Egg cohorts are based on the date of the eggs being set up post release from quarantine. Cohort 1 eggs set up 19-20/6/12, cohort 2 set up 25/6/12 etc.

Emerged F_1 beetles were paired and set up for oviposition at 22°C increasing later to 25°C with a 14:10 photoperiod. The earliest adults were mixed together for mating before being set up but later adults were kept separately and the egg production recorded (Table 13). Of 29 Group A females, no artificially-emerged females produced any eggs and naturally-emerged females produced only 1.2 eggs per female. Of 147 Group B females, 125 (71%) produced a total of 281 brood masses (2.2 brood masses or ~4.5 eggs/female overall), supporting the hypothesis that it is better to have a longer pre-vernalisation period.

Table 13. Comparison of egg production by group and type of adult emergence.

	Emergence type	Females	Brood masses	Brood masses per female	Eggs per female
A	Natural	14	8.5	0.6	1.2
	Artificial	15	0	0	0
B	Natural	125	281	2.2	4.5
	Artificial	22	1	0.05	0.09

3.4.2.1.3 2013 Rearing: 2012 Shipment 1 F_2 and 2013 Shipment 3&4 F_1 generations

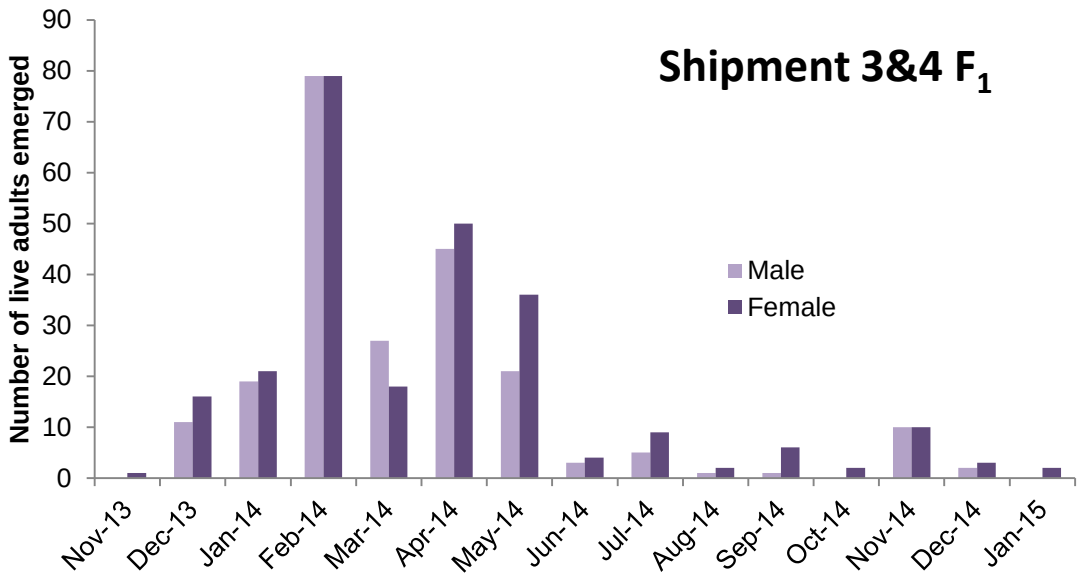
Based on the experience with the Shipment 1 (2012) F_1 immatures, larger 120 ml man-made brood balls were produced for the 2013 imports to see if a larger brood ball would help keep the larvae from escaping the balls. Deeper containers (lidded black plastic tote boxes 490 x 340 x 180 mm) were used to contain the larger brood balls. Deeper containers allowed for the brood balls to be covered more deeply in the rearing medium, in this case moist vermiculite. Lids were sealed to the containers with tape and as an added precaution containers are enclosed in a fine mesh bag to further inhibit ingress and egress of sciarid flies.

From the 2012 importation experience, it was clear that it was better to rear the larvae at high temperature until the beetles have fully developed, and then do the vernalisation protocol and subsequently set up for oviposition. From work done at the CSIRO European Laboratory, it appeared that it might be possible to shorten the overall rearing period by using a shorter (but colder) vernalisation protocol, which is what was done with the F_1 beetles from 2013.

The larvae were reared at 25°C for an average of 23 wk, before they started the vernalisation process. The vernalisation protocol used was similar to Protocol 9 for *O. vacca*, and comprised 1 wk at 22°C 12:12 L:D, 1 wk at 16°C 12:12, 6 wk at 10°C 10:14; 2 wk at 16°C 12:12, 2 wk at 22°C 12:12 and finally set up for oviposition at 22°C 14:10 L:D

Unfortunately, 40% of the rearing boxes were infested by sciarid flies. Whether this is the cause of the poor survival (Table 17.) is not clear. On dissection of the brood balls to extract the faecal shells of the beetles, many balls showed no sign of successful establishment of the young larvae and it would appear that mortality was at a very early stage (see discussion on maternal gift in section 3.4.3). Dead eggs, larvae and pupae were noted. Any remaining faecal shells were separated from the dung ball remnants and repacked into smaller containers containing moist vermiculite. Emergence continued to be monitored on a frequent basis.

Figure 23 shows the monthly pattern of emergence of live adults from these two groups. What is most obvious is the fact that emergence is spread over a very long time and that there are two main emergence periods, a primary one, followed by a much later secondary emergence.



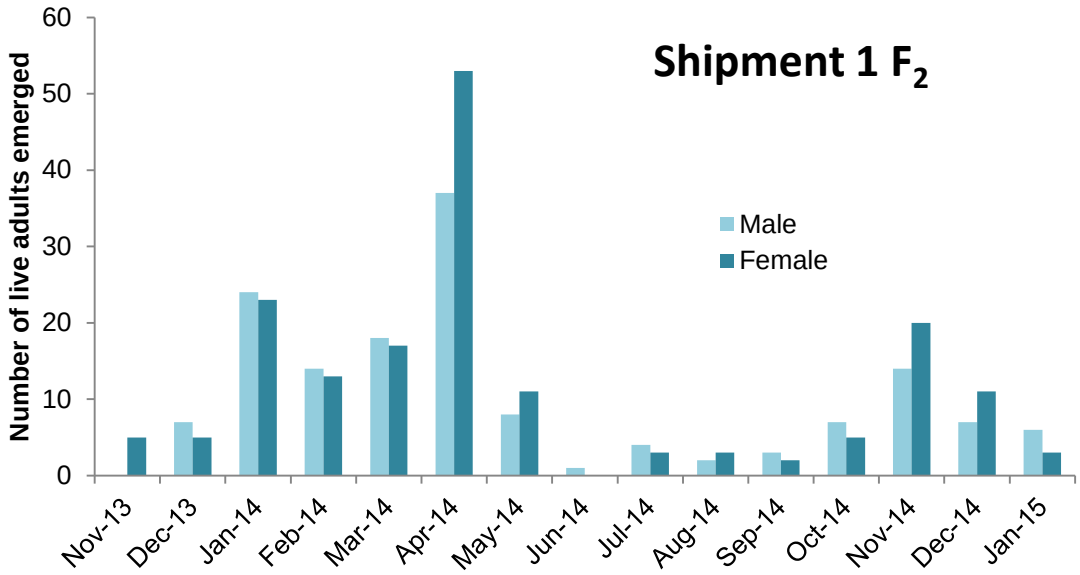


Figure 23. Natural emergence of adults from eggs laid in 2013, grouped by month of emergence.

