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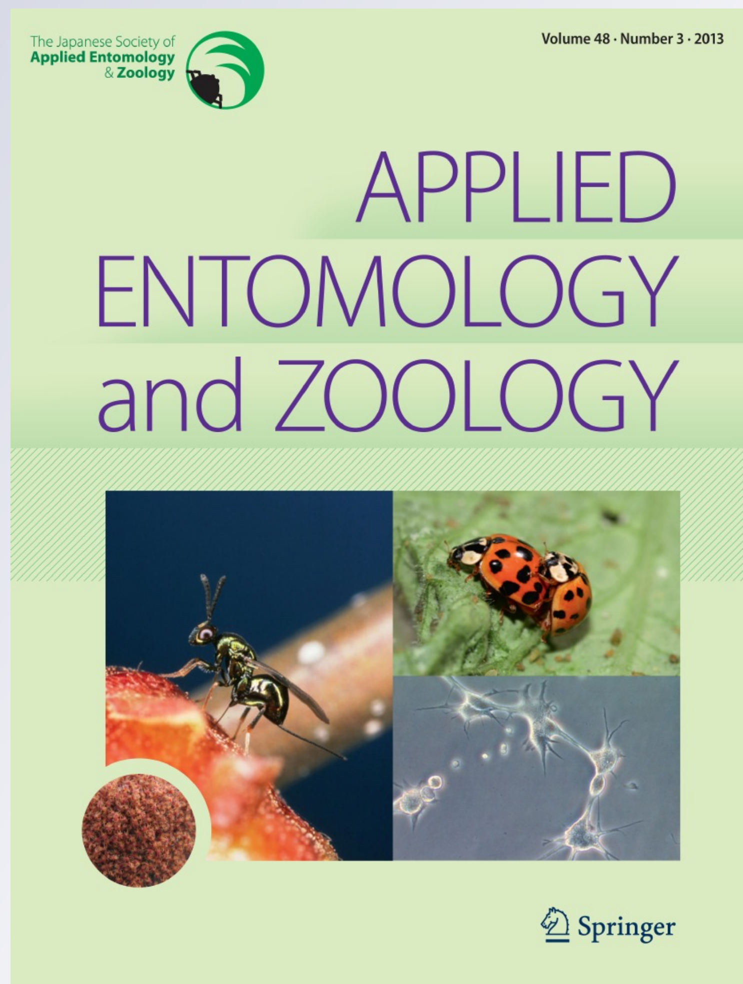

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ORIGINAL RESEARCH PAPER

Rearing bacteria and maggots concurrently: a protocol using *Lucilia sericata* (Diptera: Calliphoridae) as a model species

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Abstract Maggot debridement therapy using live *Lucilia sericata* (Meigen) larvae is an efficient and cost-effective way to treat chronic wounds. The recent increase in studies to assess the antibacterial properties of *L. sericata* has created a need for a simple, low-cost, and comprehensible rearing and investigative method for researchers with little or no entomological experience. This paper describes and evaluates a reproducible protocol for sterilising and rearing blowfly larvae utilising two sterile artificial diets (blood–yeast agar and pre-prepared blood agar plates) that is suitable for directly investigating the effect of larvae on microbial growth. Using *Lucilia sericata* as a model, the results show that larval growth on the pre-prepared blood agar diet is detrimental to larval growth and survival, whereas larval growth and survival on the blood–yeast agar diet are comparable to those of larvae raised on porcine liver. This diet is proposed as a standard for blowfly and bacteria interaction studies investigating clinical microbial strains. Developmental data are provided for *L. sericata* larvae raised on both sterile and nonsterile diets so that researchers can determine the effect of treatment based on the length of time for larvae to reach the required life stage at 25 ± 2 °C. Information on larval ageing (instars at an average of 1, 2, 3 and 4 days), oviposition times (4–5 days after adult emergence) and adult longevity on the diets (102–116 days) is also given.

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Introduction

In recent years, an increasing number of hospital and community infections caused by antibiotic-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) have resulted in an escalating number of patient mortalities (Corwcroft and Catchpole 2002; Lamagni et al. 2011). In Europe alone, excess mortality due to resistant bacterial hospital infections exceeds 25,000 per year, and associated healthcare costs and productivity losses are estimated to be more than 1.5 billion euros each year (World Health Organisation 2012).

