final report

Project code: B.NBP.0488

Prepared by: Peter James

The University of Queensland,
Queensland Alliance for
Agriculture and Food Innovation

Date published: October 2013

ISBN: 9781925045512

PUBLISHED BY
Meat & Livestock Australia Limited
Locked Bag 991
NORTH SYDNEY NSW 2059

In vitro culture of buffalo fly and infections with Wolbachia

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

Abstract

Buffalo fly, *Haematobia (irritans) exigua* infestations are consistently listed by northern beef and dairy producers as amongst their main cattle health problems. They cause irritation to cattle, reduce weight gains and decrease milk production. They also vector a filarial nematode *Stephanofilaria* sp. that causes skin lesions, downgrading skin quality and raising welfare concerns. In recent years buffalo flies have dispersed southwards and westwards and their range is projected to extend significantly with climate change.

Research into new methods of buffalo fly control has been limited by the need to maintain live cattle hosts. This is expensive, severely restricts the type of research that can be conducted and is increasingly problematic from an animal ethics standpoint.

This project has developed an *in vitro* rearing technique for buffalo fly whereby all stages can be grown in the laboratory without the need for a live animal host. The current strain has now been maintained for 33 generations. Availability of this strain will help maintain or improve the efficiency of currently available control methods, for example through provision of a characterised and consistent strain of flies for use in studies to detect and monitor resistance and for use in testing new candidate control compounds. It will also facilitate the development of potential new approaches to control such as techniques incorporating the use of *Wolbachia* and other biocontrols and the release of genetically modified strains.

We have also developed the first ever *Haematobia irritans* and *H. exigua* cell lines. Although the *H. exigua* cell line was lost during the Brisbane floods we expect that we will be able to re-establish this line using the method utilised successfully *for H irritans*. These cell lines will be critical to attempts to transfect *Wolbachia* into buffalo fly. They will also provide an important research resource in areas such as early screening and clarification of modes of action of new biocidal molecules, the use of viruses and baculoviruses for biocontrol, vaccine design, clarification of gene action and elaboration of intracellular biochemical pathways. Our finding that *Wolbachia* is not present in *H. exigua*, but is ubiquitous in closely related *H. irritans* suggests that *H. exigua* might also be a competent host for *Wolbachia* and indicates that investigation of this organism as a basis for new control strategies for buffalo fly is warranted.

Executive summary

Buffalo fly (*Haematobia exigua*) infestations are consistently listed by northern beef and dairy producers as amongst their main cattle health problems. They cause irritation to cattle, reduce weight gains and decrease milk production. They also vector a filarial nematode *Stephanofilaria sp*. that causes skin lesions, downgrading skin quality and raising welfare concerns. In recent years buffalo flies have dispersed southwards and westwards and their range is projected to extend significantly with climate change.

The main control options for buffalo fly (BF) include chemical treatments, breed choice and the use of dung beetles, but functionally control usually depends on chemical options. These suffer difficulties with the development of resistance, the potential for residues, occupational health and safety issues and environmental contamination. In addition, there are growing price premiums for commodities produced in low chemical systems. Research into the development of new and cheaper controls for BF has been limited by the need for cattle based rearing systems for the conduct of research. These are expensive, increasingly suffer animal ethics difficulties and limit the type of research that can be conducted. In this project we have developed two new tools, an *in vitro* BF colony and a *Haematobia* cell line that will significantly assist the development of new control options and in particular, the investigation of the potential use of *Wolbachia*, in novel control approaches for buffalo fly.

Buffalo fly colony
Early attempts at colonisation were based on methods used to rear closely related horn fly in the USA and a previous method used for BF in Australia. Despite more than 40 attempts over two years and change of many different parameters the colony persisted for more than 3 generations on only one occasion and in this instance only one fourth generation fly was produced. The main problem was identified as egg fertility with the hatch rate decreasing precipitously in laboratory raised flies. Dissection of female flies indicated a very low incidence of motile spermatozoa in the spermathecae of colonised flies, suggesting that failure to mate in the laboratory system was the main limiting factor. Despite extensive observation, BF were seldom seen mating in the cages whereas this is commonly observed in US colonies of closely related horn fly (HF) and in the other fly species that we maintain in our laboratory

Observations of BF on cattle indicated that mating occurred most often during low light conditions, after dawn and before dusk and most frequently on the lower flanks of cattle. A light regime simulating dusk and dawn was introduced into our rearing rooms and a curved strip of black cardboard used to provide a mating platform simulating the curvature of cattle bodies. Flies readily rested on the cardboard, particularly during the low light periods and mating was subsequently observed. These changes, in addition to a number of other modifications, most particularly the use of membrane feeders to provide a continual source of blood warmed to cattle body temperature have led to the development of a sustaining colony of buffalo flies that have now been maintained in our laboratory for more than 33 generations.

Availability of this strain will help maintain or improve the efficiency of currently available control methods, for example through provision of a characterised and consistent strain of flies for use in studies to detect and monitor resistance and for use in testing new candidate control compounds. It will also facilitate the

development of potential new approaches to control such as the utilisation of *Wolbachia* and other biocontrols and the release of genetically modified strains.

Buffalo fly cell line

Insect cell lines are now being widely used in both academia and by industry to facilitate the development of new insect control approaches. In particular, insect cell lines have been utilised in the introduction of Wolbachia into new host species In this project we have developed continuous *Haematobia* cell lines for first time in the world. *H. exigua* cells attached, grew well with a cell doubling time of 4-5 days, were grown in the laboratory for six months and were successfully frozen down and retrieved after thawing. It was considered that the cell lines were close to stability and that attempts at infection with *Wolbachia* could commence. However, this line was lost during power failures associated with the Brisbane floods in 2011-12.

In a subsequent collaboration with Dr Tim Kurtti and Dr Ulrike Munderloh at the University of Minnesota we have also developed a *Haematobia irritans (horn fly)* cell line. Eight sustaining *H. irritans* lines are currently being maintained. *H. irritans* is closely related to *H. exigua* (only relatively recently considered separate species) and *H. irritans* cells could potentially be used to transfect *Wolbachia* into *H. exigua* if necessary. However, we are continuing efforts in our laboratory to re-establish the *H. exigua* cell line using the techniques successful with *H. irritans*.

Insect cell lines, where available, are now frequently used for a range of purposes in addition to *Wolbachia* transfection. These include early screening and clarification of modes of action of potential new insecticides, production of and research into the use of viruses and baculoviruses as biocontrols, vaccine design, clarification of gene action and elaboration of intracellular biochemical pathways. Furthermore, once developed, cell lines can be frozen down and stored for extended periods. The establishment of a BF cell line will provide an important resource for future research towards new control approaches for BF.

Infection of buffalo fly with *Wolbachia*

Wolbachia is a maternally transmitted, intracellular bacterial symbiont or pathogen, currently of much interest around the world as a basis for innovative biocontrol programs. *Wolbachia* has a range of effects on reproductive success of insects as well as on a number of different fitness traits (e.g. longevity and mobility). It has also been shown to compromise the success of transmission of some insect vectored pathogens, including filarial nematodes. This may have implications for the transmission of *Stephanofilaria*, the presumed causal agents of BF lesions. We did not find *Wolbachia* in a survey of Australian buffalo fly populations, but it was widespread in closely related *H. irritans*. This suggests that *H exigua* will be a competent host and is a promising target species for the design of new control methods based on transfection with *Wolbachia*.

Table of Contents

List of Tables

List of Figures

1. Background

1.1 Buffalo fly impact

Buffalo flies (BF; *Haematobia exigua*) are a key pest in northern beef production systems, ranked first in a survey of animal health problems in 1992 (Bock et al. 1995). Similarly, in a survey of dairy farmers in the tick infested areas of Qld in 1997, BF were considered to be a greater concern than ticks with 55% considering them a significant problem, compared to 14% for ticks, and only 6% considering them not to be a problem, compared to 32% for ticks (Jonsson and Matchoss 1998). BF are functionally obligate permanent parasites of cattle living most of their lives on the backs of cattle and only leaving to oviposit in cattle dung. If uncontrolled, infestations may reach several thousands of flies, each feeding up to 40 times daily, irritating cattle and causing open suppurating lesions that potentially constitute a welfare issue (Figure 1).

Estimated reductions in growth caused by BF infestation have varied greatly, often because of difficulties in developing a robust experimental design. Bean *et al.* (1987) found that animals treated for BF gained weight 15%, 11% and 4% in the three years of their experiment and Spradbery and Tozer (1996) found that cattle treated with insecticidal ear tags gained 13 kg more body weight than untreated cattle over a 5 month period. Holroyd *et al.* (1984) reported improved rates of gain in weight from treatment in only two of four years, although they note that insecticide application in the treatment groups may have affected fly numbers in the controls and that a positive correlation between body weight and fly numbers may have obscured the beneficial effects of treatment. Results correspond approximately with those from studies with horn fly (HF; *Haematobia irritans*) in North America where improvements in weight gain of 8.3% (Kinzer *et al*. 1984), 8.7% (Harvey and Brethour 1979), 11% (Harvey and Brethour 1981) and 18% (Haufe 1982) were recorded.

Effects on skin quality have not been quantified in Australia. Lesions penetrate the dermis and render the affected areas unusable (Sutherst *et al.* 2006). They can cause significant wastage if they occur in valuable areas of the hide, but most occur near the edges of the skin where they can usually be trimmed with little loss. However, Guglielmone (1999) describes an increase in hide damage noted by tanneries that coincided with the introduction and spread of HF through Argentina and Uruguay. In experimental studies up to 50% of the hide was affected by black spots, pitted areas and semi-circular scars resulting from the feeding of BF. Similar effects have not been quantified in Australia, but could contribute to lower hide value from northern cattle (Sackett *et al.* 2006).

The lesions associated with BF infestation are often the key concern of growers, largely because of the appearance of the raw and suppurating lesions, the irritation they cause and the welfare issues they present, but also because of effects on marketability of cattle. BF lesions could also provide a prime focus for screwworm strikes in the event of a screwworm incursion. Old world screw-worm fly is endemic in a number of Australia's northern neighbouring countries and represents a major exotic pest threat to our extensive livestock industries. Dry crusty lesions as well as raw open lesions can be struck and BF lesions would provide abundant breeding sites facilitating the establishment of screw-worm in Australia following an incursion.

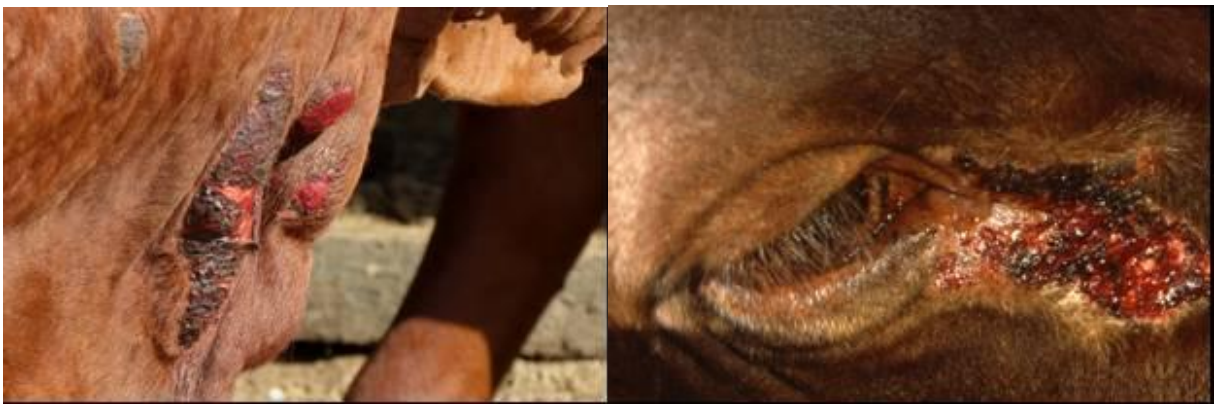

Figure 1. Buffalo fly lesions in Droughtmaster cattle

In addition, the irritation caused by BF feeding are said to make cattle with heavy fly burdens more difficult to muster than those with low fly numbers and the high fly numbers often present on bulls may interfere with mating (Holroyd *et al.* 1984). A secondary effect may be damage to infrastructure from the effects of cattle rubbing.

Sackett *et al.* (2006) estimated the cost of buffalo flies as $78m annually in an average year; clearly costs are much higher than this in wet years with high BF challenge.

1.2 Southward spread of buffalo fly

Although considered a subtropical pest, recent wet years have seen infestations spread as far south as Maitland, Dubbo and Narromine and as far west as Bourke. Climate change may see significant further spread of BF southwards with predictions using the CLIMEX model suggesting the possibility of establishment of persistent populations throughout the moist coastal belt of NSW, in southern SA and south west WA, with more extensive incursions spreading from these foci during warm wet periods (Figure 2).