To examine the emergence pattern without the complication of the spread of initial oviposition, Figure 24 shows the emergence of adults of both groups adjusted to time of egg set-up. Both groups show an early small peak of emergence, followed by the main emergence and with a small proportion emerging much later. The first emergences happened while the beetles were still at 25°C. This fits with the field observations in Europe that some *B. bubalus* adults emerge in autumn, then overwinter as adults. Because all individuals were put through the vernalisation protocol (emerged adults and immature in brood masses), it is not possible to say whether or not these early emergers were physiologically capable of oviposition without a vernalisation, but the literature would suggest not. The main peak immediately followed the rise in temperature after the vernalisation protocol and these adults went on to oviposit after a suitable feeding period. The smaller group of adults that emerged over 380 d after egg setup would correlate with a subset of the population that emerges the second spring after oviposition.

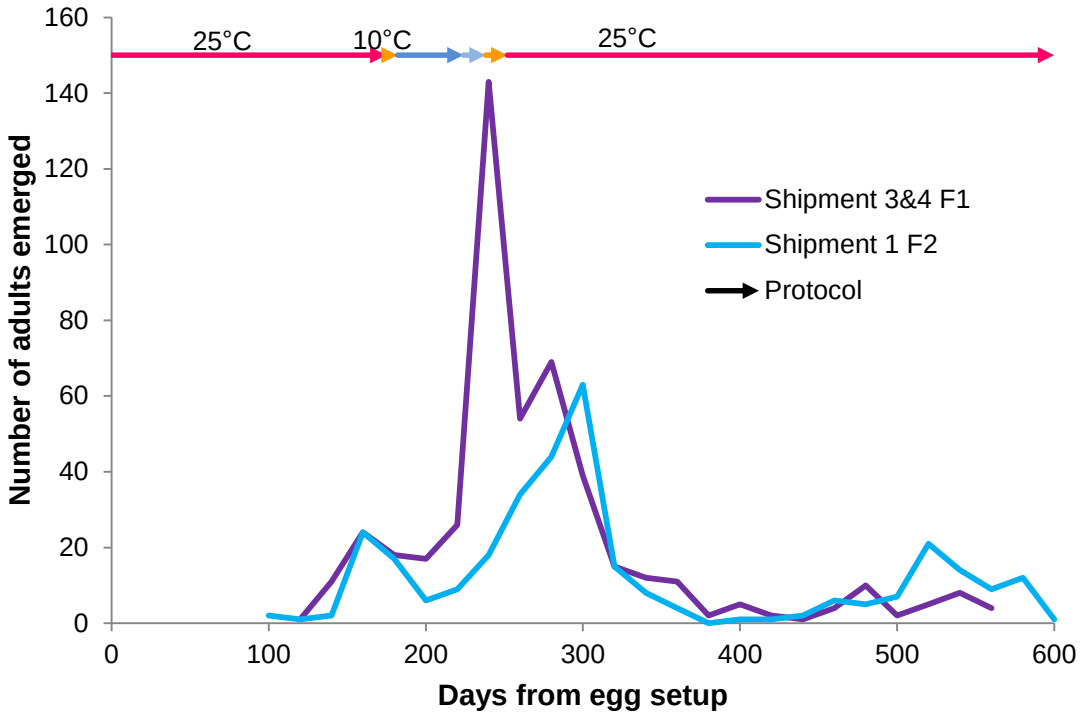


Figure 24. Emergence of adults as a function of days since egg setup.

Table 14 summarises the development of naturally-emerged adults of the Shipment 1 F_2 generation and Shipment 3&4 F_1 generation. The average development time for the bulk of population in the main peak (between 200 and 380 d) was 252 d.

Table 14. Development time of *B. bubalus* from egg to adult according to whether they are early, main or late emergers.

	n	Proportion of population	Mean . (wk)	Range days (wk)
Early	47	0.11	154 (22)	122-179 (17-26)
Main	347	0.81	252 (36)	180-379 (26-54)
Late	33	0.08	486 (69)	380-556 (54-79)

Emergence trays containing *B. bubalus* faecal shells or broods were checked every few days for adult emergences. Over the period of beetle emergences, newly emerged adults (up to 3 pairs) were either set up directly into 5 L buckets, containing sand/vermiculite mix and supplied with dung weekly in the hope that oviposition would commence shortly thereafter or set up (up to 20 beetles) into clear plastic boxes (26 x 19 x 6 cm) containing dry vermiculite and supplied weekly with dung. The latter method was also used to maintain beetles that had emerged through the vernalisation protocol period. Its advantages were that it kept beetles clean of parasites, required little energy from the beetles to access food (no tunnel building), mortality was low and reduced the weekly work load. The method was adopted for later emergences as it was found that it took considerable time for beetles in the 5 L buckets to start to oviposit and beetle mortality was increasing. It was also considered that females were not finding suitable mates and the feeding box method allowed larger numbers of males and females to mingle and potentially mate prior to being set up in oviposition containers. On analysis, the number of weeks post emergence before beetles started to oviposit was very similar between the two methods, 7 wk for the direct method as opposed to 6 wk for the feeding box method

(Table 15) but the survival was higher using in the feeding box method. For that reason, the feeding box method was adopted.

Table 15. Pre-oviposition time for *Bubas bubalus* adults after emergence. Beetles were either set up directly into oviposition containers (direct) or placed into containers to feed only post emergence (feeding box).

	Minimum weeks to oviposition	Average minimum # weeks to oviposition
Direct method	4 - 12	7
Feeding box method	4 - 9	6

Because of the wide variation in development time from egg to emergence of the adult post-vernalisation, generation time is also extremely variable. Table 16 summarises the generation times for early, main and late emergers.

Table 16. Time in weeks for a generation (first egg to first egg) of *Bubas bubalus* reared at 25°C for 23 wk, followed by vernalisation protocol (12 wk), awaiting adult emergence at warm temperatures and followed by a pre-oviposition period.

	Emergence group		
	early	mid	late
Egg to adult emergence	17-26	26-54	54-79
Pre-oviposition period	6-7	6-7	6-7
Total	23-33	32-61	60-86

3.4.2.1.4 2014 Importation Shipment 6: Rearing of F_1 generation

Given the relatively poor survival of the offspring of the 2013 importations, it was decided to attempt another importation in May 2014 to bolster the numbers of eggs in the rearing colony. However, to make that a worthwhile exercise, it was necessary to address the issues of poor immature survival and low fecundity.

Although no *B. bubalus* were reared in quarantine as happened with *O. vacca*, it is not unreasonable to think that the maternal gift (small particles + secretions + microbiome) may be important (see section 3.4.3) and indeed more important for small larvae of *B. bubalus* than it appears to be for *O. vacca*, given the apparently low survival of young F_1 larvae from the 2013 shipment (Table 17.).