Maggot debridement therapy (MDT) using live *Lucilia sericata* (Meigen) larvae was re-introduced in Europe in 1995 (Thomas 2006). It is an efficient and cost-effective way to heal chronic wounds (Wayman et al. 2000; Thomas and Jones 2001; Thomas 2006), including those infected with antibiotic-resistant bacteria (Diver and Thomas 2000; Bowling et al. 2007; Dumville et al. 2009). *Lucilia sericata* has since been used in laboratory-based experiments to explore how larvae facilitate the healing process. Such studies revealed that healing is a combined result of the eradication of necrotic tissue and its associated bacteria through larval ingestion along with the regeneration of tissue and release of specific proteases and antimicrobial compounds into the wound environment (Nigam et al. 2006).

To date, laboratory-based studies using *L. sericata* have demonstrated activity against antibiotic-resistant bacteria such as MRSA (Kerridge et al. 2005; Huberman et al. 2007b; Bexfield et al. 2008, 2010). As a result, compounds

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have been identified which have potential as future therapeutic agents (Huberman et al. 2007a; Bexfield et al. 2008, 2010; Altincicek and Vilcinskas 2009; Andersen et al. 2010; Čeřovský et al. 2010; Telford et al. 2011). Consequently, there is a need for further work in this area to explore the antibacterial spectra of this and other insect species and to identify and develop novel antibacterial compounds.

In order to properly analyse the experimental interactions between insects and microbes, the rearing substrate must be sterile (pathogen-free) so that the initial microbial load is confirmed. It is also important that standard media be utilised so that data are comparable across research groups. Whilst numerous papers have been published in the field of MDT, information about how larvae were sterilised, housed and reared is frequently absent. Papers that do outline a sterile larval growth medium (Mumcuoglu et al. 1999; Kerridge et al. 2005; Daeschlein et al. 2007; Jaklic et al. 2008; Andersen et al. 2010) show no consistency in substrate type and provide no larval developmental data at the temperatures chosen. This makes a comparison of the effects of the research presented in the literature difficult.

Entomological studies have outlined artificial diets for *L. sericata* larvae (Murdoch and Smart 1931; Michelbacher et al. 1932; Hobson 1932; Barlow and Kollberg 1971; Tenquist 1971; Mandeville 1988; Daniels et al. 1991; Sherman and Tran 1995; Tachibana and Numata 2001; Wolff and Hansson 2005), and more recently for adult blowflies (Zhang et al. 2009). These studies have added significant insight into the effects of blowfly larval diet on development. However, the majority use diets that cannot be heat-sterilised and would not support concurrent growth of both larvae and microbes, as is required in some antibacterial studies (Lerch et al. 2003; Daeschlein et al. 2007; Kawabata et al. 2010; Margolin and Gialanella 2010; Barnes and Gennard 2011). There is also minimal information in the literature on how to house and breed *L. sericata* (Sherman and Wyle 1996; Wolff and Hansson 2005) for maggot therapy (MDT) research, and a lack of vital information for researchers breeding blowflies for the first time, such as how to determine larval age and when to provide oviposition substrates.

Therefore, the aim of this paper was to outline a simple, low-cost, and reproducible protocol for sterilising eggs and rearing *L. sericata* larvae which could be used as a standard procedure for insect–microbial interaction studies within the area of MDT, and in particular to investigate antibacterial activity against those microbes that colonise the human body detrimentally. The paper also provides larval developmental data on a sterile artificial medium which could sustain the microbial growth of a test species if necessary, at a temperature suitable for bacterial and larval growth (based on length), that has not been published to date. To achieve these aims, *Lucilia sericata* were sterilised at the egg stage and then reared on either of two sterile artificial diets. Nonsterile control larvae were also reared. The larvae on the two diets and the control larvae were then compared in order to determine the suitability of the diets for laboratory use. Developmental data in terms of larval length is provided in this report, along with the lengths of time taken for the larvae to reach the required life stage. This information is valuable when investigating the effect of antimicrobial chemicals as a means of controlling bacterial growth (Bexfield et al. 2004; Thomas 1993). Information on larval ageing, oviposition times, and adult longevity is also presented.