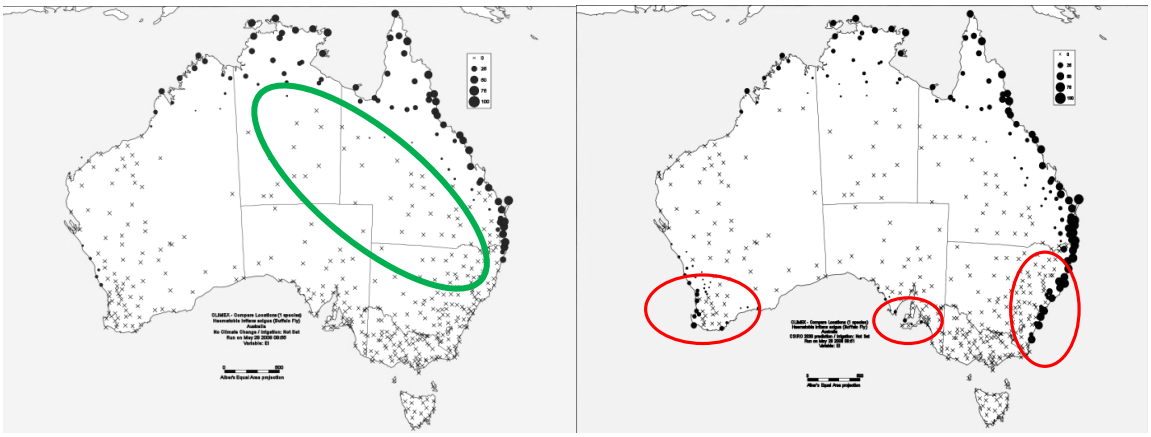

Figure 2. Current location and potential spread of resident populations of buffalo fly by 2030 under current predictions for climate change (CLIMEX model, Sutherst and Maywald)

1.3 Control

Recommendations for BF control normally include the use of dung beetles, BF traps where practicable, breed selection in some instances, culling allergic cattle and chemical treatments. Functionally fly control generally depends mainly on chemical options. However, control of BF is increasingly compromised by the development of resistance and there is an ever growing market demand for meat and milk produced in low-chemical systems. Alternative means of controlling BF are needed.

1.4 Wolbachia

Biological control, particularly classical inoculative biocontrol control where the agent is introduced and is expected to spread and persist, has many advantages. Biocontrol avoids the potential disadvantages associated with chemical approaches such as resistance, residues, occupational health and safety issues and environmental contamination. Biocontrols are more specific, usually targeting only the pest insect and avoiding non-target effects. In addition, cost is generally lower, particularly for producers. Initial research and release entails a cost, but after introduction the agent is self perpetuating and is expected to persist and spread without further cost inputs.

Wolbachia is a maternally transmitted, intracellular bacterial symbiont or pathogen, currently of much interest around the world for use in innovative biocontrol programs. The success of the high profile *Wolbachia* research program toward the control of dengue fever, transmitted by the mosquito *Aedes aegypti* (McGraw and O'Neill 2013), has heightened this interest. *Wolbachia* has a range of effects on reproductive success of insects as well as on a number of different fitness traits in some insects (e.g. longevity and mobility). It has also been shown to compromise the success of transmission of some insect vectored pathogens, including filarial nematodes (Kambris *et al.* 2009). This may have implications for the transmission of *Stephanofilaria*, the causal agents of buffalo fly feeding lesions. Importantly *Wolbachia* is able to 'drive' itself through insect populations, removing the need for multiple or widespread releases often required for the successful establishment of other biocontrol agents.

As *Wolbachia* is transmitted maternally in the eggs of infected females, establishment of a stable infection requires infection of germinal tissue. Natural horizontal spread between insect species is not known to occur. However, successful transfection and establishment of *Wolbachia* in new hosts has been successfully achieved by adaptation in cell lines derived from the intended host and then microinjection of infected cells. Surveys of buffalo fly populations reported here and published in Zhang *et al.* (2009) indicate that *Wolbachia* is not present in *H. exigua*. However, to date, all populations of *H. irritans* tested have been positive for *Wolbachia* (Jeyaprakash and Hoy 2000; Floate *et al.,* 2006; Zhang *et al.* 2009), suggesting that closely related BF would also be a competent host. This suggests potential for utilising *Wolbachia* in new strategies for controlling BF directly or, alternatively, the transmission and effects of *Stephanofilaria* nematodes vectored by BF.

1.5 Buffalo fly rearing

Historically most research with BF has been conducted with field collected flies or a semi-*in vitro* system using live cattle hosts to rear the adult stage of the fly and an *in vitro* system for the off-host (egg/larvae) stages. A cattle based colony was

maintained for 8 years at CSIRO in Brisbane (Stegman *et al.* 1996) and later at DPI at Yeerongpilly. Flies produced by this method have been used for purposes such as screening new chemicals, resistance assessment (Farnsworth 1997) and to examine cattle immune response to BF (Kerlin and Allingham (1992). The methods used are described by Thomas and Davis (1984) and Stegman *et al.* (1996). Cattle based rearing systems can provide a source of age defined flies but are extremely expensive, can suffer difficulties with gaining animal ethics approvals and are difficult to use for long term maintenance of defined strains of flies (for example strains characterised for resistance/susceptibility to insecticides, strains transfected with *Wolbachia*). It is also extremely difficult to carry out well structured studies of the effects of potential control strategies on the biology and population dynamics of BF with cattle based systems. To investigate the effects of transfection with *Wolbachia* on BF biology and survival prior to release into the field, a laboratory contained culture system will be necessary.

A completely *in vitro* system for BF (*H. exigua*) was developed in Australia and reported by Anderson *et al.* (1995). This appears to be the only successful completely *in vitro* system developed for BF to date and was maintained for at least 23 generations. What happened to it after this time is unclear. There is quite an extensive literature around development of a laboratory culture of horn fly (*H. irritans irritans*) at the USDA Livestock Insects Laboratory at Kerrville in the USA (Harris 1962, Schmidt *et al.* 1976, Kunz and Schmidt, 1985; Burg *et al.* 1993). This colony was initiated approximately 50 years ago and it seems that most subsequent studies reporting use of a laboratory strain of *H. irritans* have used flies derived from the Kerrville colony. In addition Doube *et al.* (1982) were able to develop a laboratory culture of a closely related species (*H. thirouxi potans*) a parasite of cattle in Africa, acting as vector of the subcutaneous filarial nematode, *Parafilaria bovicola*.

1.6 Cell culture

Insect cell lines are now being implemented to facilitate a wide range of research outcomes in areas such as early screening and clarification of modes of action of new biocidal molecules, effects of viruses and baculoviruses, vaccine design, clarification of gene action and elaboration of intracellular biochemical pathways (Smagghe *et al.* 2009). Once developed cell lines can be frozen down and stored for extended periods.

Successful transfection of *Wolbachia* strains into a new host requires infection of host ovaries. This has been successfully achieved in other species by passaging in host cell lines to provide prior adaptation and subsequent microinjection of infected cells into host germinal tissue. The second objective of this project was to establish a BF cell line towards this end. Establishment of a BF cell line will also provide a valuable resource for other approaches to better controlling BF.

2. Project objectives

1. Develop and optimise a reliable *in vitro* culture method for BF.

2. Use the *in vitro* system to determine whether experimental *Wolbachia* infection can be established in BF.

3. Prepare further applications for related work towards better control of BF, exploiting the *in vitro* systems.

3. Methodology

3.1 Establishing an in vitro culture of buffalo fly

We chose initially to base our efforts on the methods reported by Anderson et al. (1995) The system used 40 cm x 15.3 cm diameter cardboard cylinders with mesh tops resting on Wettex® sponge to maintain humidity. The flies were held at 32ºC and 70-80% RH with light:dark regime of 14:10 h. Flies were fed with cotton wool pads saturated with citrated bovine blood containing130 µg/L gentamycin sulfate replaced at 8 am and 4 pm daily. A sugar cube and vitamin/mineral supplement was also provided at the top of cage. Larvae were reared at 25°C on dung from pastured cattle or an extended dung based medium incorporating hammer milled bagasse and a number of other ingredients. A number of modified and scaled down versions were also tested (Figure 3).

Figure 3. (a) Rearing equipment based on the method of Anderson *et al.* (1995): large cardboard cylinder resting on moist Wettex sponge, blood pads used for fly feeding; vitamins provided in absorbent pads. (b) Modified system with cardboard tube and faeces provided '*ad-lib*' at the base of the cage; (b) Plastic cages enabling observation of flies and easy cleaning and disinfection. Fly pupae at base of cage.

However, we had little success with these systems with no flies surviving past the first two generations. In addition the method was considered too labour intensive for long term maintenance, requiring that blood pads be provided morning and night daily, including weekends. The cylinders became moist, were difficult to clean and required frequent replacement. In addition it was not possible to observe fly behaviour with the cardboard cylinders.

A number of different inputs and rearing practices were compared or tested over 2 years before the current sustaining colony was eventually established. Different

factors investigated and changes made on the way to development of the current sustaining colony are described below.

- **Different cage systems:** A number of different cage systems were used including those based on Anderson *et al.* (1995) (Figure 3). A system of plastic cages consisting of a top plastic container with mesh top and bottom to maintain the flies with a second plastic container containing moist sponge positioned below was tested. The bottom container and sponge assisted maintenance of humidity for flies and could be easily detached to collect eggs without the need to anaesthetise or remove the flies (Figure 4).

  The long standing *Haematobia irritans* colony at the USDA Kerrville Livestock Insects Laboratory has been successfully maintained over approximately 50 years using conventional glass and mesh sided cages and after a number of tests this was the cage system we eventually used (Figure 5).

Figure 4. An iteration of rearing system incorporating membrane feeders providing ad lib blood at the top of the cage with blood heated to 37ºC by water recirculated from a water bath. Dung pats for egg laying (and feeding if required) is provided in a Petri dish in the bottom plastic container with mesh of top cage pushed down on top. Bottom cage also contains saturated Wettex® sponge to maintain humidity and keep faeces and eggs moist

Figure 5. Rearing system with larger glass/ mesh sided cages, heated blood feeders on top, and fabric-covered heat mats to stimulate mating, held at 30ºC and 80% relative humidity

- **Membrane feeders (Figure 6):** The membrane feeders we use are glass, water-jacketed containers (Rutledge *et al.* 1964), 40 mm or 60 mm diameter, with blood contained by an attached membrane through which the flies can feed (Figure 6). These were used to provide an ongoing source of warmed blood. It was considered that using warmed blood enhanced feeding success, particularly of newly colonised and newly emerged flies, and could provide a reservoir of blood, removing the need for twice daily feeding. The membranes currently used are silicone supported with nylon netting rolled to approximately 0.06 mm thickness using an Atlas 150® pasta maker. A number of thicknesses and methods of preparing the silicone membranes were tested. Any thicker than 0.06 mm caused difficulties for feeding and making the membranes too thin increased likelihood of leakage. Recently we have moved to finer-meshed nylon to support the membranes. This has reduced the frequency of membrane rupturing and reduced the frequency of replacement of membranes. For day to day rearing on weekends, blood pads (once daily) are also provided as a backup for ruptured membranes.

Figure 6. A membrane feeder used in BF culture. Blood is provided *ad lib* at the top of the cage with blood heated to 37ºC by water recirculated from a water bath. Flies feed through the silicone-nylon membrane attached at the bottom of the feeder.

- **Different populations of BF:** The majority of BF used were sourced from the University of Queensland Pinjarra Hills Veterinary Research Farm (PJH). However, over the course of the project flies were also sourced from the DAFF Brian Pastures Research Farm, private properties at Cudgera Creek, Beaudesert, Rockhampton and a number of properties near Townsville. The final sustaining colony was derived from flies collected from PJH and near Townsville.

  Flies were collected by hand netting from cattle into purpose-built cylindrical cages and provided with blood pads during transport to the laboratory. Flies from Rockhampton and Townsville were contained in the same way in checked luggage. Although some flies died, most survived this means of transport and were successfully transferred to cages in the laboratory at Dutton Park.

- **Blood anti-coagulant:** Heparin and sodium citrate were compared for their effect on fly survival and egg production. There appeared to be no clear difference between the two over the concentration range tested in two experiments, the results of one experiment shown below (Figure 7). Therefore citrate (5 g/L) was used as it is cheaper and was used successfully by both Anderson *et al.* (1995) and in the Kerrville HF colony (Kunz and Schmidt 1985).

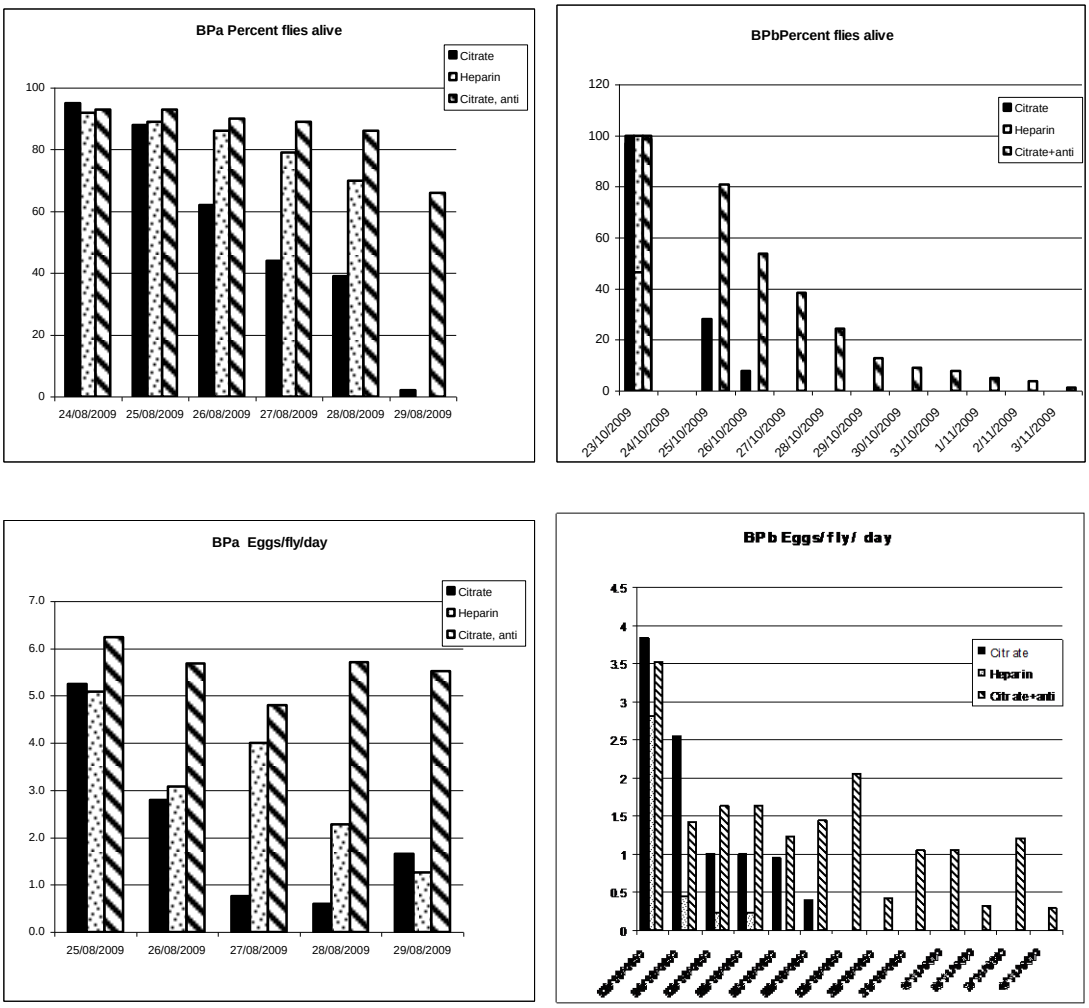

Figure 7. Effect of heparin (10 units/ml) and citrate (5 g/L) as anticoagulants and the addition of antibiotic (kanamycin 60 mg/L) in two generations of flies collected from Brian Pastures Research Farm.