Therefore the assumption for rearing the larvae from the 2014 importation was that the maternal gift is important, and so maternal gift from Australian dung beetles was provided for each egg released from quarantine. This was done by putting a small amount of thawed dung (prepared for man-made brood balls) into each well of a 48-well tissue-culture plate onto which was put a portion of the brood cell wall of *O. binodis*, or if those were not available, some of the gut contents of older *B. bubalus* larvae that had previously been fed *O. binodis* maternal gift. From mid-August 2014 the eggs were provided with maternal gift from *Bubas bison*, which had been brought into culture for the purpose. Only the larvae that had hatched and fed well (Figure 25) were then transferred into the man-made balls (60 ml) along with the contents of the well. The hole in the ball was then sealed and the balls buried in moistened vermiculite.

The boxes were closed and put into a fine mesh bag to exclude sciarid flies. The boxes were then placed into a constant temperature room at 25°C and 14:10 LD photoperiod. When it became clear that the *B. bubalus* brood balls would have to buried in the field in the spring of 2014 rather than being kept until release as adults in spring 2015, the temperature was reduced to 20-22°C on 5/9/2014 to slow down the development of the larvae, so that they would be developmentally closer in synchrony to the season when they were released.

Figure 25. Forty-eight-well tissue culture plate used for inoculating larvae with maternal gift. Note egg at top left which is dead, many well-fed larvae and the well in bottom right showing the dung material that is being repositioned by the larva to cover itself. Larvae were transferred to man-made brood balls at this stage of development.

A total of 4940 eggs were set up in the 48-well plates, from which 3491 larvae were transferred to artificial brood balls (78%). The first 203 eggs that were not given any maternal gift were set into artificial balls but left open to watch development. Of these 78 developed to larvae that could be sealed into the ball and buried (38%).

Brood balls were then allocated to different release sites, with a range of ages of larvae sent to each location. The brood balls were buried in the field between September and November 2014 (see section 3.5) so whether or not the maternal gift made a difference could not be determined.

3.4.2.1.5 Mass Rearing Colony: 2012 F_2 and 2013 F_1 adults

As with *O. vacca*, adults from different importation years were combined to form the mass-rearing colony.

To decrease the workload compared to rearing by pairs, up to 20 (10 male, 10 female) adults were put into 20 L buckets (Figure 8) filled with tamped down moist

vermiculite and fed twice-weekly as needed. After 3 wk on average, the buckets were tipped out and the natural brood masses were collected. An estimate of the number of brood masses was made, but this was complicated by the fact that after 3 wk, the bottom third of the buckets was packed with brood masses. The brood masses were transferred to the standard black tote boxes and covered with moist vermiculite. The boxes were then closed and put into a fine mesh bag to exclude sciarid flies. The boxes were then placed into a constant temperature room at 25°C and 14:10 L:D photoperiod.

When it became clear that the *B. bubalus* brood masses would have to buried rather than keeping them until 2015, the room was turned down to 20-22°C on 05/09/2014 to slow down the development of the larvae, so that they would be developmentally closer in synchrony to the season when they were released.

The brood masses were buried in the field between September and November 2014 (see section 3.5).

3.4.2.1.6 Cross-generational comparisons of laboratory rearing

Table 12 and Figure 26 show the basic data for the rearing of *B. bubalus* over the life of the project. A few key things stand out. Firstly, the fecundity of imported beetles is much higher in quarantine than in the laboratory, a situation mirrored by *O. vacca*. However, the number of brood masses produced by the mass rearing colony in 2014 was a great improvement over the prior years with eggs per female approximately half that of quarantine parents. Secondly, survival from egg to adult was only 13-25%, with infestation by sciarid flies probably an important factor. Because it was necessary to bury the brood masses in 2014, it was not possible to see whether or not the improvements in sciarid fly control and the microbiome treatment had a positive effect. However, it was noted when burying the brood masses, that there were a great many faecal shells present, suggesting that survival was good.

3.4.2.1.7 Generation Time for *Bubas bubalus*

As discussed above, there great variability in the time for emergence of adults after development and vernalisation. Nevertheless, some generalisations can be made. Using the rearing protocol of 23 wk at 25°C followed by vernalisation period of 12 wk, 80% of adult emergence (the main group) occurs 26-54 wk (mean = 36 wk) after egg lay. It then takes an additional 6-7 wk of feeding before oviposition starts, for a minimum of 32 to a maximum of 61 wks (mean 42-43 wk) from egg to first egg. It appears impossible to rear tightly-defined generations and so the only way to rear is to accept that the colony will consist of all stages at all times and if a synchronised population is required, for example for field release, then it should be done by lengthening the cold period to slow down some of the population.

Table 17. Cross-generational comparisons of rearing of *Bubas bubalus.*

Generation	Dates (1st egg to last adult)	Eggs set up	Faecal shells	Emerged adults	Survival egg - adult	Avg. Days egg – adult (range)	Adults set for oviposition	Brood masses	Production per female	Comments
2012 Importation (Shipment 1)										
F_0	31/5/2012 – 27/9/2012	-	-	-	-	-	385 *186* ♀ *199* ♂	2769	14.9 brood masses ~29.8 eggs† 21.0 eggs actual	400 beetles imported
F_1	3/4/2013 - 4/4/2014	3802	na	493 *258* ♀ *235* ♂	13%	304 *(149-456)*	all	650	2.5 brood masses ~5.0 eggs†	Includes vernalisation treatments A & B. Some F_1 brood masses fly infested. Not all females became fecund (esp Group A) see Table 9
F_2	na	650 broods ~1300 eggs†	500	326* *174* ♀ *152* ♂	25%*	314 *(84-589)*	na	-	-	4% of brood boxes infested by sciarid fly so faecal shells retrieved. 92 faecal shells still awaiting emergence*. Adults set for oviposition and egg production under Mass Rearing Colony
2013 Importation (Shipments 3&4)										
F_0	27/5/2013 - 10/10/2013	-	-	-	-	-	447 *210* ♀ *237* ♂	1688	8 brood masses ~16.1 eggs† 13.1 eggs actual	469 beetles imported, of which 447 survived to set up
F_1	na	2680	861	483* *259* ♀ *224* ♂	18%*	262 *(113-556)*	na	-	-	40% of brood boxes infested by sciarid fly. 33 faecal shells still awaiting emergence*. Adults set

										for oviposition and egg production under Mass Rearing Colony
Generation	Dates (1st egg to last adult)	Eggs set up	Faecal shells	Emerged adults	Survival egg - adult	Avg. Days egg – adult (range)	Adults set for oviposition	Brood masses	Production per female	Comments
Mass Rearing Colony: Combined 2012 F_2 and 2013 F_1 adults										
2012 F_2 and 2013 F_1 adults	28/3/2014 - ongoing						658 *362* ♀ *296* ♂	1646*	4.5 brood masses ~9.1 eggs†	No rearing containers with sciarid flies. 1583 brood masses released into field. 281 retained because they were insufficiently synchronised.
2014 Importation (Shipment 6)										
F_0	12/5/2014 – 21/10/2014	-	-	-	-	-	779 *377* ♀ *402* ♂	3917	10.4 brood masses ~20.8 eggs† 15.1 eggs per ♀ dissected out	785 beetles imported, 773 set up for oviposition. 5867 eggs released from quarantine
F_1	na	5710	-	-	-	-	-	3983	-	Eggs set up in 48-well plates, 3983 larvae were transferred to brood balls. Only 1 container of 66 was infested with sciarids. 3696 brood masses released into field. 287 retained because they were insufficiently synchronised.