Materials and methods

Insect housing

Breeding colonies of *L. sericata* adults were maintained under a lighting regime of 16:8 (L:D) hours to mimic the longer photoperiods of the summer (breeding) months in 30 × 30 × 30 cm Bugdorm® breeding cages (Watkins and Doncaster, Cranbrook, UK) at room temperature (25 ± 2 °C). Light was supplied by full-spectrum light bulbs controlled by plug-in timers. Larvae were maintained at 25 ± 2 °C in Stewart® electric propagators (52 × 42.5 × 28 cm) with plug-in thermostats (TLC Electrical Supplies Direct, London, UK) in the same room as the adult flies. The propagator environment ensures sufficient humidity for the immature stages to grow satisfactorily and prevents desiccation.

Insect rearing

Adult flies were provided with two individual plastic pots, one containing granulated sugar and the other water. Newly emerged adult flies were provided daily with 25 g of fresh porcine liver, since a protein source is necessary for ovary development and oocyte maturation (Kamal 1958; Daniels et al. 1991; Wall 1993). After 4–5 days, the matured females were able to oviposit on fresh liver.

Determination of larval stage

Lucilia sericata goes through complete metamorphosis. Eggs hatch into larvae which pass through three feeding growth stages (instars). To determine the larval instar in this study, a binocular light microscope (magnification ×30) was used to examine the posterior spiracles and determine the number of posterior spiracle slits and hence the instar.

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Sterilisation of *L. sericata* eggs

A deposit of approximately 300 *L. sericata* fly eggs was collected from porcine liver placed in the adults' cage as an oviposition site. Egg clumps were agitated in 1 % sodium sulfite (Sigma Aldrich, Gillingham, UK) in a 90 mm Petri dish and the individual eggs were separated using a small, sterile paintbrush. The 1 % sodium sulfite solution was removed by pipette from around the eggs, which were then washed in 70 % ethanol solution. The 70 % ethanol solution was removed by pipette, and the eggs were rinsed with sterile deionised water. Excess water was removed by pipette and the Petri dish was left in a tub overnight for the eggs to hatch in a previously sterilised propagator with appropriate levels of humidity. Approximately 16 h later, sterile larvae were transferred to a heat-sterilised artificial diet to continue their development to adult eclosion.

Absence of bacteria

In order to confirm the sterility of the eggs, 10 % of the eggs from both treatments and the control were crushed in one pipette drop of sterile deionised water using a sterile pestle and mortar. A loop of this mixture was spread onto warmed nutrient agar and incubated at 37 °C for 24–48 h. An absence of bacterial growth after this time confirmed that the egg surfaces were sterile compared to eggs treated solely with deionised water (a control to confirm the effectiveness of the sterilising treatment) and that the method had therefore worked satisfactorily. Sterility was maintained during growth by retaining larvae in an enclosed sterile environment (Petri dishes) and ensuring that good laboratory etiquette was upheld.

Artificial diets for blowfly larvae

The artificial diets were chosen as they were both reflective of the natural larval blowfly diet, easy to source from common laboratory suppliers, odour-free in use and contained blood, which is a common component of agar plates used to grow clinically important microbes. Blood may be added to a number of microbial growth media as, in small quantities, it also improves microbial growth—such as that of streptococci (Russell et al. 2006), which is one of the most frequently investigated microorganism genera with regard to maggot therapy investigations, where microbes are predominantly human pathogens. In addition, blood provides both larvae and microorganisms with a nutrient base, and its inclusion ensures an artificial diet for fly larvae as near to a natural diet of flesh as possible.

The first diet consisted of 20 % horse blood–5 % yeast agar (HBYA) and was modified from the population studies growth medium used by Daniels et al. (1991); 500 ml of blood agar base were made up with the addition of 5 % of yeast extract and autoclaved at 121 °C for 15 min. When the agar had cooled to 50 °C, 20 % of the defibrinated horse blood (Oxoid, Basingstoke, UK) was added. The mixture was poured into sterile Petri dishes (20 ml per dish) and left to cool at room temperature.

The second diet consisted of pre-poured 5 % blood agar plates (BA, Oxoid) without additional supplements, and did not involve any further preparation. To facilitate larval food consumption, grooves and dips were made on the surfaces of both artificial diets with a sterile pipette tip. The diets were stored in the fridge at 4–6 °C until required.

Blowfly development

An experiment was conducted at the University of Derby in July 2010 to assess the suitabilities of both artificial diets as low-larval-density rearing substrates for sterile *L. sericata* compared to a control of porcine liver. The porcine diet was prepared as follows: liver (suitable for human consumption) was chopped up into 20 g units (to equal the weight of the HBYA and BA diets) and added to each of ten sterile Petri dishes. The Petri dishes were retained in sterilised plant propagators under controlled conditions in terms of temperature and light regime (see "Insect housing").