- **Addition of antibiotics to blood.** Antibiotics added to the blood appeared to have beneficial effects and 60 mg/L kanamycin is currently used. Use of antibiotics is most important to prevent deterioration of blood during storage. Both Anderson (1995) and Kunz and Schmidt (1985) reported successful rearing of colonised flies without antibiotics so it is expected that antibiotic will be able to be removed for the studies with *Wolbachia*.

- **Using excess tick fever blood:** The possibility of using left over blood from the Tick Fever Vaccine Production Centre was investigated. However the blood tested appeared to have detrimental effects and this option was not pursued.

- **Addition of glucose and potassium chloride to blood**: Glucose and potassium chloride added to the blood was shown to increase egg viability in *Haematobia thirouxi potans* (Doube *et al.* 1982) but showed no beneficial effect with *H. exigua* in our tests (Figure 8).

- **Addition of "beef juice":** This was fluid expressed from beef by freezing, thawing and applying pressure. Beef juice was a key ingredient used by Harris (1962) in the initial establishment of the Kerrville HF colony, but appeared to give little benefit in aiding establishment in our studies.

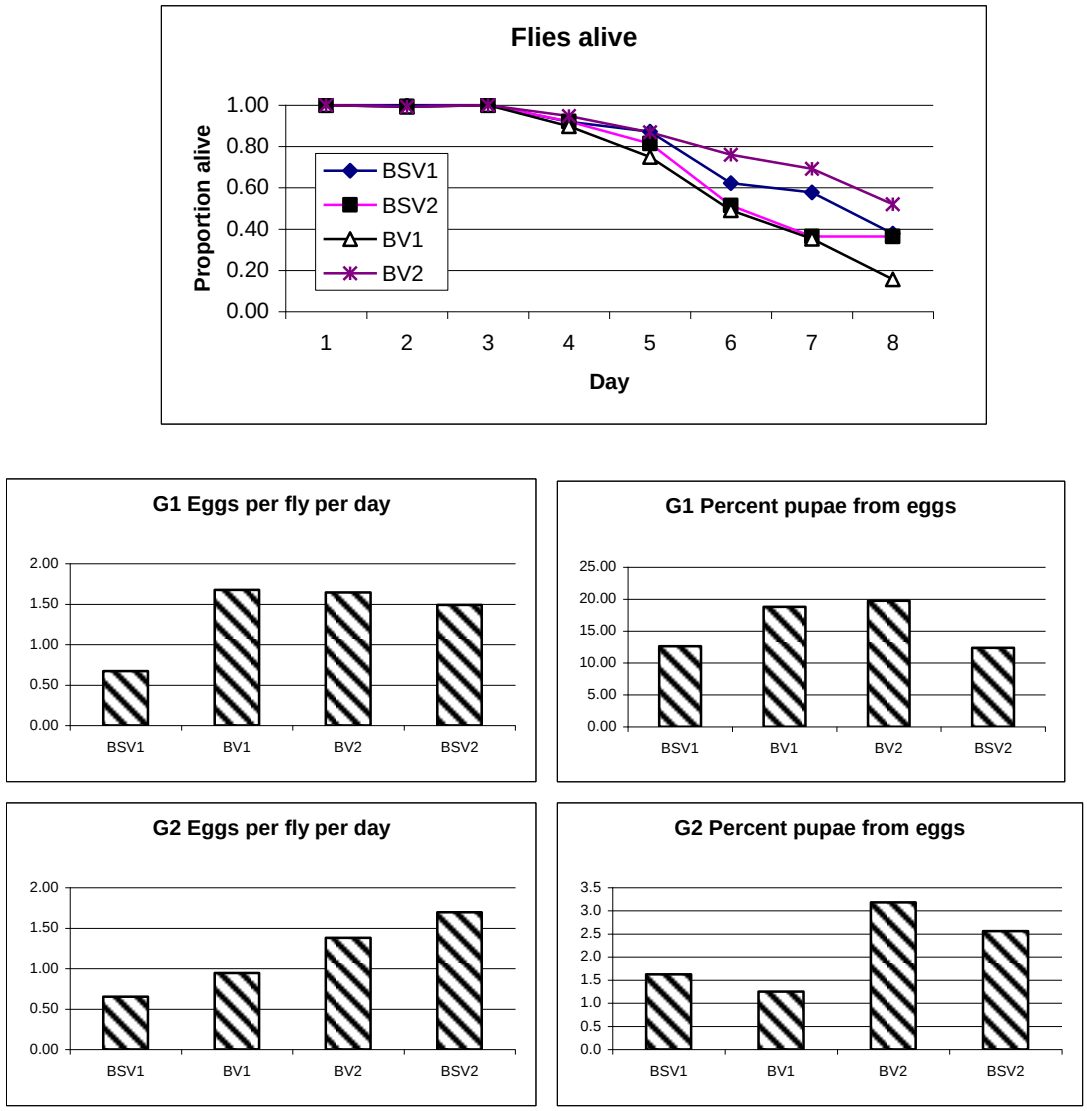

Figure 8. Effect of provision of 5% glucose and 1% potassium chloride (BSV), together with blood and a vitamin supplement, compared to blood and vitamin supplement alone (BV) on fly mortality, eggs/fly per day and percent pupae developing from eggs in first (G1) and second (G2) laboratory generation buffalo flies.

- **Provision of vitamin supplements**. Although no clear effect of a vitamin supplement could be distinguished, blood feeding insects often have micronutrient deficiencies and a solution of 'Hair Skin and Nails' (Herron Pharmaceuticals) is provided *ad lib* to address any possible imbalances.

- **Access of flies to bovine manure:** This was tested to enable dung feeding as well as to provide an oviposition medium. Flies were seldom observed to visit the manure and were not seen either feeding or ovipositing in the manure. Flies randomly deposited eggs in the bottom of the cages with no apparent orientation to dung. However, although flies did not utilise dung for either feeding or ovipositing, as eggs can hatch within 12 hours, we continued to provide dung in the cages as a food source for newly hatched larvae. First instar larvae are commonly seen in significant numbers in the dung, suggesting that this increases the survival rate of the larvae after eclosion from eggs.

- **Provision of cattle skin (warmed and unwarmed) and cattle hair within cages to stimulate mating**. – Flies remained mainly near or on the underside

of the top of the cages and showed little attraction to cattle skin. Only occasional flies were seen to land on the provided skin or hair.

- **Provision of heat pads, covered with black cloth, with and without cattle skin washings and cattle hair applied** (Figure 5). The heat pads were used to provide a potential surface for flies to mate on. Flies were sometimes seen resting on the heat pads, although the frequency of flies resting on the pads was generally low. Mating was observed on the pads on at least two occasions. Addition of skin sebum washings or cattle hair (collected from Droughtmaster cattle) had no discernable effect.

- **Use of carbon dioxide.** CO2 is a well documented attractant for blood feeding arthropods, including *Haematobia irritans* (Dalton *et al.* 1978). Carbon dioxide was provided at a range of flow rates close to the heat pads to attract BF and potentially, to help stimulate mating.

- **Different shading patterns within cages to change distribution of flies.** This was tested along with the provision of heat pads and sometimes, but not regularly, appeared to affect the numbers of flies resting on heat pads. Orientation to light also seemed to influence attractiveness of the pads.

- **Provision of black card board 'artificial cattle backs'.** Following field observations of fly behaviour, black cardboard strips approximately 7 cm in width were bent into a half parabola shape running from near the top of one side of the cage to the bottom of the cage to provide a resting platform and a surface for fly 'courting' behaviour and mating. This mimicked the contours of the side of cattle and allowed the flies to orient to light as was observed on yarded cattle. Flies were seen to rest on the card in significant numbers, particularly in lower light intensity and mating was frequently observed (See section 3.2.2 below).

- **Different lighting regimes** Different light intensities, wavelength and light to dark transitions were tested. BF showed extremely strong positive phototactic behaviour in most of these studies but did respond slightly differently with different wavelengths of light. This has been utilised in the final rearing system (See section 3.3.3 below).

- **Dung mixtures and additives**: Cattle dung, collected fresh from cattle pastures and frozen for storage and to kill larvae of other fly species and the mixture of Anderson (1995) were tested with no clear advantage from the mixture. As egg powder and lucerne powder were found to be the most important additives when attempting to rear *H. irritans* larvae on agar media (Perotti and Lysyk 2003) these were also tested as manure additives. As we have ready access to fresh manure from pastured cattle we now use frozen manure with lucerne powder (3 g/Kg) added to compensate for seasonal fluctuations in dung quality.

Figure 9. Artificial dung pats for oviposition and larval growth; dung on sand in containers for larval growth and pupation. Pupae are subsequently separated from sand and dung by sifting and flotation, dried and placed in new cage for hatching.

3.2 Buffalo fly colony: supporting observations

Consideration of the results from early attempted colonisations of flies showed that the numbers of eggs deposited per fly were relatively consistent across generations and the percent emergence of flies from the pupae that developed was relatively similar for the first two generations. However, the percent pupae developing from eggs decreased rapidly after the first generation and appeared to be the major factor limiting persistence. This suggested that either viability of the eggs was poor or newly hatched larvae were failing to survive. A series of observations and experiments was conducted towards identifying and overcoming the reasons for the low fertility rate of colonised flies.

3.2.1. Egg viability

Eggs were collected fresh after deposition by newly field-netted flies held in the laboratory for 2 days and by first and second generation laboratory bred flies. They were then allowed to hatch at 25°C on moistened filter paper and the number of eggs that hatched recorded. Eggs collected from field caught flies held in the lab for two days on two occasions had 72% and 74% viability compared to 40% and 38% on days 5 and 7 respectively. However the hatch rate of F1 flies was only 12 % on both occasions and no eggs from F2 flies hatched. This suggested that low viability of eggs was the major factor limiting persistence of the colony. Reasons for this could be either nutritional or other necessary factors limiting successful egg development, or a low fertilisation rate. We had not observed flies mating in the cage, whereas this is frequently observed in the other fly colonies that we maintain. Failure to observe flies mating also contrasted with the experience at the USDA Kerrville laboratories with the with the HF colony where mating was frequently observed (M Waldon pers com)

3.2.2. Fertilisation of female flies

Many species of *Diptera*, including BF, have paired organs called the spermathecae. These organs are used to store sperm after mating. The stored sperm are used to fertilise subsequently produced eggs. Therefore the spermathecae of field collected and laboratory reared flies were compared for the presence of motile sperm to determine the relative frequencies of mating.

Testes of male flies were dissected to ensure confident recognition of viable spermatozoa and to establish an optimal inspection protocol. Motile sperm were found in 80% of male flies examined. Recently field collected female flies and F1 flies were then selected and anaesthetised with carbon dioxide. Their spermathecae were removed by ventral dissection and mounted on a microscope slide in saline under a

coverslip. The spermathecae were observed at 400x magnification for the presence of motile sperm within, or for sperm in the proximal portion of the spermathecal ducts. If motile sperm were not seen, pressure was applied to the coverslip to rupture the spermathecae and the slide re-inspected. Female flies were examined on 5 occasions.

The proportions of flies with motile sperm found in their spermathecae at the 5 inspections of field collected flies were 100%, 87.5%, 45%, 100% and 89%, compared to 0%, 50%, 12.5%, 0%, and 12.5% in F1 flies. This suggested that failure to mate when held in the cages was a major factor in the low fertility rate and consequent poor persistence in the colonies. It is of note that the inspection with 50% fertility was from one of the most successful early colonisations.

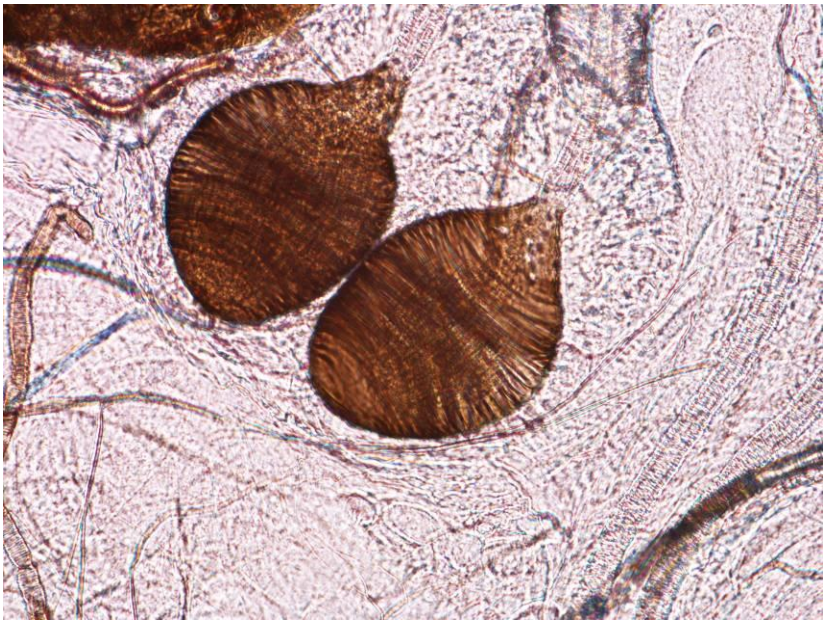

Figure 10. Spermathecae dissected from female BF. Spermatazoa can be seen as the parallel lines within the spermathecal duct at the opening of the spermathecae (more clearly apparent when motility can be observed).