* = data to 5/01/2015; †= estimate based on 2 eggs per brood mass; na = not applicable

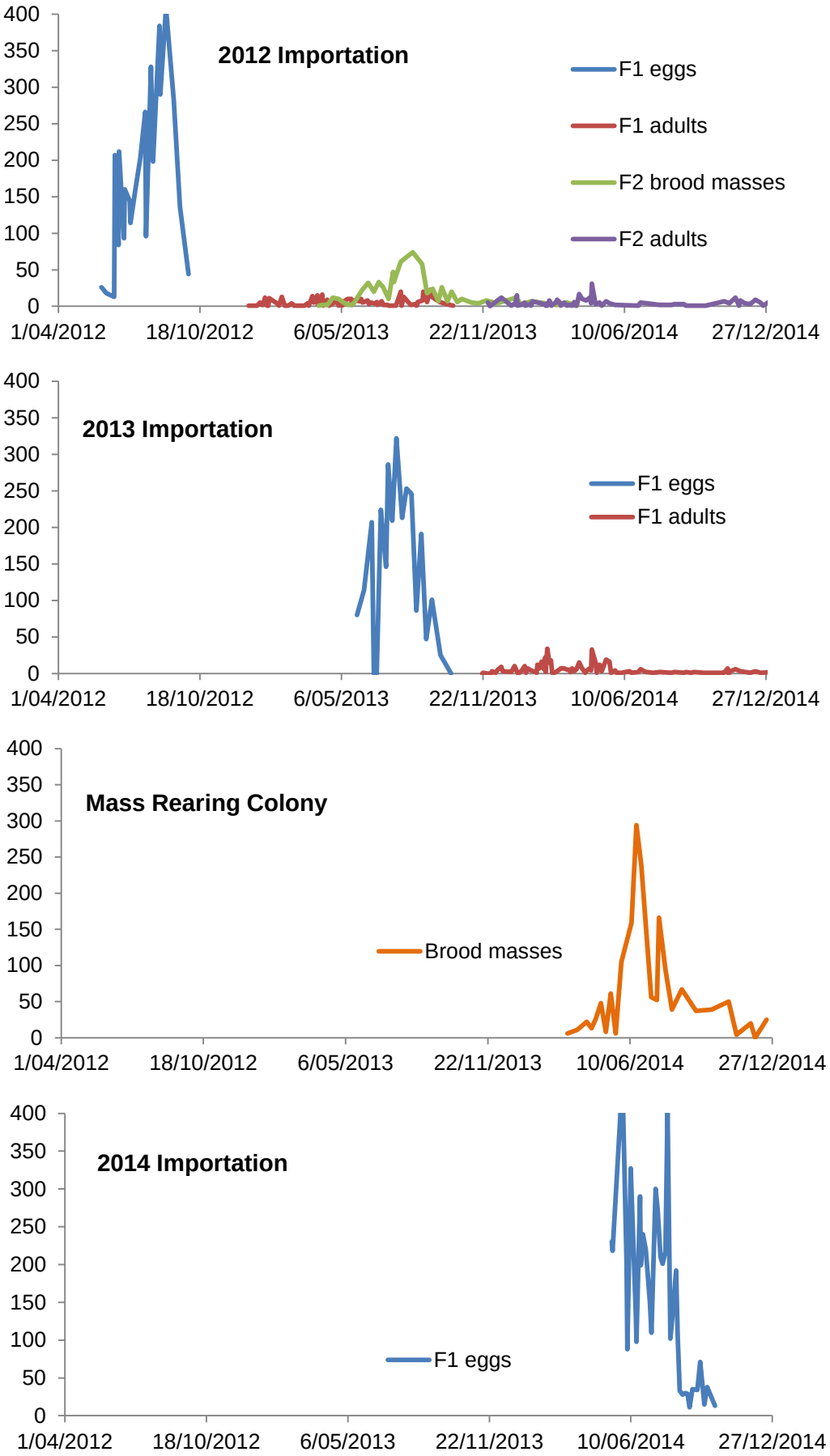


Figure 26. Plots of eggs and adults of each generation of *Bubas bubalus.*

3.4.3 The question of the microbiome

It is now known that gut microbes assist all manner of species to digest their food. Dung beetles also have a suite of microorganism in their guts, termed the gut microbiome, that are assumed to assist in digesting cellulose and other components of dung. Dung beetles transfer this microbiome from mother to offspring by lining the walls of the brood chamber with secretions from the mouth, anus or both. These secretions (termed the maternal gift) are then fed upon by the newly hatched larva and the gut microbiome is established in the next generation. The maternal gift is composed of small partly-digested particles, as well as microbes and other unknown components.

Figure 27. Egg of *O. vacca* in its natural brood chamber, perched upon some maternal gift provided by the mother beetle.

Due to the strict quarantine protocol that surface-sterilises the outside of the dung beetle egg, all of the beetles reared from those eggs lack the maternal microbiome. The question was whether this would explain the poor longevity and fecundity of the lab-reared beetles, especially *O. vacca*.

Two recent papers explore the question of the dung beetle microbiome. Estes *et. al.* (2013) analysed the DNA from maternal gift and larval gut contents from *Onthophagus taurus* and showed that the same kinds of microbes were present in both, indicating a specific transmission from mother to offspring via the brood ball. However, Byrne *et. al*. (2013) showed that for *Euoniticellus intermedius*, it was the small particle size that was important to the developing larva rather than the microbiome itself. Unfortunately, adult longevity and fecundity were not recorded in the latter study, so that particular question was not addressed.

Two experiments were performed to investigate the issue.

3.4.3.1 Genomics Experiment

Maternal gift samples were collected from brood balls made by

- *O. vacca* in quarantine (which would have the natural European microbiome) (samples from 2 females)
- *O. vacca* in the laboratory culture (which would be lacking the European microbiome due to the surface-sterilisation of the eggs)
- *Onthophagus australis* in the laboratory (an Australian native species collected from the field in Yass, NSW and presumably having the native Australian microbiome)
- *Onthophagus taurus* in the laboratory (an introduced but established species, collected from the field in Yass, NSW and presumably having a microbiome that it acquired since its introduction via surface-sterilised eggs many years ago)

All five maternal gift samples and a control dung sample (from dung that had been fed to the beetles and used by them to construct the brood balls) were sequenced. All DNA fragments were sequenced in two directions, and the two datasets from each sample were analysed separately as pseudo-replicates to check that consistent results were obtained. For this first pass analysis, 100,000 reads were taken from each of the twelve samples and assigned taxonomy against a widely-used bacterial 16S rRNA gene database. These taxonomic assignments were converted into taxon-by-sample matrices at the rank of Phylum, Class, Order and Family (i.e. there is one matrix for each rank that can be analysed separately). The relative abundances of taxa across samples were visualised through heatmaps and the beta-diversity relationships shown with non-metric multidimensional scaling (NMDS) ordinations on untransformed Bray-Curtis distances.