One hundred first-instar larvae were used per treatment and placed on Petri dishes containing either artificial diets or liver and left overnight to feed. Ten first-instar larvae were transferred to each Petri dish; this number enabled unlimited growth of larvae without impeding development (Daniels et al. 1991). Once the larvae had ceased feeding and dispersed from the food source, they were termed "post-feeding". At this stage, lids were removed from the artificial diets and sawdust was added to the base of the propagators to provide a suitable, dry, pupariation site. Once they had entered pupation, the insects were transferred from propagators to Bugdorm cages to complete their life cycle.

A positive relationship has been confirmed between larval nutrition and insect size (Daniels et al. 1991; Honěk 1993; Thomas 1993). Insect size and life stage were used as measurable variables in this experiment, as these are of most value to researchers within the field of MDT. Larval lengths were measured daily (mm) from a random sample in each treatment group and the life stage recorded. This was achieved by driving larvae to the bottom of the Petri dish using light, turning the dish over, and on each occasion measuring 10 larvae per treatment. Larvae were stretched linearly when using electronic callipers, the accuracy of which was determined to be ±0.5 mm.

The oviposition interval, size (mm), male:female ratio and longevity were recorded for emerging adult flies in each treatment group. Porcine liver was placed in the cages of newly emerged flies daily to assess the interval between

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emergence (eclosion) and oviposition, and a count of the resulting eggs was made to determine fecundity for each treatment group. Mean values were analysed in repeated measures ANOVA (RM ANOVA) tests using the statistical package SPSS (version 16), and Scheffé tests were used as post hoc tests to determine significance.

Results

The 70 % ethanol solution sterilised the surfaces of all 300 fly eggs and was therefore deemed a suitable sterilising agent for the protocol. Controls using nonsterile larvae demonstrated growth of >100 microbial colonies per egg batch. These microbes were commonly *Staphylococcus* sp., *Micrococcus* sp. and *Streptococcus* sp. No microbes were noted on the sterile agar plates during larval growth.

For the purpose of statistical analyses, life-stage descriptors were assigned numerical values (0, egg; 1, first instar; 2, second instar; 3, third instar; 4, post-feeding larvae; 5, pupae; 6, adult). Developmental data showed that there was a significant difference in development rates (egg to adult eclosion) between diet groups (RM ANOVA: $F(2, 34) = 4.71$, $p = 0.019$). Overall development rate was significantly slower on 5 % blood agar (BA) compared to the liver control ($p = 0.05$).

The type of diet significantly influenced the size (mm) of *L. sericata* from first instar to adult eclosion (RM ANOVA: $F(2, 27) = 9.70$, $p = 0.001$) (Fig. 1). Overall, there was no significant difference between the size of *L. sericata* reared on the HBYA diet compared to the size of the liver diet controls ($p = 0.441$). However, the size of *L. sericata* on the BA diet was significantly reduced compared to those on the HBYA diet ($p = 0.001$) and the liver diet controls ($p = 0.021$).

When size was investigated within the 15-day development period, results showed that there was no variation between treatment groups on day 2 when all insects were in the second instar (RM ANOVA: $F(2,16) = 0.63$, $p = 0.534$). However, from day 3 to day 13 when insects were in the third instar, post-feeding stage or puparial stage, there was a significant size difference between *L. sericata* larvae in the BA group compared with those in the HBYA and liver diet groups ($p < 0.05$). On day 15, when the adult flies had emerged from each treatment group, there was a highly significant difference in the size of the flies in the BA group compared to those in the HBYA and liver diet groups ($p < 0.0001$) (Table 1). In each treatment group there was a near 50:50 ratio of males to females in all populations.