3.2.3. Observations of fly mating on cattle

There has been little discussion of the mating habits of BF in natural situations in the literature and no reports of structured observations. Hill (1916) commented that the process of mating in BF was not recorded on the host or elsewhere. A number of authors have noted that mating must occur on the host on the basis that the flies seldom leave the host, except to oviposit. With HF, the most detailed observations were carried out by Kuramochi (1989) who noted that HF mated during the day on cattle surfaces, mainly on the side of the animal and in pectoral and thoracic areas. Despite intensive observation over three years, in the study of Kuramochi (1989) mating was observed relatively rarely compared to the rate at which it must have occurred as determined from spermathecal dissections. He observed a total of 15 matings; 11 of these on the sides of standing cattle and 4 on the udder of recumbent cattle.

We conducted two sets of intensive observations on relatively heavily infested single black Angus bulls held in open, unshaded pens at PJH. These bulls were accustomed to human handling and could therefore be observed from close proximity. One set of observations was made in February when BF populations were

high (more than 300 flies per side) and one in April when fly numbers were lower (approx 50 per side). The first set of observations were relatively unstructured with monitoring of the bull, cattle pats after the animal defaecated and of any flies seen resting on the sides of the yards or vegetation. Observations were made over a 24 hour period, starting at about 11:00 am and continuing to dusk on the first day, then re-commencing at sunrise and finishing at 11:00 am on the second day. The second set of observations took place over a similar 24 hour period, but was more structured with intensive observation of cattle for15 minute periods at hourly intervals from first light in the morning until last light in the evening. Opportunistic observations were also made on a herd of Droughtmaster cattle when they were yarded for other purposes. These observations were generally during the day between 8:00 am and about 3:00 pm.

In the first observations, despite a relatively heavy infestation, no flies were seen mating through the first afternoon. However, in the late afternoon at approximately 5:30 pm, a different behaviour was observed on the lower flanks of the recumbent bull. Flies exhibited 'milling' behaviour, walking around on the surface of the bull with infrequent short flights. This behaviour was not noted during the day when the flies did not walk but generally relocated on the animals by quick short flights. A number of pairs of mating flies were subsequently observed on the flanks on the shaded side of the recumbent bull. A further mating pair was later observed on the lower flanks of the (then standing) bull. Further observations commenced the following morning at approximately 15 minutes after sunrise when two pairs of mating flies were seen on the lower flanks of the bull. No further matings were observed during the period to 11:00am when observations concluded.

In the second set of observations, the distribution of flies on the cattle appeared to change markedly through the day. This may have been more noticeable on the second occasion as fly numbers were lower. At the commencement of observations at 11:00 am by far the largest proportion of the BF population was resting on the ventral areas of the animal, most on the ventral midline anterior to the prepuce. Some flies were also resting on the lower flanks and this was particularly so on the shaded side of the animal. For some observations the bull was laying down. At these times most flies were under the chin, along the neck and on the lower parts of the animal's flanks, generally in shaded areas. Many flies were seen feeding on or near *Stephanofilaria* lesions near the animals' eyes.

Relatively low numbers of flies were seen resting on the back even though at the temperatures were only moderate (max 28°C). Through the middle of the day flies were generally quite stationary on the animal and when they relocated it was generally by quick short flights with rapid return to the animal. No mating or anything with the appearance of courtship behaviour was observed during these times and despite the many observations of the yarded PJH Droughtmaster herd during the period from about 8:00 am to 3:00 pm, we did not ever observe mating flies.

No mating of flies was observed during the middle of the day and the same general distribution and behaviours of flies continued until the sun started to fall between 5:00 pm and 6:00 pm. It should be noted that it was almost impossible to observe the flies along the ventral aspects of the animals and whether or not mating occurred here during these times could not be assessed.

As light reduced, flies became more evident on lower flanks and gradually moved up the sides of the animal. The flies were seen more frequently to walk over the surface of the body than during the day and the 'milling' behaviour seen during the first observations was again observed on the lower flanks. A characteristic head to head

configuration of pairs of flies was also noted, usually with one fly with wings spread and the other with wings folded. When the 'head to head' flies were disturbed they usually subsequently returned to the head to head position. This was one of the pre-mating behaviours noted by Anderson et al (1995), although we did not see it progress to mating in our observations. Between 5:00 pm and 5:30 pm four pairs of flies were observed mating, all on the lower flanks. We observed no further matings after about 5:30 pm, suggesting that BF probably do not mate at night. Kuramochi (1989) states that HF do not mate at night, but how his night time observations were conducted is unclear.

Early in the morning, at sunrise when the bull was recumbent, nearly all of the flies were on the dorsal aspects of the animal, particularly near the head, on the highest and most brightly lit areas. Many flies were seen feeding on or near the *Stephanofilaria* lesions near the animal's eyes at this time. As the sun came up, more flies returned to backline and lower flank where they rested, usually with wings spread and not walking as much as those on the lower flanks. Characteristic milling and head to head behaviours were observed on lower flanks. Between 6 am and 6:30 am, 4 pairs of mating flies were observed. Most were the on lower flanks but one mating was observed on the back. No more matings were observed that morning, despite that the number of flies on the bull increased markedly following yarding of the Droughtmaster herd.

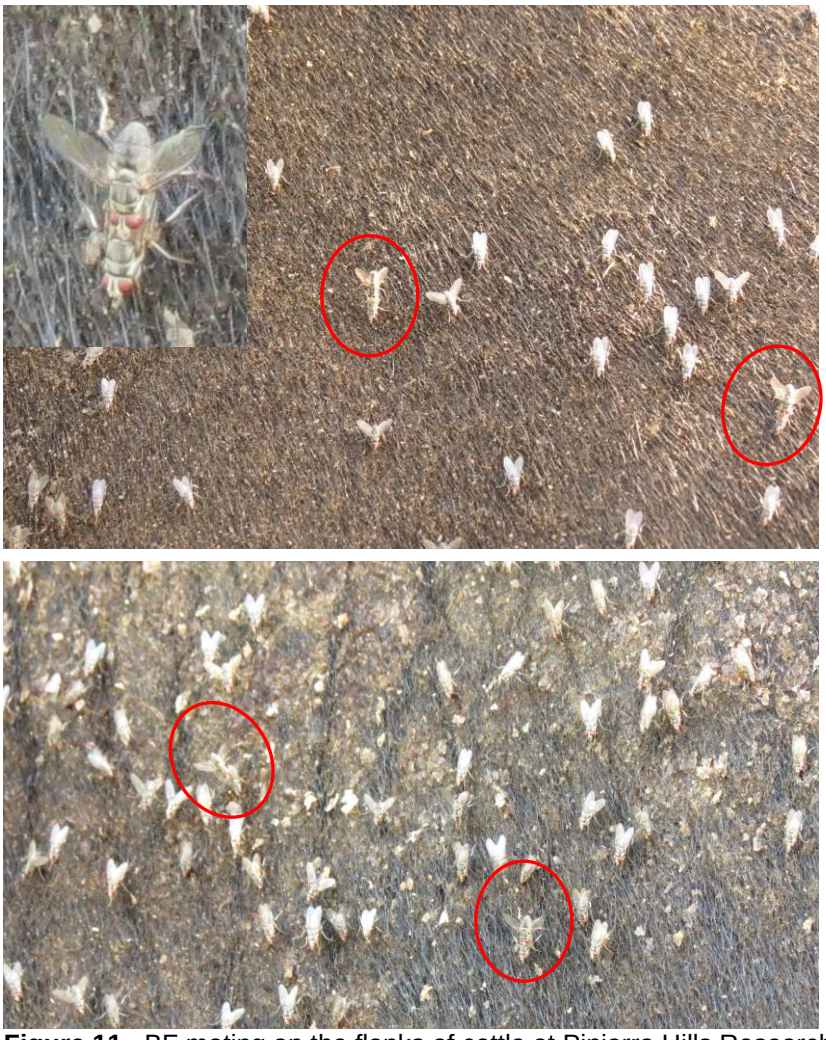

Figure 11. BF mating on the flanks of cattle at Pinjarra Hills Research farm

In summary, no BF matings were observed through the middle of the day. However, it was not possible to observe the flies on the ventral midline at these times and we cannot be certain that no mating occurred there. Most matings were observed close to dusk and early in the morning, and most matings were observed on the lower flanks of the animal. Matings seemed to be preceded by a change in behaviour of flies from where most flies were stationary when not disturbed and relocated to new positions by quick short flights, to a 'milling' behaviour where many flies were mobile on the host, particularly on the lower flanks, and movement was predominantly by walking.

3.2.4. Light effects on buffalo fly

Different light configuration, wavelengths and intensity: BF in the colony continually rest on or near the tops of the cages. There appears to be a strong orientation to light, overwhelming other potential forms of attraction, including blood placed in the bottom of the cage, bovine manure, heat pads, cattle skin and cattle odours which all seemed to have little effect. To further characterise the response to light and potentially develop a lighting regime that had a less overpowering effect a range of light configurations, wavelengths and intensities were tested (Figure 12). Configurations tested included lights placed above the cages, on one side of the cages directly beneath the mesh of the cage, below a table on which the cages rested so that there was no direct light. Different types of light included white light (both incandescent and fluorescent) (100 W, 35 W) infrared (100 W, 75 W) and moonlight (100 W, Reptile One®, Kong's Aust Pty Ltd). In addition as Harget and Goulding (1962) concluded that flies responded to areas of contrast and that visual stimuli were probably the main cue by which horn flies located their host, a black spot applied to the side of the cage in front of a light to produce silhouette effect was also tested. Tests were conducted using clear plastic cages with mesh bottoms with 30 flies per cage and two replicates for each test. Flies were observed for an hour in each test after they were allowed to stabilise for 15 minutes before tests commenced. Six counts of numbers of flies on each side and the top and the bottom of the cage were conducted at 10 minute intervals over the hour period of each test.

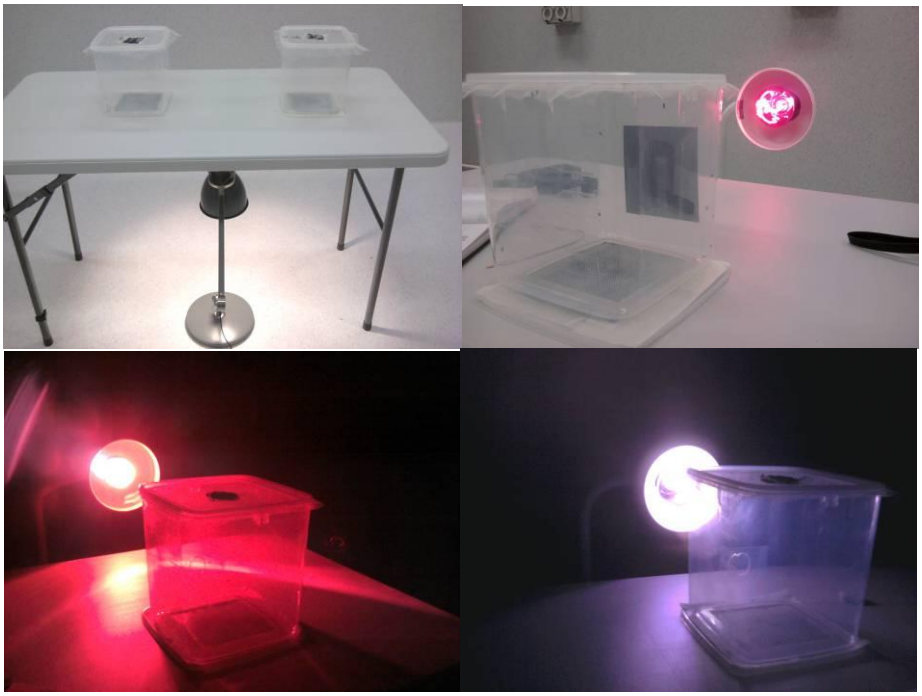

Figure 12. Some of the light configurations tested: a) light beneath the table; b) silhouette and light to the side; c) infrared lamp; d) moonlight

Response of flies: The study confirmed the phototactic response of the flies with the distribution of flies changing depending on positioning of the light. This was most marked when the light was placed directly below the netting base of the cage (Figure 13). However there was also clearly a negative geotropism involved and even when light was to the side or directly below the cage there were significant numbers of flies resting near the top of the cage. With the light directly below the cage only c. 20% of the flies rested on the cage bottom. Using a silhouette may have slightly increased attraction, in comparison to when no silhouette was used but the effect was not large. Changing light intensity, from fluorescent to incandescent light had little obvious effect and flies also appeared to be relatively strongly attracted to ultra violet light. In addition, in contrast to the findings of Hargett and Goulding with HF, BF also appeared to be relatively strongly attracted to red light (Figure 13). However, in our studies there was no heat filter and this could equally well have been a heat response. Using the moonlight lamp appeared to change distribution in the cage somewhat (Fig 13f) but an additional interesting observation was that with the moonlight lamp, the behaviour of the BF changed. The flies appeared to form groups and a milling behaviour of the flies, similar to that observed on cattle at dusk and dawn and associated with mating was observed.