The classes of bacteria found in each of the samples of maternal gift and dung is shown in the heatmap illustration (Figure 28). The dung ball samples contain bacterial DNA, and these communities are dominated by ruminant gut micro-organisms. All of the mother's gift samples are more similar to each other than to the dung sample. There are large differences in abundance of specific Classes between mother's gift samples. In particular, *Cytophagia* and *Deltaproteobacteria* appear to be more abundant in the quarantine samples, i.e. the European microbiome. Bacterial communities in the two field-collected species (*O. taurus* and *O. granulatus*) are the most similar, followed by the two quarantined samples. The *O. vacca* sample brought out of quarantine shares similarities with both pairs of samples.

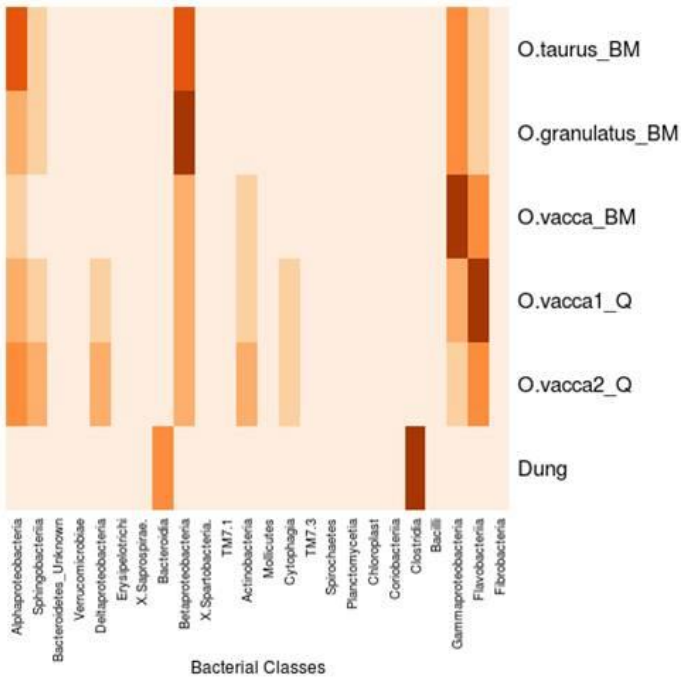


Figure 28. Heatmap showing the classes of bacteria found in each of the maternal gift samples.

Figure 29 shows the ordination plot of the data. In this figure, the placement of samples represents the difference in bacterial community composition. In general, the closer two samples are, the more similar the bacterial communities. The two replicates from each sample appear close to each other, which is a useful check that the results are somewhat representative of the sample diversity. The dung sample was not included in this figure because it is so distinct that it obscures the relationships between the remaining samples.

The x-axis represents the vector that explains the most variance in beta-diversity. This shows that the *O. vacca* samples brought out of quarantine is more similar to the two “wild” species (Figure 29). The y-axis explains the second-most variation, and it separates the laboratory-reared *O. vacca* sample from the four other samples, indicating that it has some similarities and differences to both pairs of samples.

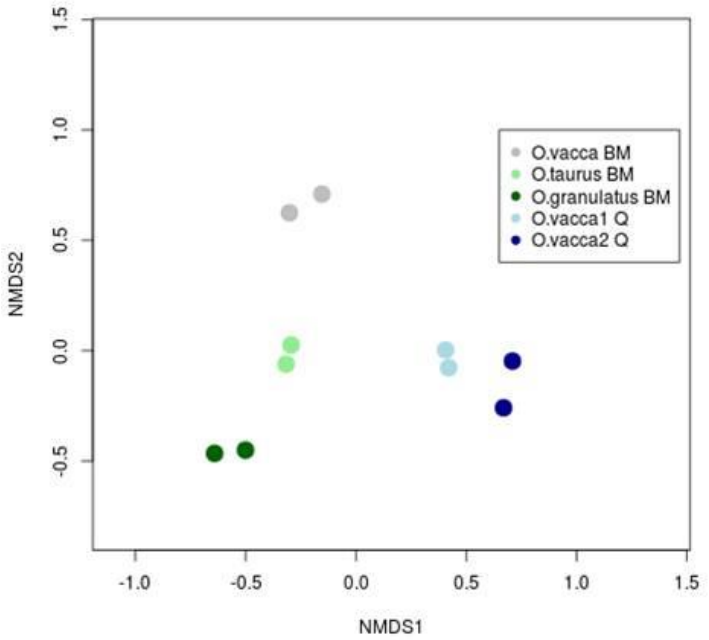


Figure 29: Ordination plot of the bacterial community composition

3.4.3.2 Life history experiment using *Onthophagus binodis*

This experiment was done using *O. binodis* because it is much faster to rear than *O. vacca* and does not need vernalisation and therefore could produce a quick answer to whether or not the microbiome made a large difference to life history traits.

O. binodis eggs from recently collected field beetles from the field were dissected from natural brood masses and surface-sterilised according to the quarantine protocol. They were then set up in man-made brood masses (thawed pressed/mixed brood dung not autoclaved) with the following treatments:

1. Control: man-made balls only, eggs inserted into brood hole
2. Maternal Gift: Half a hemisphere of *O. binodis* brood chamber wall inserted into brood hole in man-made brood ball, egg put on top.
3. Small Particles: Each man-made ball with 250µL of dung ball particles <150µL (prepared by blending dung ball dung and passing through a series of fine mesh sieves) added to the brood hole, egg put on top.

Balls were set up in flat black trays in moist vermiculite, with holes open to see hatch of eggs. Once all had hatched, note was taken of dead eggs and balls with live larvae were sealed and buried. Beetles were incubated at 25°C for development of immatures and emergence of adults. Emerging adults were sexed and pronotum widths measured. Adults were set up in 5L buckets according to treatment and fed. Once the beetles were feeding actively, and were ready to oviposit, they were set up in oviposition containers and adult longevity and fecundity were measured. The experiment was repeated 5 times.

Table 18 and Figure 30 summarise the data for the 5 replicates of the experiment. There was so much variability that none of results are statistically different except for eggs per female and longevity of adult females. In these cases, the control was significantly different from the particle and maternal gift treatments. The survival of the control dropped very quickly compared to the other treatments and the brood

mass production therefore also fell. This is similar to the situation with the short survival of *O. vacca* adults.

Table 18. Summary of results from *O. binodis* microbiome/ life history experiment.