Eclosion data showed that the emergence of adult flies was staggered for each treatment group (Fig. 2). *Lucilia sericata* reared on the HBYA and liver diets emerged as adults on day 14; those reared on BA emerged on day 15. Males emerged during the first 24–36 h, after which female emergence occurred (Murdoch and Smart 1931). Eighty-six percent of the adults emerged from the liver diet group, 81 % from the HBYA diet group and 46 % from the BA

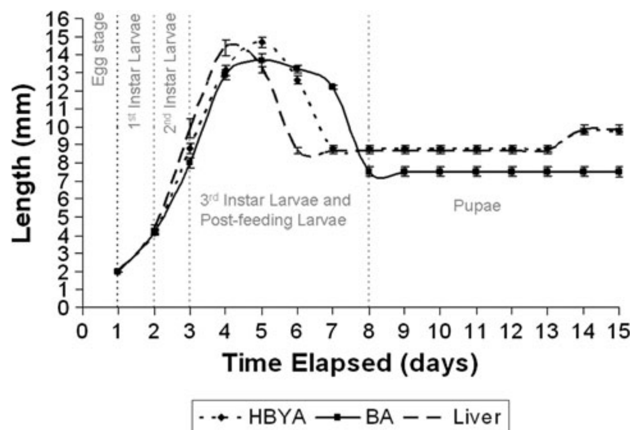

Fig. 1 Mean length of 100 individuals of *Lucilia sericata* from egg to adult eclosion reared on HBYA (20 % horse blood–5 % yeast agar), BA (5 % blood agar) or porcine liver (control) over a 15-day period at 25 ± 2 °C. *Error bars* indicate standard errors. *Life stage markers* show when flies in all treatment groups had reached the indicated life stage

Table 1 Mean sizes (±standard deviation) of male and female *Lucilia sericata* adults reared on HBYA (20 % horse blood–5 % yeast agar), BA (5 % blood agar), or porcine liver (control) at 25 ± 2 °C

Diet	Male length	Female length
HBYA diet (mm)	8.5 ± 0.5	9.8. ± 0.6
5 % BA diet (mm)	7.3 ± 0.7	7.5 ± 0.8
Liver diet (mm) control	9.2 ± 0.5	9.9 ± 0.6

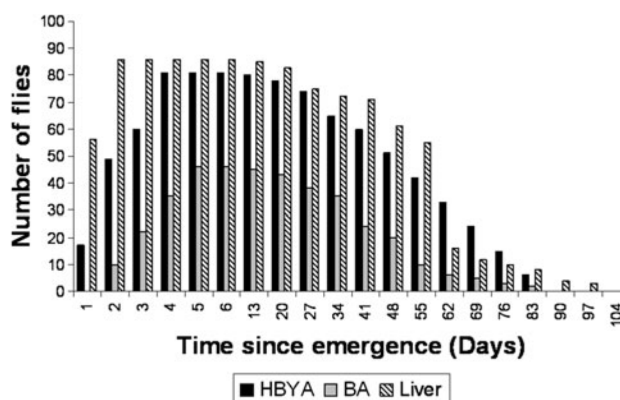

Fig. 2 Comparison of longevity of *Lucilia sericata*—adult eclosion (completed by day 15) to final death (day 116) on three diets: horse blood yeast agar (HBYA; 20 % horse blood–5 % yeast, agar), blood agar (BA; 5 % blood agar) or porcine liver (control) at 25 ± 2 °C. The time since emergence represents the number of days spent in the adult stage for flies in each treatment. Barnes and Gennard (*Top*)

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diet group. The first females reared on HBYA and liver diets were able to oviposit 5 days after emergence, and females reared on BA diets after 4 days. Adults reared on liver diets lived for a maximum of 116 days; adults reared on the HBYA diet or the BA diet lived for a maximum of 102 days at 25 ± 2 °C.

Discussion

The results confirm that 70 % ethanol solution is an efficient and cost-effective sterilising agent for *L. sericata* eggs. Ethanol is commonly stocked in standard biological laboratories and so is easy to source by most researchers working with blowflies. An examination of the microbes on the fly eggs showed that they were *Staphylococcus* sp, *Micrococcus* sp. and *Streptococcus* sp. The microbes on the porcine liver were also from these genera, so those on the fly eggs were most likely transferred from the porcine liver through oviposition.

Lucilia sericata satisfactorily completed development (first-instar larvae to adult eclosion) on all three diets tested. The developmental rates on porcine liver were comparable to those reported by Grassberger and Reiter (2001) at the same temperature (25 °C). There was no significant variation in *L. sericata* developmental rate and size (mm) when reared on a sterile 20 % horse blood–5 % yeast agar (HBYA) diet compared to a control diet of porcine liver. However, developmental rates were slower and flies were considerably smaller on a sterile 5 % blood agar (BA) diet. These results support data published by Daniels et al. (1991) on population studies, who also found that a 20 % blood–yeast agar diet was able to sustain normal growth of *L. sericata* at this density (2 g per larva) at least as well as lamb's meat did. However, Daniels et al. (1991) did not investigate larval success with the effectiveness of the diet at maintaining microorganism growth, as was shown by Barnes and Gennard (2011).