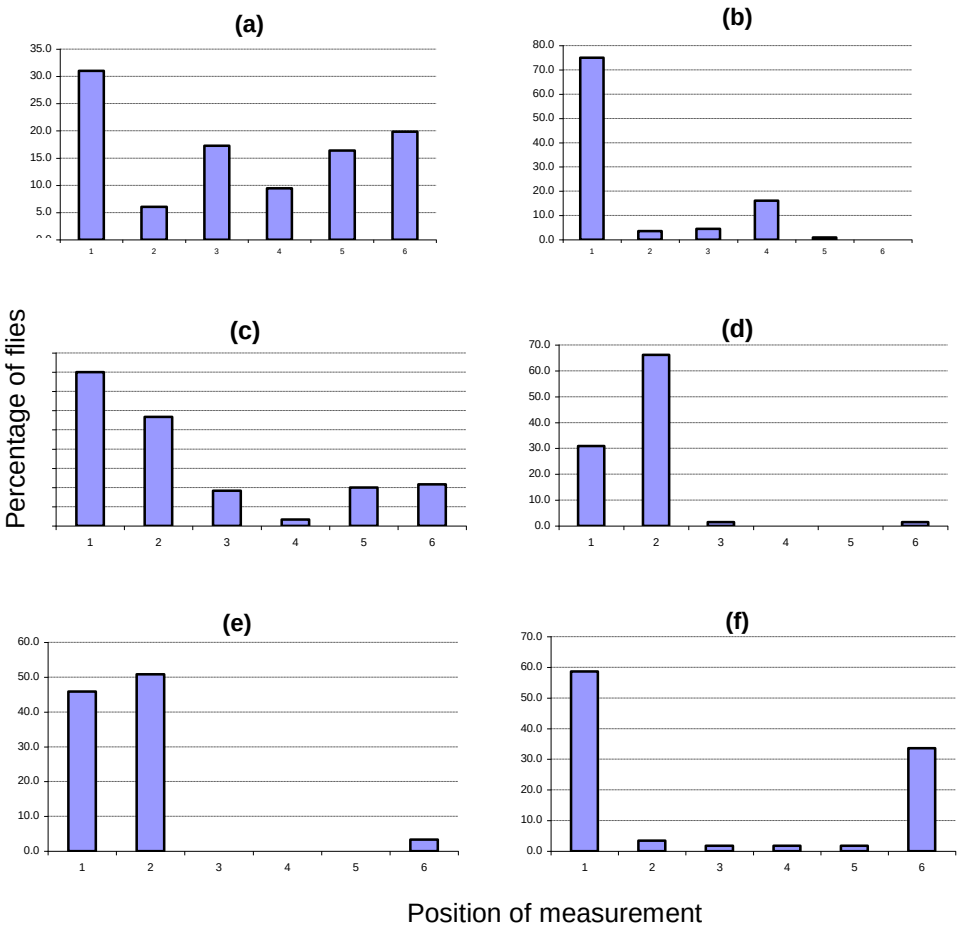

Figure 13. Distribution of flies under different light regimes. a) Light below netting, b) Light under table (no direct light), c) White light on side 1; d) White light from side 1 with silhouette on side 1; e) Infra red on side 1, f) Moonlight on side 1. Positions on the x axis are: 1 = top of the cage; 2 -5 = sides; and 6 = bottom.

3.3 Cell culture

3.3.1. Haematobia exigua

Establishment of BF cell lines from eggs freshly collected from buffalo flies has been attempted at QAAFI St Lucia Laboratories on 16 separate occasions prior to 2012 and on 30 occasions from eggs and 5 times from pupae since then. Eggs from field collected flies from a number of sources, most commonly from the Droughtmaster herd at the University of Queensland PJH research farm have been used on most occasions although eggs from the laboratory BF colony have also been used recently. The establishment method and growing media and conditions have been optimised over the course of the project with the following technique currently used: Field collected or laboratory strain flies are held at 28ºC and ≈80% RH with access to bovine blood and eggs collected overnight on moistened filter paper placed at the base of the cage.

Groups of about 100 eggs were washed in a 10.5 g per litre solution of sodium chloride followed by a 20-min treatment with 2.5% sodium hypochlorite solution to surface-sterilize and de-chorionate the eggs. Eggs were then rinsed three times with the culture medium and mechanically dissociated in small, conical glass homogenizers. Embryonic tissue was collected and centrifuged at 150g for 10 min at 8°C. The pellet was resuspended and cultured in complete culture medium consisting of 3% yeast hydrolysate, 20% foetal bovine serum, 100IU/mL penicillin and 100ug/mL streptomycin in the mixture medium of 50% Dulbecco's Modified Eagle Medium and 50% Schneider's medium. The media were held at 26°C, changed weekly and early subcultures performed when the cells were confluent.

3.3.2. Haematobia irritans

A collaboration with Professor Tim Kurtti and Dr Uli Munderloh at the University of Minnesota was undertaken to facilitate establishment of a *Haematobia* cell line. The Kurtti-Munderloh laboratory has had considerable success in the development of a wide range of cell lines, including recently a world first honey bee cell line (Goblirsch et al. 2013). Dr Munderloh has also previously collaborated with DAFFQ scientists to assist the establishment of a cattle tick cell line for use in vaccine studies.

Establishment of embryonic cell lines (from eggs) was attempted on 22 occasions during May 2013. Tissue from larvae and pupae was also used but we had no success in keeping these tissues free of bacteria and all successful cultures originated from embryonic tissue.

Eggs were sourced from the *H. irritans* colony maintained at USDA Livestock Insects Laboratory in Kerrville,Texas, shipped refrigerated overnight to Minnesota and stored at 5°C until used (no eggs stored for more than seven days were used). Approximately 0.5 ml of eggs were transferred to sterile 15 ml centrifuge tubes and surface disinfected in 5 ml of bleach containing 50 µl of Tween 80 for 5 minutes. The bleach-Tween solution was then removed and eggs sequentially washed with benzalkonium chloride and 70% ethanol, each for 5 minutes. This was followed by 3 washes in 5 ml of sterile water and 3 washes in the culture media. Approximately 0.2 ml of eggs were then transferred to 1.5 ml Eppendorf tubes where they were crushed using a sterile plastic pestle. Three washes of the crushed material were conducted with 0.5 ml of medium each time, resuspended and transferred to a 10 ml centrifuge tube. The volume was made up to 5ml and the tube spun at 170 RCF for 2 min. The pellet was then resuspended in 2.5 ml of media and transferred to a small (2.5 ml) tissue culture flask, inspected for the presence of cells and egg yolk and transferred

to an incubator at 30°C. Cultures were generally inspected daily and media changed weekly.

3.4 Buffalo fly infection with *Wolbachia*

BF was collected from 12 locations in Australia (Table 6) and from Bali, Indonesia. Specimens were immediately stored in 20% DMSO/0.25 M EDTA solution (Seutin *et al*. 1991). Horn flies were collected from three locations in southern Alberta, Canada, stored in 95% EtOH and held at -20^{o}C until needed.

Testing for *Wolbachia* was carried out in laboratories in both Australia and Canada. To confirm the test method, HF also were tested in each laboratory. In Australia, genomic DNA was extracted from individual flies using Puregene Tissue Core Kits (Qiagen) according to the manufacturer's instructions. Extracted DNA was tested for the presence of *Wolbachia* using polymerase chain reaction (PCR) tests and *wsp* primers. These primers amplify *Wolbachia* DNA (if present) to detectable levels that can be visualized as bands of characteristic size (ca. 600 bp) on agarose gels (Braig *et al*., 1998). DNA from a single *D. melanogaster* known to be infected was used as a positive control. PCR was performed in a 20 μl volume containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 2.5 mM $MgCl_2$, 0.1 mM dNTPs, 0.5 μM primers, and 0.8 unit Taq DNA Polymerase (Roche Molecular Biochemicals, Indianapolis, IN). The thermal cycling regime was as follows: 3 minutes denaturation at 94°C, 35 cycles of 0.5 min denaturation at 94°C, 0.5 min annealing at 55°C and 1 min extension at 72°C, followed by an extra 10 min extension step at 72°C. The PCR reaction was run in a Mastercycler (Eppendorf, Westbury, NY, USA). PCR products were separated by agarose gel electrophoresis, and stained with ethidium bromide. DNA bands of ca. 600 bp were extracted and purified from gels. Sequencing confirmed the presence of *Wolbachia*. Similar methods were used in Canada, with specific details provided in Kyei-Poku *et al*. (2003).

4. Results and Discussion

4.1 Buffalo fly rearing

Most past research into BF biology and control has been conducted with field collected flies. This presents difficulties because of problems of strain definition, particularly for studies testing new insecticides and resistance where the past history of chemical exposure, strain and susceptibility status will not be known and a susceptible reference strain, critical for resistance definition or monitoring, will not be available.

This has been overcome in the past by using a semi *in vitro* system with stanchioned cattle held in insect proof rooms as hosts and flies reared through artificial manure substrate in the laboratory (Stegman *et al.* 1996). However, this is extremely expensive, unsuitable for long term strain maintenance, is increasingly problematic from an animal ethics standpoint and is subject to difficulties resulting from variability both between and within hosts.

In addition, as our knowledge of and ability to manipulate insect genomes grows and there is increased aversion to chemical methods, there is growing interest in the use of genetic modifications (see McGraw and O'Neill 2013 for discussion) and biological agents (for example *Wolbachia*) in novel control systems. Such agents generally

need to be able to spread through a population once released, require assessment of their effect at the population level prior to release into the environment and present significant difficulties for assessment in systems requiring large animal hosts.

Despite a long-term desire for an *in vitro* system of rearing BF, this has been previously achieved only once (Anderson 1995). However, there are no subsequent studies reported using this colony and its ultimate fate is unclear. *In vitro* rearing of closely related HF has been similarly difficult and most reports noting research with laboratory reared flies have used flies derived from the from the USDA Kerrville colony. Perotti and Lysyk (2003) also investigated laboratory rearing of HF in Canada and reported the successful development of a synthetic larval agar-based medium. However they were not able to rear HF completely *in vitro* and maintained strain integrity of their flies by rearing them through small cages attached to the backs of cattle.

We have now established a completely *in vitro* colony of BF that has been successfully reared in our laboratory without the use of an animal host for 33 generations.

As described earlier, we initially used methods based on those of Anderson (1995), but had little success with the colonies failing to persist past the second generation. There were also other undesirable features of this method. The colonies were labour intensive to maintain with fresh blood pads required twice daily. Even with twice daily changing the pads sometimes became completely depleted of blood or dried out and it was thought that this adversely affected the flies. In addition, the cardboard cylinders used by Anderson rapidly became moist and soiled and were hard to clean and it was difficult to observe fly behaviour.

Therefore, as described above we moved to using clear plastic cages and ultimately more standard glass and mesh sided cages. In addition, we changed from using blood pads to using membrane feeders to provide blood. These water-jacketed feeders used heated water recirculated from a water bath held at 37°C to provide blood. They only required filling once daily, maintained a reservoir for flies throughout the day and it was felt that heating to cattle body temperature aided the location of the food source and feeding success of newly emerged flies. We also tested a number of different manure additives, but none showed clear advantage over field collected manure. However, as seasonal differences in manure quality have been shown to influence maggot survival and growth, we routinely add (3 g/Kg) lucerne powder to compensate for seasonal fluctuations in dung quality.

Adult flies were seldom seen to visit dung pats placed in the bottom of cages. Female BF randomly deposited their eggs from the top of the cage, the eggs falling to the sponge matting in the base of the cage from where they were subsequently collected every two days. BF eggs can hatch in as little as10-12 hours and although artificial dung pats were not used for oviposition by female flies, provision of the dung in the base of the cage provided a food source for hatching larvae and reduced labour by avoiding the need for daily collection of eggs.

Even with all of the inputs investigated above, our early attempts to establish a sustaining colony were unsuccessful. Tables 1 and 2 give a summary of the first 18 attempts at colonisation. On 6 occasions the colonies did not survive past the first generation and on 15 of the 18 attempts they did not survive past the second generation. On only 3 occasions did any flies survive to the 3rd generation and on only one occasion did any 4th generation flies emerge (1 fly) (Table 1). Table 2 summarises population parameters for the early colonisations of flies collected from

PJH and Table 3 similar parameters from a strain collected from Brian Pastures that was the most successful of the first 18 attempts

Table 1. Maximum generation achieved in 18 colonisations of flies

Generation	Number of colonisations
1	5
2	9
3	3
4	1

Table 2. Summary of egg, pupal and fly parameters from different generations of flies established in the laboratory (first 18 generations)

Generation	Flies	Eggs	Eggs/ fly/day	Pupae	Pupal wt (mg)	Flies emerged	% Pupae from eggs	% Fly emergence
1	685	3135	1.5	652.5	3.5	303.2	19.6	51.3
2	422	2040	1.5	23.8	3.4	23.5	1.3	57.4
3	23	295	2.9	5.0	3.3	1.0	1.0	

Table 3. Fly survival in successive generations in flies initially collected from Brian Pastures Research Station

Generation in laboratory	Flies	Eggs	Eggs/ fly/day	Pupae	Pupal wt (mg)	Flies emerged	% Pupae from eggs	% Fly emergence
1	280	4683	3.5	621	3.6	390	13.3	62.8
2	390	7083	3.7	32	3.3	26	0.5	81.3
3	21	526	3.9	10	3.3	1	1.9	10.0
4	1	0						

Consideration of these results suggested that the numbers of eggs deposited per fly were relatively consistent across generations and the percent emergence of flies from pupae was relatively similar for the first two generations.