Treatment	Eggs	Larvae	Adults			Survival	
			Female	Male	Total	Egg - Larva	Egg- Adult
Control	114	82	30	35	65	0.72	0.57
Particle	107	79	42	22	64	0.74	0.60
Maternal gift	107	86	37	39	76	0.80	0.71

Treatment	Thorax width (mm)		Mean Development time (d)		♀ set for ovipos-ition	Egg / ♀	Mean d survival of adult ♀
	♀	♂	♀	♂			
Control	6.4	7.0	57.3	56.9	28	2.8	39.6
Particle	6.6	6.9	54.8	56.6	32	6.6	53.6
Maternal gift	6.8	7.2	53.2	55.7	34	7.1	49.9

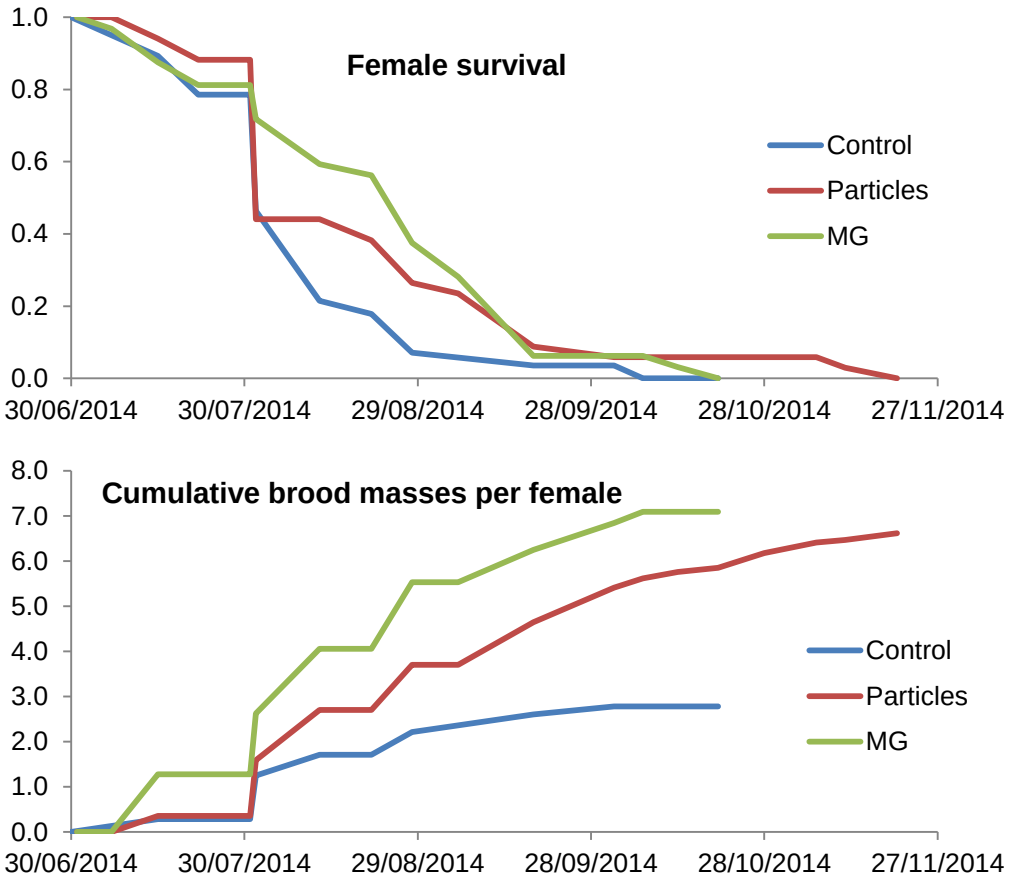


Figure 30. Female survival and cumulative brood mass production of adult *O. binodis* reared from surface-sterilised eggs reared in artificial brood balls with the addition of *O. binodis* maternal gift or small dung particles compared to the control.

3.5 Field releases

3.5.1 Selection of sites

Although there were many offers of release sites from interested property owners, many were located in climatic areas not well suited to the beetles. To maximise the chances of success, sites had to be selected in areas predicted to be most suitable. Sites were chosen in consultation with local experts; WA Department of Agriculture, Bernard Doube (Dung Beetle Solutions) for SA, John Feehan (Soilcam) for NSW. The chosen sites were on properties whose owners had previously collaborated in dung beetle research and were prepared to forgo the use of parasiticides (except for moxidectin), and ensure that cattle were in the release paddocks during beetle emergence period in early spring.

3.5.2 Beetle life stage at release

The plan was to release adult beetles that were sexually mature and physiologically synchronised with the local season and this was done with *O. vacca*. However, to complete the project, it was necessary to release *B. bubalus* and some *O. vacca* as immatures in their brood masses (Table 19), but still moderately-well synchronised to the season. In the case of *O. vacca*, eggs that had been released from quarantine September – November were buried as brood masses because they would be expected to be brood masses in Australia spring. In the case of *B. bubalus*, brood masses from eggs laid from May to October 2015 were buried in between September and November 2014. The earlier ones were somewhat advanced in development compared to the expected life cycle in Australia, but most of them were close to synchrony. Given the long developmental period and the fact that larval development time in this species is extremely variable, it is expected that the advanced ones will still survive and emerge on schedule on the spring of 2015.

Table 19. Release data for both species 2014.

Species	State	Location	Stage	No. *	Date	Releases made by
B bubalus	NSW	Cootamundra	immature	996	24/09/2014	CSIRO
	NSW	Howlong	immature	619	9/09/2014	CSIRO, J Feehan
	WA	Jingalup 2	immature	1185	22/09/2014	WA Dept Agric
	SA	Bool Lagoon	immature	390†	26/11/2014	B Doube
	SA	Port Elliott	immature	425†	20/11/2014	B Doube
	SA	Strathalbyn	immature	500†	14/11/2014	B Doube
O vacca	NSW	Cootamundra	adult	242	24/09/2014	CSIRO
	NSW	Yass	immature	161	18/10/2014	CSIRO
	SA	Bool Lagoon	adult	80†	18/10/2014	B Doube
	SA	Port Elliott	adult	80†	16/10/2014	B Doube
	SA	Strathalbyn	adult	76†	22/10/2014	B Doube
	SA	Strathalbyn	immature	150†	22/10/2014	B Doube
	WA	Jingalup 1	adult	132	19/08/2014	WA Dept Agric, CSIRO

* Number of adults or number of brood masses (B. bubalus generally having 2 eggs per natural brood mass); † Releases made into field cages.

3.5.3 Method of release of adult *Onthophagus vacca*

Beetles were released onto a fresh pad of dung and covered with a beetle-proof cage that was pegged down (Figure 31). After 1 week, additional fresh dung was added to each cage. After another week, the cage was removed and beetles were free to disperse.

Figure 31. Method of releasing *Onthophagus vacca* adults onto fresh natural dung pads and covering with a beetle-proof cage.

3.5.4 Method of release for brood masses

For release of immature beetles, trenches were dug to a depth of 40 cm for *B. bubalus* and 20-30 cm for *O. vacca* (Figure 32). The trenches were placed on a slope of ground where they were unlikely to be inundated in heavy rain. Details of the trenches varied with site, but were about 2 m long for *B. bubalus* and only 40 cm long for *O. vacca*. In some cases, agricultural pipe was laid into the bottom of the trench with a tail projecting past the main trench to draw any excess water away. When this was used, soil was added to cover the pipe before the brood masses were placed on top. The hole was then filled in with field soil to about 10 cm below the ground level. Heavy wire mesh was placed over the top to discourage foxes from digging up the brood masses and filling of the trench was completed (Figure 32).

In some cases, particularly for *O. vacca* which were buried less deeply, heavy open-mesh cages were placed over the trench to prevent cattle from walking over the trench.