However, the percent pupae developing from eggs declined dramatically from the first generation to subsequent generations. This suggested that the fertility of eggs was low, conditions were unfavourable for egg development and hatching, or that larvae were not surviving from hatching to pupation.

As there was a relatively high hatch rate of eggs, percent pupae developing from eggs and flies emerging from pupae from eggs laid by newly collected field flies incubated under the same conditions as colony flies, it seemed unlikely that effects of the rearing conditions on egg, larval or pupal survival were the main reason for the marked decrease in flies emerging in later generations. This suggested either failure to mate or low viability of spermatozoa or ova as the likely reason for the low hatch rate of eggs. Dissection of mature female flies usually showed oocytes developing in the ovarioles and often eggs in various stages of development. Dissection of the testes in male flies revealed motile spermatozoa in most instances. However, dissection of spermathecae of colonised female flies showed a low frequency containing spermatozoa (in most cases <15%) compared to a relatively high frequency in field collected flies (>85%). In addition, we had only very rarely observed mating or attempted mating in the cages, whereas this is frequently observed with other colonised *Muscidae*. It therefore appeared that failure to mate in our rearing cages was a key reason for the low reproductive rates observed in later colonising generations

However, it should be noted that the percent pupae developing from eggs also declined with increase in time for which field collected flies were held in the laboratory (Table 4). This could simply have been a sampling effect as flies that were older at collection died and younger flies that were not mated at the time of collection increased in proportion. However it could also have resulted from the stress of colonisation or change in nutritional regime and effects other than failure to mate may have contributed to population decline.

Table 4. Mean percent pupae from eggs laid by wild caught flies by day after laboratory colonisation

Day	1	2	3	4	5	6	7	8
% pupae from eggs	49.2	33.9	17.2	12.8	14.3	4.9	4.6	4.3
%flies from pupae	59.1	80.1	66.7	76.5	83.3	100		

Observations on cattle suggested two main things. First, most mating took place early in the morning soon after dawn or in the late afternoon, shortly before dusk. At these times there was a sort of 'milling' behaviour where many flies walked in a rapid and apparently undirected motion. This motion was not seen during the day when flies were largely motionless, resting or feeding. When they did change positions it was usually by means of short flights rather than walking. We saw no mating in the middle part of the day, although we could not completely discount the possibility of BF mating along the ventral midline of standing cattle. Secondly, most mating was seen on the lower flanks of the animal.

Studies of response to different light regimes were conducted for two main reasons. First, it was considered that the phototactic response of the flies under the standard light regime in the rearing room may have been overwhelming other mating cues such as fly pheremones (Bolton et al 1980) or host volatiles. A different light regime may reduce this effect. Second, it is well known that light intensity influences mating behaviour of other blood feeding Diptera, most notably mosquitoes (Baerg 1971).

The light studies confirmed the strong attraction of light of most wavelengths to BF, although they also suggested an element of geotropism in the flies' response. In contrast to the results of Hargett and Goulding (1962) with HF we found that red light was as attractive as white light. However, as no heat filter was used in our studies heat effects may have contributed to attraction to the red light. When the moonlight lamp was used, there was both a change in the distribution of the flies, and in their behaviour. The flies formed into groups and a milling behaviour, similar to that noted on cattle at dawn and dusk and associated with increased mating frequency, was observed.

As a result two changes were made to the rearing procedure:

1. The lighting regime in the rearing room was altered: We attempted to provide a change in both intensity and wavelength with our current regime that approximated transition from daylight through dusk to night time and then from night to dawn to daylight (see below)

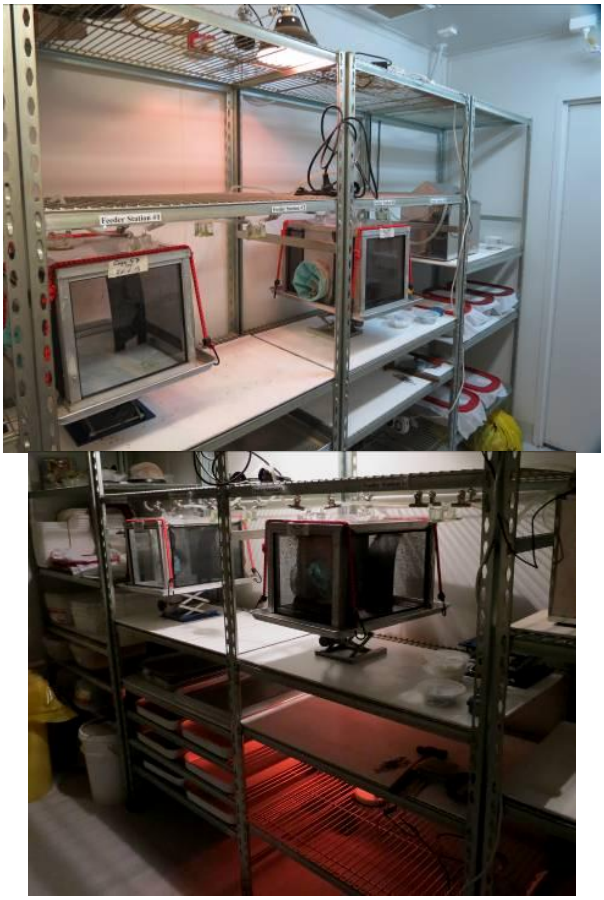

Figure 14. Daylight and dusk lighting regimes

2. We provided a half parabola of black cardboard in the cages to simulate the contours of cattle and provide a platform on which flies could mate. This was attractive to the flies which rested on the cardboard in a configuration similar to that seen on cattle, especially in the evenings as the light intensity decreased.

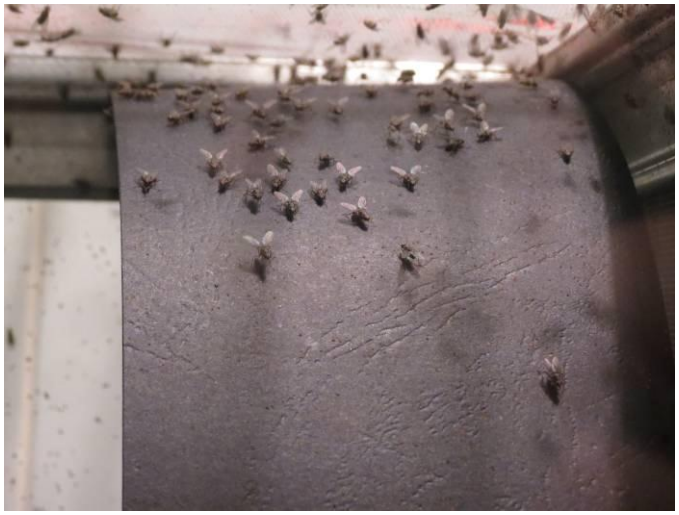

Figure 15. Flies resting on black cardboard platform

We consider that these changes, together with a degree of subsequent laboratory adaptation, have stimulated increased mating and been critical in the development of a sustaining, completely *in vitro* laboratory colony of BF.

The current rearing method is given in Appendix A.

A comparison of the life table parameters for rearing success in the current colony with those for earlier colonisations is given in Table 5. In the first 18 attempts, F2 flies were produced on only 16% of occasions and an F3 fly (one fly only) on only one occasion. Only 0.5% pupae developed from eggs in the F1 generation from these colonisations. In the middle 14 generations, following a range of modifications to technique, but before the current light regime and addition of the cardboard platform, the percent pupae from eggs had improved somewhat to 2.5%, but F2 flies were only produced once (7.1%) and no F3 flies were produced.

Table 5. Life table parameters for buffalo fly reared in early colonisations and in the 10 most recent generations of the current colony

Generation		Eggs*	Eggs/ fly/day	Pupae	Flies emerged	% Pupae from eggs	% Fly hatch	% Flies from eggs
First 18	F0	4683	3.5	621	390	13.3	62.8	8.4
	F1	7083	3.7	32	26	0.5	81.3	0.4
Middle 14	F0	9476	1.2	1189	730	14.5	61.4	8.9
	F1	2051	1.0	55	42	2.4	78	1.9
Last 10	F22-F32	23848*	2.4	4730	1935	20.4	41.0	8.6

* Eggs used. Excess eggs were discarded.

However, with the current method (see appendix) the colony has been reared through 33 generations. The percent pupae produced from eggs is now 20.4% (based on last 10 generations), much higher than the 0.5% and 2.4% rates measured for F1 flies in earlier colonisations and certainly high enough to sustain or grow the laboratory population.

Figures 16a-d show the changes in life table parameters over the 32 generations of the established colony. Note that the first point in the graphs is from the field collected generation of flies. These points have been plotted to provide a point of comparison.

Figures 16a and 16b show that after the initial drop from the field collected flies, when the different life table parameters are regressed over generation there has been a significant increase in percent pupae from eggs, percent flies from eggs and eggs per fly per day ($P<0.001$, $P=0.006$. $P=0.002$ respectively). There has been no change in the death rate of ($P=0.662$, data not presented), but a significant decrease in percent flies hatching from pupae ($P<0.001$).

The improvements noted have probably resulted largely from changes to the rearing system, which have stimulated mating, followed by ongoing adaptation of the colonised strain to the laboratory conditions. The reason for the decline in percent flies developing from pupae is somewhat concerning and we are currently looking at various dung fortifications to reduce the impact of seasonal fluctuations in dung quality. However, it is encouraging that the last four generations have seen a continuing increase in this parameter and that the most recent value for percent flies from pupae (Figure 16c) is similar to that in early generations of the colony.

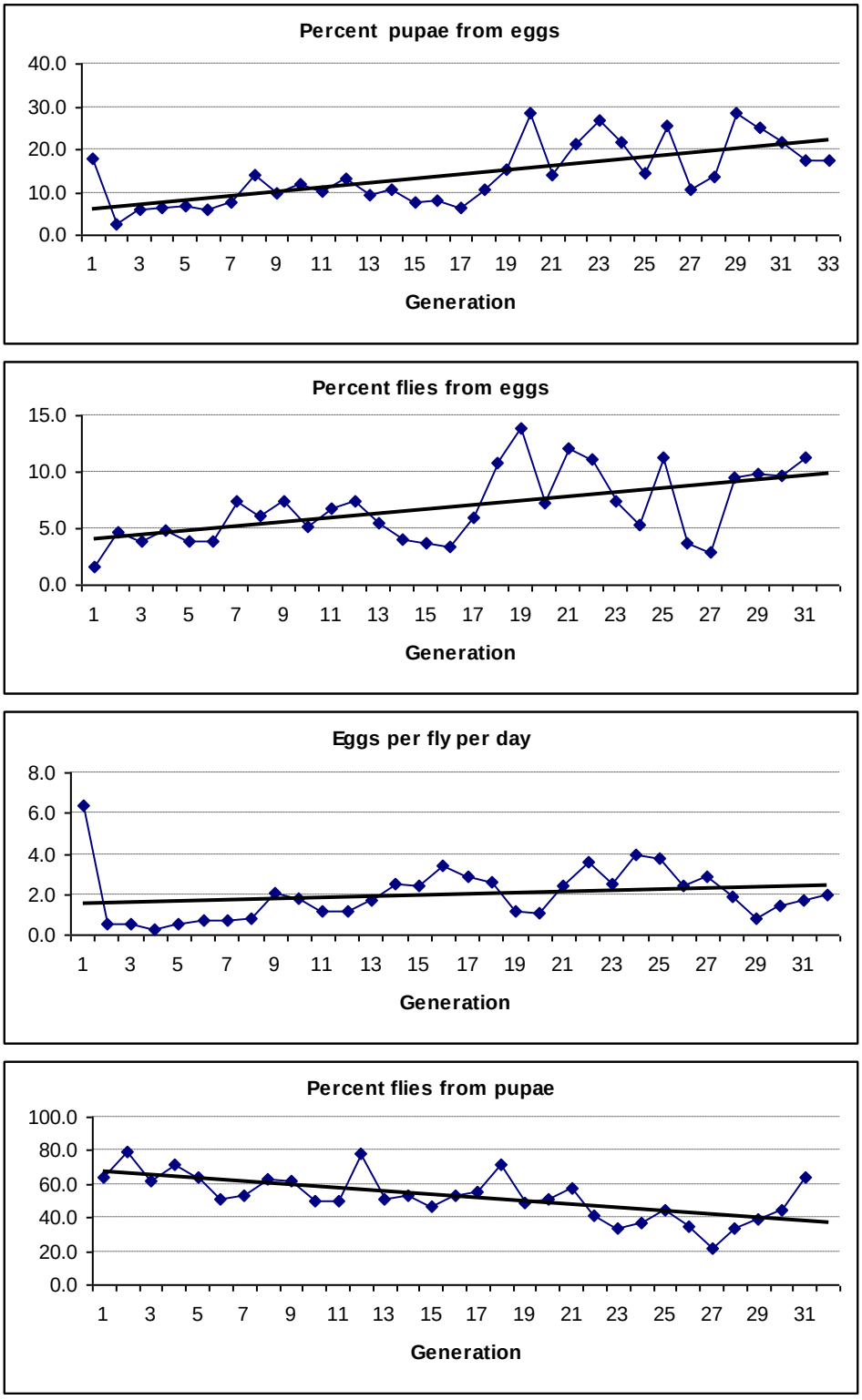

Figure 16. Change in reproductive and survival parameters over the 33 generations of the current colonised strain (a) Percent pupae developing from eggs; (b) Percent flies developing from eggs (c) Eggs laid per fly per day (d) Percent flies from pupae

4.2 Cell lines

4.2.1. Haematobia exigua

Four sustaining cell lines were developed at St Lucia in 2010. Of the four cell lines established, BFEC#12 showed the best growth. The cells attached, grew well with a cell doubling time of 4-5 days, and were grown in the laboratory for six months. It was considered that the cell lines were close to stability and that attempts at infection with *Wolbachia* could commence. The culture was sub-divided to test different growth conditions and one of the resultant sub-cultures was subsequently transferred to the Animal Research Institute (ARI), grown through a number of generations and stored in liquid nitrogen. Unfortunately the incubator in the St Lucia laboratories malfunctioned three to four times from the end of October to mid-November, because of power supply interruptions. When checked in November the cell lines appeared unhealthy with low cell numbers and slow proliferation (cell doubling time of two to three weeks). All four cell lines in this laboratory were subsequently discarded.