Figure 32. Burial of *Bubas bubalus* brood masses. Top left, trench showing brood masses being laid in the trench and the tail containing agricultural drainage pipe to draw excess water away; Top right, trench partly filled in with weldmesh laid over before final back-filling; Bottom, the team with property owners lined up behind boxes of brood masses to be buried at the Cootamundra site.

3.5.5 Monitoring and follow-up

In the original proposal for this project there was no provision for follow-up monitoring because previous experience suggests that no beetles will be recovered for several years after release. The exception to this would be the field rearing being undertaken by B. Doube, of Dung Beetle Solutions. Nevertheless, there is great interest in seeing when the beetles have established and the cooperating landowners and collaborators will be watching carefully. The project leader will make a point of contacting all cooperators annually and making site visits whenever possible during early spring.

3.6 Handover to private concerns

The proposal was to give some starter colonies to private companies, landowner groups or government departments, once a number of strategic releases had been made across the predicted ultimate distribution of the beetles.

Adult *O. vacca* and larval *B. bubalus* were taken to WA to be released with/by the WA Department of Agriculture.

Adult and immature *O. vacca* and immature *B. bubalus* were given to Dung Beetle Solutions (Dr Bernard Doube) to initiate field rearing near Penola and on the Fleurieu Peninsula in SA.

In both cases, there was a good day of discussion between the CSIRO team and collaborators before the handover.

Discussion / Conclusion

3.7 Success of methods and rearing

The system of surface-sterilisation of eggs was very successful, with very low mortality of the eggs from the process, compared to the very high mortality incurred in previous work due to the use of formalin as a sterilant (e.g. Steinbauer and Wardhaugh 1992).

Rearing in quarantine was also successful, despite the cramped conditions. Egg production by imported adults was excellent and adult longevity far exceeded what would have occurred in nature.

A number of methods were tested for inoculating surface-sterilised eggs into man-made brood balls. It was clear very quickly that brood balls had to be buried as soon as possible to avoid fungal growth. Although it was labour-intensive, the system of setting up eggs in 48-well plates to hatch and then transferring only well-fed larvae to brood balls meant a smaller number of balls was required, thus reducing the time and effort needed for their production of balls. Also, the survival in the balls was higher, resulting in fewer brood containers being needed and less space required in the CT rooms.

After some experimentation, the 1:1 mix of plasterers' sand and fine vermiculite was found be the best medium for oviposition because, the beetles could dig self-supporting tunnels in this medium, when at a suitable moisture content, and the abrasive nature of the medium assisted in suppressing populations of nematodes.

Sciarid flies were especially destructive to *B. bubalus* brood masses, and caused significant mortality to the immatures in 2012 and 2013. The adoption of multiple pest control measures and scrupulous adherence to hygiene procedures meant that almost no brood containers were infested in 2014.

3.8 *Onthophagus vacca*

The key elements of the most successful vernalisation protocol (9, (Table 6) were an ample time at 25° for newly-emerged adults to feed and build up fat body before the cooler temperatures, a substantial cold period at 10°C, and a period of feeding at 25°C in dry vermiculite before set-up for oviposition.

In an attempt to maximise mass-rearing potential, many different options were tried to speed up the development time and reduce the period of the vernalisation protocol to achieve 2 generations per year. Taking into account trade-offs such as larval survival, adult size, longevity and fecundity, the best outcome achieved was to rear *O. vacca* from egg to first egg in 27-32 wk by rearing the larvae at high temperature (25°C) and using vernalisation protocol 9. The substantial variability in time for development from egg to adult meant that each succeeding generation became less and less well-defined, until most stages were present at all times (Figure 15, top graph). From a mass-rearing perspective, this is not necessarily a problem, as it evens out the workload associated with colony maintenance. However, if it is necessary to synchronise all the individuals of a colony, for example when preparing for field-releases, the way to do it would be to lengthen the cold period, as attempts to shorten the generation time further were not successful.

The unsolved issue with rearing *O. vacca* remains the longevity and fecundity of adults. Whether this has to do with being stripped of its native microbiome is unresolved, as almost all *O. vacca* put into surrogate *O. binodis* brood masses were released into the field to make up the numbers to assist establishment. However, the microbiome experiment with *O. binodis* (see section 3.10), suggests that at least part of the answer lies there. Clearly, more work is required to solve this problem.

O. vacca field releases were made across southern Australia. Unfortunately, the number of adults synchronised to the season was not great, but all of them were released. In addition, all immature beetles were released that would be expected to emerge in the summer of 2014-15 then feed and dig underground to overwinter.

3.9 *Bubas bubalus*

B. bubalus has a much longer developmental time than *O. vacca*. It appears to have an inbuilt propensity for some individuals to complete development and emerge as adults quickly, whereas others take very much longer, up to two years. The data to date implies that it is related, in part, to the stage of development reached before the onset of cooler conditions in autumn and winter. The 2012 F_1 individuals that had an extra 60 d (Group B) at 25°C showed that longer development at high temperature produced a higher proportion of adults emerging quickly after vernalisation. In nature, these would equate to the eggs laid early in the season.

Data from rearing in 2013 (Figure 24) showed that when all individuals had a long period at 25C pre-vernalisation, there was still a range of development times, with some adults emerging before the vernalisation, most emerging immediately after the vernalisation and some that emerged very late. The small population of early emergers fits with the field observations in Europe that some *B. bubalus* adults emerge in autumn (if the soil conditions are suitable), then overwinter as adults. The main peak of emergence following the rise in temperature after the vernalisation protocol corresponds to the major emergence of adults in early spring which go on to oviposit after a suitable feeding period. The small but consistent group of adults that emerged 500-600 d after egg setup would correlate with a subset of the population that emerges the second spring.

The rearing programme used with development at 25°C for 23 wk followed by a vernalisation protocol of 12 wk produced a large emergence of beetles (over 80% of the population) that were ready to oviposit after a pre-oviposition feeding period of an additional 6-7 wk. This results in a minimum of 32 to a maximum of 61 wks (mean

42-43 wk) from egg to first egg. It appears impossible to rear tightly-defined generations and so the only way to rear is to accept that the colony will consist of all stages at all times and if a synchronised population is required, for example for field release, then it should be done by lengthening the cold period to slow down some of the population.

3.10 Microbiome

Results of the preliminary genomics experiments suggest that the European *O. vacca* microbiome is distinctly different to that of dung beetles in Australia, and that the microbiome that was picked up by the *O. vacca* in the laboratory culture was substantially different from the other two field-collected species tested (Figure 29). The rearing experiment with *O. binodis*, itself an introduced but very successful species in Australia, were suggestive of a role of the microbiome and/or initial feed of small particles in increasing fitness. As found by Byrne *et al.* (2013), there was no difference between development of larvae fed on small particles compared to maternal gift in relation immature survival, development time or adult size but there was a substantial difference between these treatments and the control in a number of parameters. Most importantly, the survival of adults and fecundity was much higher for either treatment compared to the control. This suggests that some of the difficulties with adult longevity and fecundity in the *O. vacca* culture may be explained by the fact that eggs were put into balls equivalent to the control in this experiment. This finding has relevance to the methods for rearing surface-sterilised eggs from quarantine in future.