Half of the BFEC#12 cell lines stored at ARI were retrieved successfully from liquid nitrogen storage and grew vigorously enough for new reserve cell lines to be frozen for liquid N_2 storage. These cells were again successfully thawed and retrieved after three weeks storage. Unfortunately, during the Brisbane floods in January, power was cut at both UQ and ARI. Attempts to rescue the cells by addition of conditioned media and/or fly egg extract to the culture were partly successful, such that the cells continued to grow slowly from mid-February. However, bacterial contamination of these revived lines led to them being subsequently discarded.

Unfortunately the 35 subsequent attempts to re-establish a cell line (30 embryonic tissue, 5 larval or pupal tissue) using the previously successful methods have not resulted in persisting lines. A significant problem has been ongoing bacterial contamination. In 2012 the main bacterial contaminant was identified as *Stenotropomonas maltophilia*. Despite a wide range of technique modifications including changing egg collection protocols, feeding antibiotics to flies prior to egg collection, tests with a range of different antibiotics and antibiotic combinations, different disinfection regimes and confirming sterile conditions we have not been able to re-establish a cell line.

However, our previous success, together with success in optimising methods and developing an *H. irritans* line (see below), suggests that establishing the BF cell line is an achievable future goal. Future attempts will use the techniques successful for the *H. irritans* line, which differ in a number of significant aspects from that used to date for *H. exigua.*

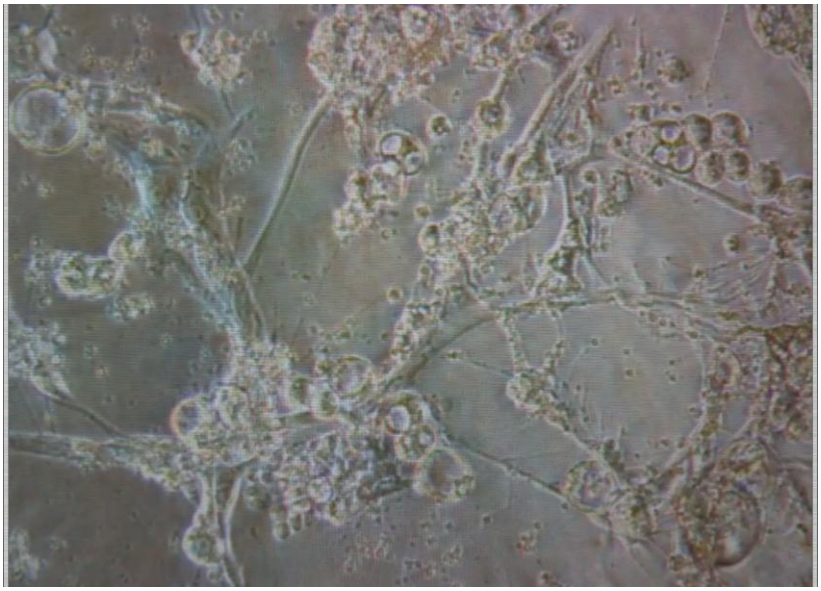

Figure 17. *H. exigua* cells from a line developed at University of Queensland in 2010

4.2.2. Haematobia irritans

Establishment of a total of 22 embryonic lines, 2 lines from neonate larvae and 2 from pupae were attempted between 7 May and 3 June 2013 at the University of Minnesota. The lines from neonate larvae and pupae showed contamination within 2 days of preparation and contamination also became evident in 5 embryonic lines. These lines were all discarded. Nine off the seventeen embryonic lines that were established without indication of contamination were discarded on the basis of poor cell attachment, failure to grow, morphology that suggested unhealthy cells or slow cell multiplication. The eight healthiest and most vigorous lines were chosen for retention and are currently being maintained at the University of Minnesota. Figure 18 shows two of the remaining cell lines photographed on 3rd of June and Figure 19 shows a subculture from one of the healthiest lines photographed nearly a month later. There are basically 2 cell types showing in this culture, those that grow in clusters of round cells and those that flatten themselves to the bottom of the flask. Our Minnesota collaborators have started to make subcultures and will select a few for continuation and freeze the remainder down for storage in liquid nitrogen. Once we are certain that healthy continuing lines have been established we will apply for permits to import these cells to our Brisbane laboratories. As *H. irritans* and *H. exigua* are extremely closely related (only relatively recently considered separate species) the *H. irritans* cell lines will probably be suitable for use in transfection of BF with *Wolbachia*. However, continuing efforts, using these techniques, are currently underway to establish a *H. exigua* cell line in our laboratory

Cell lines are now being implemented to facilitate a wide range of research outcomes in areas such as early screening and clarification of modes of action of new biocidal molecules, use of viruses and baculoviruses as biocontrols, vaccine design, clarification of gene action and elaboration of intracellular biochemical pathways. Furthermore, once developed cell lines can be frozen down and stored for extended periods. The establishment of a buffalo fly cell line will provide an important resource for future research towards new control approaches for buffalo fly.

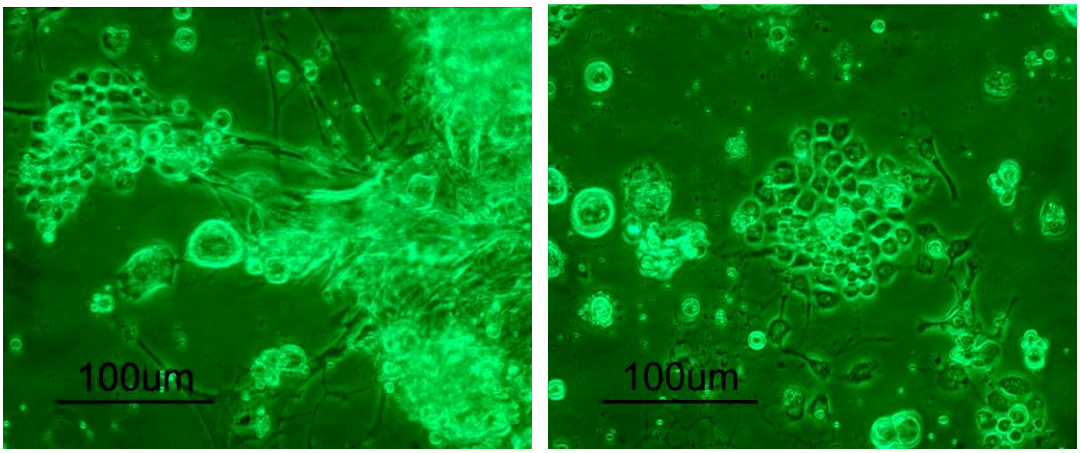

Figure 18. *H irritans* cells from a line at University of Minnesota approximately one week after establishment

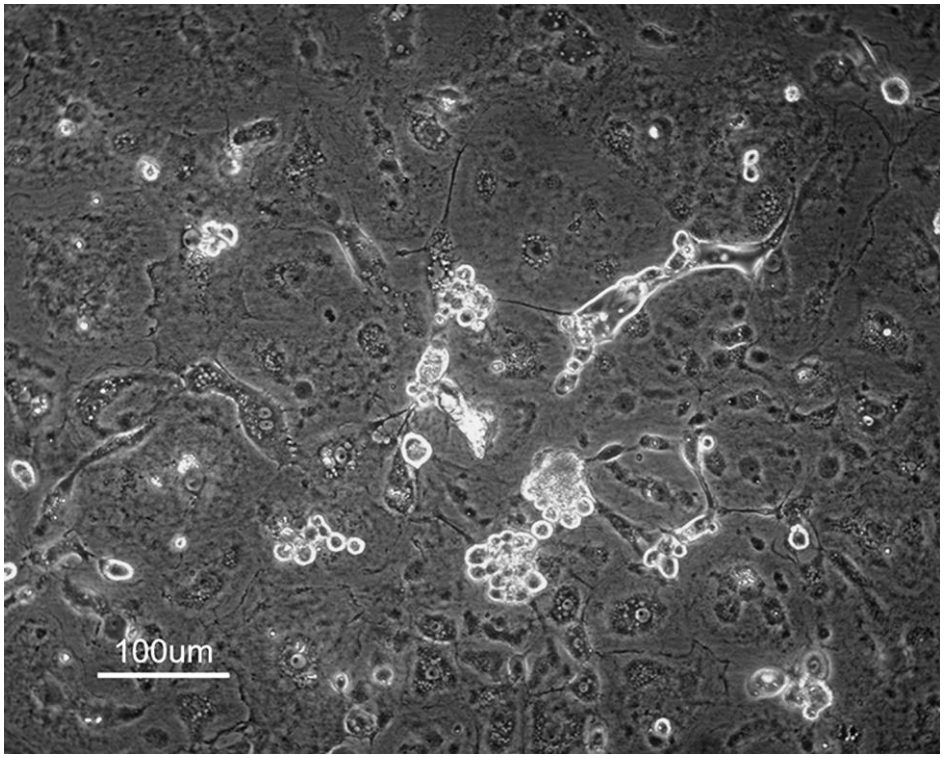

Figure 19. *H. irritans* cells from a subculture from the University of Minnesota line one month after establishment

4.3 Infection of buffalo fly with Wolbachia

Although *Wolbachia* occurs in many Australian insect species and is apparently widespread in North American *H. irritans* populations (Jeyaprakash and Hoy, 2000; Floate *et al*., 2006) no infections were detected in BF (Table 6). *Wolbachia* were detected however in each positive control and in 69 of 70 horn flies individually tested. The absence of *Wolbachia* in a sample of *H. exigua* collected from Bali may suggest that this is a more general characteristic of *H. exigua*.

Table 6. Summary of screening for *Wolbachia* infection in buffalo flies and horn flies

Species	Source	Where tested	No. flies testing positive
Buffalo fly	Gatton, Qld	Australia	0 of 33
	Berrimah Farm, NT	Australia	0 of 8
	Douglas Daly, NT	Australia	0 of 8
	Beatrice Hill Farm, NT	Australia	0 of 8
		Canada	0 of 2
	Yeerongpilly, Qld	Australia	0 of 10
	Mutdapilly, Qld	Australia	0 of 8
		Canada	0 of 4
	Rochedale, Qld	Australia	0 of 6
	Pinjarra Hills, Qld	Australia	0 of 4
	Duck Creek Research Farm, NSW	Australia	0 of 8
	Warrawagine, WA	Australia	0 of 8
		Canada	0 of 4
	Camballin, WA	Australia	0 of 8
	Ruby Plains, WA	Australia	0 of 8
	Gianyar, Bali, Indonesia	Australia	0 of 5
		Canada	0 of 2
Horn fly	Lethbridge Research Centre colony	Australia	4 of 5
		Canada	50 of 50
	Coaldale, AB	Canada	5 of 5
	Onefour, AB	Canada	10 of 10

Successful transfection of *Wolbachia* across genus and species barriers and establishment of stable infections in previously naïve insect populations has been reported in a number of instances (Bourtzis 2008). The widespread occurrence of *Wolbachia* in *H. irritans* suggests that *H. exigua* will be a competent host and is a suitable target species for novel control approaches based on *Wolbachia* infection.

5. Conclusion

Buffalo fly infestations are consistently listed by beef and dairy producers as amongst their main cattle health problems. In addition buffalo flies are spreading southward into areas where they have never been previously seen and the rate of this increase in the range of buffalo flies is expected to increase with changing climate.

Control options include chemical treatments, breed choice and the use of dung beetles, but functionally control usually depends on chemical options which suffer difficulties with the development of resistance, the potential for residues, occupational health and safety issues and environmental contamination. In addition, there are growing price premiums for commodities produced in low chemical systems. Research into the development of new and cheaper controls for BF has been limited by the need for cattle based systems for the conduct of research. These are expensive, limit the type of research that can be conducted and increasingly suffer animal ethics difficulties. In this project we have developed two new tools that will be critical to furthering buffalo fly research.

We have developed a completely *in vitro* BF colony that has now been reared through 33 generations in the laboratory without the use of live animal hosts

We have also developed the first ever *Haematobia irritans* and *Haematobia exigua* cell lines. Although the previous *H. exigua* cell line was lost during the Brisbane floods, we have determined methods that we expect will enable the re-establishment of a *H. exigua* cell line in our Brisbane laboratories

These two new experimental tools will be important in facilitating research towards new and better controls for buffalo fly and maintaining or improving the efficiency of currently available methods. In particular, our finding that *Wolbachia* is not present in *H. exigua*, but is ubiquitous in closely related *H. irritans* suggesting that *H.exigua* would also be a competent host, indicates that investigation of this organism as a basis for new control strategies for BF is warranted.

6. Appendix

6.1 Appendix. *Haematobia exigua* rearing method

A.1 Rearing conditions

Adult flies are housed in 30 x 30 x 45 cm aluminium framed cages, with glass ends, mesh sides and cloth sleeve entrance. They are held in a constant environment room at 28^{o}C and 70%RH, with a 15 hour light and 9 hour dark light regime. The dawn light regime, commencing at 4am consists of one hour moonlight (Reptile One® 100W night heat lamp), followed by one hour red light (Philips Party Light, red, 40W) positioned below the cages, one hour, incandescent white light (Exoterra Glow Light® with 100W Sun Glo neodymium daylight lamp) above the cages and then full fluorescent room lighting from 7am until 6pm. The dusk regime is similar to the dawn regime, but applied in reverse order, with room lights off at 6pm transitioning to complete darkness from 9 pm.

Prior to use cages are cleaned by soaking in a weak detergent solution for about 5 minutes, scrubbed clean and rinsed with water. They are then rinsed with 100% ethanol and allowed to dry. The cage entrance sleeve is removed, soaked in dilute detergent solution overnight, rinsed with 100% ethanol and allowed to dry prior to use. Mesh sides are removed, washed and rinsed with 100% ethanol and allowed to dry. Tulle is placed over the top of the clean cage and held in place with rubber beading, the bottom of the cage is left open

A Wettex® sheet (300 mm x 500 mm) covered with a piece of blotting paper (520 mm x 350 mm) is laid on an aluminium tray. The Wettex and paper are saturated with tap water and the cage placed on top. This provides extra humidity in the cage, as well as a place for the flies to lay eggs. Excess moisture is required to stop the eggs from drying out and dying. Cages are secured in trays using two elastic ‘octopus’ straps.

Manure is placed onto the paper towelling in the cage when flies are expected to lay eggs (flies greater than 5 days old). For eggs collected over one night, no manure is placed inside the cage. For collection over two or more nights, 2 x 500 ml quantities of manure are place on the bottom of the cage. If collecting over 3 nights (e.g. weekend), 6 x 500 ml pats of manure are placed on the bottom of the cage. If manure is left in cage for more than one day, the top of the manure is moistened with a little water daily.

A piece of black cardboard (with wire taped along centre) is placed in the cage curving from the base to the top to form a “cattle back” platform for the flies.

A.2 Feeding flies

Flies are fed bovine blood, collected in bottles containing 4.5 g sodium citrate and 60 mg kanamycin sulfate mixed with 50 ml water per litre blood. This is stored in the refrigerator (up to 4 weeks) or frozen in 100 ml or 200 ml lots in plastic containers and thawed in refrigerator as needed. The antibiotic can be omitted, but blood longevity is reduced

A continuous supply of blood is provided at the top of the cage from two silicon membrane blood feeders. These feeders are water jacketed and have water heated to 36^{o}C circulated through them from a water bath. Blood is contained by silicone membranes (c. 0.06 mm thickness) supported by polyester membrane mesh through

which the flies feed. Membranes rest on the tulle top of the cage to provide access for feeding flies.

During periods when the flies can not be regularly checked (e.g. weekends) blood soaked cotton pads (20 mm x 100 mm) covered with Parafilm® are also provided as a backup.

Blood feeders are cleaned daily and a new supply of blood added. New silicon membranes are used at each change although a membrane configuration that allows repeated uses is currently under test.

Flies are also provided ad lib with a vitamin/mineral supplement prepared by dissolving 1 tablet of Hair Skin and Nails vitamin and mineral supplement (Herron Pharmaceuticals) in 500 ml Mount Franklin water. This is delivered from an inverted 200 ml container with small holes in the lid covered by a piece of Wettex (40 mm diameter circle) attached to the lid.

A.3 Egg collection

Eggs are generally collected Monday, Wednesday and Friday of each week from flies greater than 5 days old. They are visible as 1 mm long brown eggs spattered on the paper under the cage and on the manure. The dung from the base of the cage is used in larval rearing and eggs on the paper are washed into a container with a wash bottle. When precise estimates of rearing success are required, no dung pats are used in the base of cages and eggs are collected from the paper in the base of cages. Numbers of eggs are estimated volumetrically or by counting under a Maggylamp® and the required number added to fresh 700-1100 ml dung pats.

A.4 Larval rearing

All dung used in the colony is collected from pastured cattle that have received no chemical treatments and is uncontaminated with urine. Manure is frozen prior to use for storage and to kill any insects present. A sub-sample is tested by rearing *Musca domestica* as a further check on suitability. Lucerne crumbs (3 g/kg) are added to provide for fluctuations in dung quality.

Dung pats for rearing are prepared by mixing defrosted manure in a 2 litre ice-cream container. Moisture content is adjusted to 80-85% by using extra lucerne to soak up excess moisture if the manure is too wet, or adding water if the manure is too dry. The dung pats (700 to 1100 ml) are then placed onto clean, sieved sand poured into a 10 L plastic container.

The smaller pats of manure from inside the cage are ‘seeded’ with eggs are then placed on top of the larger pats and pressed into the pat. The dung is covered with moist paper towelling and a 250 ml disposable ‘take out’ plastic food container to reduce the rate of drying. The 10 litre container is covered with the gauze lid and held at at 28°C and c. 70% humidity for 7 days to allow larval feeding and pupae formation.

A.5 Collecting pupae

Six days after addition of the eggs the manure pat is removed from the sand and either washed (see below) or covered with paper towelling, then placed in a 5 L container with a gauze lid. The sand is spread onto an aluminium tray and held in an air conditioned room (23°C) overnight to dry.

On day 7, the sand is sieved to collect pupae and the manure pat is broken up and soaked in a container of water to remove pupae remaining in the pat by flotation. Waste water/manure is collected and passed through the coarse sieve to collect any remaining pupae and the solids discarded.

Pupae are briefly rinsed in a weak bleach solution, followed by rinsing in water. They are then dried and placed into containers covered with a gauze lid where they are held until flies begin to emerge. Alternatively, if a record of fly numbers is not required they can be allowed to emerge directly into fly cages.

7. Bibliography

Anderson JME (1995) Culturing Haematobia exigua de Meijere (Diptera: Muscidae) in the laboratory. *Journal of the Australian Entomological Society* **34**, 17-21.

Anon (2005) Recommendations for integrated buffalo fly control. Meat and Livestock Australia, Sydney.

Baerg DC (1971) Colonization of Anopheles pseudopunctipennis in Panama. *Journal of Medical Entomology* **8**,180–182.

Bean KG, Seifert GW, Macqueen A, et al. (1987) Effect of insecticide treatment for control of buffalo fly on weight gains of steers in coastal central Queensland. *Australian Journal of Experimental Agriculture* **27**, 329-334.

Bock RE, Blight GW, Kingston TG and de Vos AJ (1995) A survey of cattle producers in the Boophilus microplus endemic area of Queensland to determine attitudes to the control of and vaccination against tick fever. *Australian Veterinary Journal* **72**, 88-92.

Bolton HT, Butler JF, and Carlson DA (1980) A mating stimulant pheromone of the horn fly Haematobia irritans(L). *Journal of Chemical Ecology 6*, 951-964.

Bourtzis, K (2008) *Wolbachia*-based technologies for insect pest population control. Advances in *Experimental Medicine and Biology* **627**, 104-113.

Burg JG, Knapp FW and Silapanuntakul S (1993) Feeding Haematobia irritans (Diptera: Muscidae) adults through a nylon reinforced silicone membrane. *Journal of Medical Entomology* **30**, 462-466.

Curtis, CF and SP Sinkins (1998) *Wolbachia* as a possible means of driving genes into populations. *Parasitology* **116** Suppl: p. S111-5.

Dalton LW, Kinzer HG, Reeves JM and Atmar JW (1978) Host location in the horn fly: roles of heat, CO2, water vapor, and cow-produced odors in attraction. *Southwestern Entomologist* **3**, 147-153.

Doube BM, Fay MAC and Aschenborn HH (1982) Rearing the blood feeding fly Haematobia thirouxi potans in the laboratory *Onderstepoort Journal of Veterinary Research* **49**, 255-256.

Farnsworth WR; Collett MG and Ridley IS (1997) Field survey of insecticide resistance in Haematobia irritans exigua de Meijere (Diptera: Muscidae). *Australian Journal of Entomology* **36**, 257-261.

Floate KD, Kyei-poku GK, Coghlin PC (2006) Overview and relevance of Wolbachia bacteria in biocontrol research. *Biocontrol Science and Technology* **16**, 767-788.

Goblirsch MJ, Spivak MS, Kurtti TJ (2013) A Cell Line Resource Derived from Honey Bee (Apis mellifera) Embryonic Tissues. *PLoS ONE* **8** e69831.

Guglielmone AA, Gimeno E, Idiart J Fisher WF, Volpogni MM, Quaino O, Anziani OS, Flores SG and Warnke O (1999). Skin lesions and cattle hide damage from

Haematobia irritans infestations. *Medical and Veterinary Entomology* **13**, 324-329.

Harget LT and Goulding RL (1962) Studies on the behaviour of horn fly ,Haematobia irritans (Linn.) Tehnical Bulletin 61 Agricultural Research Station, Oregon State University. 27 pp.

Harris RL (1962) Laboratory colonisation of horn fly Haematobia irritans (L) *Nature* **196**, 191-192.

Harvey TL and Brethour JR (1979) Effect of horn flies on weight gains of beef cattle. *Journal of Econonomic Entomology* **72**, 516-18.

Harvey TL and Brethour JR (1981) Control of the horn fly, Haematobia irritans (L) (Diptera: Muscidae) on cattle by partial herd treatment with fenvalerate impregnated ear tags. *Protection Ecology* **2**, 313-317.

Haufe WO (1987) Host parasite interaction of blood feeding dipterans in helaht and productivity of mammals. *International Journal for Parasitology* **17**, 607 -614.

Hill GF (1916). Some notes on the bionomics of the buffalo fly (Lyperosia exigua de Meijere). *Proceedings of the Linnean Society o of New South* Wales **41**, 763-768.

Jeyaprakash A and Hoy MA (2000) Long PCR improves Wolbachia DNA amplification: wsp sequences found in 76% of 63 arthropod species. *Insect Molecular Biology* **4**, 393–405.

Jonsson NN and Matschoss AL (1998) Attitudes and practices of Queensland dairy farmers to the control of the cattle tick (Boophilus microplus). *Australian Veterinary Journal* **76**, 746–751.

Jonsson NN and Mayer DG (1999) Estimation of the effects of buffalo fly (Haematobia irritans exigua) on the milk production of dairy cattle based on a meta-analysis of literature data. *Medical and Veterinary Entomology* **13**, 372-376.

Kambris Z, Cook PE, Phuc HK and Sinkins SP (2009) Immune activation by life-shortening Wolbachia and reduced filarial competence in mosquitoes. *Science* **326**, 134–136.

Kerlin RL and Allingham PG (1992). Acquired immune response of cattle exposed to buffalo fly (*Haematobia irritans exigua*) *Veterinary Parasitology* **43**, 115-129.

Kinzer HG, Houghton WE, Reeves JM et al (1984) The influence of horn flies on weight loss in cattle, with notes on prevention of loss by insecticide treatment *Southwestern Entomolgist* **9**, 212-217.

Kunz SE and Schmidt CD (1985) *Haematobia irritians*. In Handbook of Insect Rearing, vol 2. Singh P and Moore RF editors. Elsevier New York. Pp 113-117.

Kuramochi K (1989) Studies on the reproductive biology of the horn fly, Haematobia irritans (L.) (Diptera: Muscidae) III. Mating behavior of the fly in the field. *Applied Entomology and Zoology* **24**: 326-333.

McGraw EA and O'Neill SL (2003) Beyond insecticides: new thinking on an old problem. Nature Reviews: Microbiology **11**, 181-193.

Perotti MA and Lysyk TJ (2003) Novel growth media for rearing larval horn flies, Haematobia irritans (Diptera: Muscidae) *Journal of Medical Entomology* **40**, 22-29.

Rutledge LC, Ward RA and Gould DJ (1964) Studies on the feeding response of mosquitoes to nutritive solution in a new membrane feeder. *Mosquito News* **24**, 407-419.

Sackett D, Holmes P, Abbott K, Jephcott S, Barber M (2006) Assessing the economic cost of endemic disease on the profitability of Australian beef cattle and sheep producers. Final report of project AHW-087 Meat and Livestock Australia, Sydney, 119 pp.

Schmidt CD; Dreiss JM, Eschle JL; et al. (1976) Horn fly: modified laboratory rearing methods..*Southwestern Entomologist* **1**, 49-51.

Sinkins SP and O'Neill, SL (2000). *Wolbachia* as a vehicle to modify insect populations, in Insect Transgenesis: Methods and Applications, AM Handler and AA James, Editors. CRC Press: Boca Raton. p. 271-288.

Smagghe G, Goodman CL, and Stanley D (2009) Insect cell culture and applications to research and pest management. *In Vitro Cellular and Developmental Biology - Animal* **45**, 93-105.

Spradbery JP and Tozer RS (1996) The efficacy of diazinon impregnated ear tags against buffalo fly and resulting weight gains and diazinon residues in meat and milk. *Australian Veterinary Journal* **73**, 6-10.

Stegeman DA, Tozer RS, Sutherst RW (1996) Procedures for mass rearing the buffalo fly, Haematobia irritans exigua de Meijere (Diptera: Muscidae). *Australian Journal of Entomology* **35**, 77-79.

Sutherst RW, Bourne AS, Maywald GF and Seifert GW (2006) Prevalence, severity, and heritability of Stephanofilaria lesions on cattle in central and southern Queensland, Australia. *Australian Journal of Agricultural Research* **57**, 743-750.

Thomas PL, Davis NC, (1984) A method for rearing the buffalo fly, Haematobia irritans exigua de Meijere. *Journal of the Australian Entomological Society* **23**, 81-82.

White N; Sutherst RW, Hall N, Whish-Wilson P (2003) The vulnerability of the Australian beef industry to impacts of the cattle tick (*Boophilus microplus*) under climate change. *Climatic Change* **61** 157-190.

Williams JD, Sutherst RW, Maywald GF and Petherbridge CT (1985) The southward spread of buffalo fly (Haematobia irritans exigua) in eastern Australia and its survival through a severe winter. *Australian Veterinary Journal* **62**, 367-369.

Zhang B, McGraw E, Floate KD, James PJ, Jorgensen WK and Rothwell JT (2009) Wolbachia infection in Australasian and North American populations of

Haematobia irritans (Diptera: Muscidae). *Veterinary Parasitology* **162**, 350–353.