3.11 Field release and follow up

Although the original intention was to release sexually mature, synchronised adults in spring, it was not possible during the life of this project and so all beetles that were even moderately synchronised with the season as adults or immatures were either released into the field by CSIRO and collaborators or set up in field cages by Dr Doube (Dung Beetle Solutions). Dr Doube will be following the field cage populations closely as part of MLA project ERM.0214.

Previous experience suggests that no beetles will be recovered for several years after release into the field. Nevertheless, there is great interest in seeing when the beetles have established and the cooperating landowners and collaborators will be watching carefully. Once populations have reached a sufficient size, a program of cropping and redistribution would be useful to speed up the dispersal across the predicted range. This may take up a decade; cropping and redistribution of *Bubas bison,* the introduced species most similar to *B. bubalus*, started 9 years after the original release. *O. vacca* is also univoltine, so it would be expected to take a similar time.

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Appendices

4.1 Communication

There were 3 key main periods of communication activity.
The first followed the media release on 6 June 2012 about the beetles being brought into quarantine. It generated many articles in country papers and interviews. The second was following the MLA Feedback Magazine article in May 2013. It generated a number of enquiries and dung beetles and offers of properties as release sites. The last was following the first release of adult *O. vacca* in WA in August 2014, which generated newspaper, TV and radio stories, as well as more offers of properties as release sites.

4.1.1 Articles/Media Releases prepared by CSIRO/MLA

Where	When	Detail
CSIRO's Rural Press insert	31/5/2012	They've dung it again. http://www.csiro.au/en/Outcomes/Climate/Rural-Press.aspx.
CSIRO MLA Media Release	6/06/2012	Far flung beetles here to finish the job
MLA Feedback Magazine	May 2013	Article: Dung Disposers' Debut
The Conversation (Online news analysis and commentary)	18/08/2014	French beetles flown in to clean up Australia's cattle dung https://theconversation.com/french-beetles-flown-in-to-clean-up-australias-cattle-dung-30367
CSIRO News Blog	25/08/2014	Spring forth and eat dung http://csironewsblog.com/2014/08/25/spring-forth-and-eat-dung/

4.1.2 Print and Internet

Where	When	Detail
Geeko System	07/06/12 01:05	Australia has Too Much Cow Poop, Imports Dung Beetles to Curb Crap Levels
Cowra Community News	07/06/12 10:50	Far-flung dung bugs to round
Daily Telegraph, Sydney	8/6/2012	Aussie flies are dung and dusted thanks to French beetles' bon appetit
Queensland Times, Ipswich QLD	8/6/2012	Improved job tipped with beetle
Daily Examiner, Grafton NSW	General News: 11 June 2012	Dung-hungry beetles spring out for a feed
Courier Mail, Brisbane	Insight: 09 June 2012	Dung their best
Fiji Times Online	09/06/12 08:58	French dung beetles to be unleashed in Aust

Countryman, Perth	General News: 14 June 2012	New dung beetle force
Meat & Livestock Australia	14/06/12 15:30	New dung beetle species roll up into Australia
Stock and Land	16/06/12 04:30	Foreign dung beetles improve pasture
Straight Furrow	16/06/12 04:30	Foreign dung beetles improve pasture
The Cattle Site	19/06/12 19:40	New Dung Beetle Improves Cattle Pastures
Warrnambool Standard, Warrnambool VIC	21/06/2012	New dung beetle species here
Waste Management & Environment, National	01/07/2012	New species of dung beetle could help store carbon
Cosmos Magazine	17/07/2012	What to do with too much poo http://www.cosmosmagazine.com/features/online/5803/on-a-roll?page=0%2C0
ABC News	18/08/2014	New dung beetle release to tackle fly problem in WA's Great Southern http://www.abc.net.au/news/2014-08-18/dung-beetle-to-tackle-fly-problem-in-wa-great-southern/5678864
West Australian	18/08/2014	Beetle faces crap job in WA wine region
Phys Org	26/08/2014	French beetles tackle Great Southern cattle dung http://phys.org/news/2014-08-french-beetles-tackle-great-southern.html
News.net	19/08/2014	New Species of dung beetle to be released in Great Southern, WA http://www.news.net/article/1781658/?referid=137
Sydney Morning Herald	21/08/2014	http://www.thesydneynews.net/index.php/sid/224964849/scat/88f7d0d02bea1b33
WA Today	21/08/2014	http://www.watoday.com.au/national/french-dung-beetle-release-in-wa-20140821-105v4p.html
The Tribune News (Indonesia)	19/08/2014	http://www.tribunnews.com/australia-plus/2014/08/19/kumbang-tinja-kurangi-jumlah-lalat-di-australia
Queensland Country Life	18/08/2014	Beetles spring into action in WA dung
Canberra Times	22/08/2014	In praise of the dung beetle
Countryman, Perth	21/08/2014	Countryman, Perth
Albany Advertiser	21/08/2014	Bug Release
Serpentine Jarrahdale Examiner	21/08/2014	Dung beetles to control flies
The Great Southern Weekender	21/08/2014	New beetle for fly battle
Esperance Express	22/08/2014	New beetle to beat the flies

Harvey Waroona Reporter	26/08/2014	High Hopes for dung beetle
Entomology Today	18/08/2014	New Dung Beetles are Coming to Australia http://entomologytoday.org/2014/08/18/new-dung-beetles-are-coming-to-australia/

4.1.3 Radio and Television

ABC 666 Canberra	15:00 News: 07 June 2012 03:03PM
ABC New England North West, Tamworth	06:30 News: 08 June 2012 06:33AM 08:30 News: 08 June 2012 08:33AM
ABC 666 Canberra	Breakfast: 11 June 2012 06:42AM
ABC North and West SA, Port Pirie	SA Country Hour: 11 June 2012 12:53PM
ABC New England North West, Tamworth	Rural Report: 08 June 2012 06:46AM
ABC Goulburn Murray, Wodonga	07:30 News: 08 June 2012 07:33AM
ABC Upper Hunter, Muswellbrook	Breakfast: 08 June 2012 06:46AM
ABC 720 Perth, Perth	07:45 News: 08 June 2012 07:54AM
ABC Ballarat, Ballarat	National Rural News: 08 June 2012 12:10PM
Radio National, Canberra	PM: 08 June 2012 05:16PM
ABC Central Victoria, Bendigo	Breakfast: 12 June 2012 06:40AM
ABC Central Victoria, Bendigo	Breakfast: 12 June 2012 06:40AM
ABC 612 Brisbane, Brisbane	Afternoons: 12 June 2012 02:26PM
8 regional ABC station in WA	18/08/2014 With Rob Emery, WA Dept Ag
720 ABC Perth	Drive radio 18/08/2014 16:34
ABC News Perth (TV)	TV, Early Edition 18/08/2014 17:12
GWN7 Perth (TV)	Golden West News 18/08/2014 17:40
ABC Perth (TV)	News 18/08/2014 19:18
ABC News 24 (TV) Capital cities except Darwin + regional WA, VIC, QLD	19/08/2014 13:29

4.1.4 Miscellaneous

Australian Science Teachers Association	June 2013	"A Century of Australian Science" poster and a web-based digital book for Australian teachers to promote participation in National Science Week Image of *Onthophagus vacca* on the cover and information on the dung beetle project in the resource material.