In the present study, the percentage of *L. sericata* that emerged as adults was similar for those on the HBYA diet (81 %) and the liver diet controls (86 %). These emergence percentages are in line with previous studies which reported that 69–91 % of *Lucilia* (*Phaenicia*) *sericata* emerged after being reared on beef liver (Kamal 1958). However, the percentage that emerged from the BA diet was significantly reduced (46 %). The percentage of mortality has been shown to be dependent upon whether larvae obtain the minimum food requirements needed to satisfy the physiological level of their metamorphosis and further successful development (Kamal 1958). Adult survival in most insects is positively correlated with size (Vogt et al. 1985). Flies reared on a BA diet were significantly smaller from third instar through to adult eclosion than those raised on HBYA or porcine liver. The most significant size difference was in the adult stage, where female adults reared on the BA diet were much smaller (an average of 7.4 mm) than those reared on the HBYA and liver diets (Table 1).

Previous research has shown that adult size of *L. sericata* varies in proportion to overcrowding intensity experienced at the larval stage (Wall 1993). However, larvae were not reared in an overcrowded environment in this experiment (Kamal 1958). Therefore, the size difference demonstrated above must result from a difference in larval diet. This view corresponds with previous research in which larval food/nutrition restriction produced undersized puparia, from which small-sized adults emerged (Webber 1955; Kamal 1958).

Results from the developmental study show that adult flies emerged 1 day later when reared on BA than those reared on a HBYA diet or liver diet controls. However, after being supplied with porcine liver from the day of emergence, female flies were able to oviposit after 4 days when reared on a BA diet and 5 days when reared on a HBYA diet or liver control. These times support previous data indicating that *Lucilia* (*Phaenicia*) *sericata* is able to oviposit 5–14 days after adult emergence when supplied with a daily protein source and oviposition substrate (Kamal 1958). Kamal (1958) also demonstrated that the oviposition period was reduced in underfed populations compared with normal populations, suggesting that a lack of nutrition in the BA diet may have played a part in reducing the oviposition period.

The number of eggs produced were markedly reduced for flies reared on BA (130) compared to those on the HBYA diet (>1,000) and liver diet controls (>1,000). However, the ratio of males to females remained approximately 50:50 for populations reared on all three diets. These results support a lack of effect of competition or food shortage on sex ratio (Kamal 1958; Andersen 1961).

The egg numbers produced do not appear to be the result of there being half the number of female flies in the BA treatment compared to the HBYA and liver treatments. More than double the number of eggs were produced in the HBYA and liver treatments (>1,000) than in the BA treatment (130). Therefore, the reduced size of the adult flies reared on 5 % blood agar (Table 1) most likely influenced their fecundity. Previous research shows that the number of oocytes matured by *L. sericata* is significantly related to female size (Wall 1993), as is the number of ovarioles in adult female *L. cuprina* (Webber 1955). Fly fecundity depends upon size, which in turn is dependent upon the quantity of food consumed during the larval stage (Webber 1955; Kamal 1958; Vogt et al. 1985). However, undersized female *Lucilia* species, whilst having small ovaries and a reduced number of ovarioles, are still able to produce normal-sized eggs, which produce normal-sized

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adults (Webber 1955; Kamal 1958). This was confirmed in the present study; eggs from all treatments were approximately 1.5 mm in length.

The adult *Lucilia (Phaenicia) sericata* lifespan in laboratory cultures is 40–59 days (Kamal 1958). In the field, the longevity of adult female *L. sericata* is dependent on a range of factors, such as the time of year—flies live three days fewer on average in August than in July (Wall 1993).

In summary, this experiment demonstrated that the HBYA diet fulfils the criteria for an artificial diet for sterile laboratory cultures of *L. sericata* which can also support bacterial growth studies of species of microorganisms that are of clinical importance. It uses a medium that is readily available in the majority of laboratories, has a simple, replicable methodology for preparation, and produces no malodorous component at any stage of development. It also enables *L. sericata* to complete its life cycle relatively normally, as the larvae that fed on the artificial HBYA diet developed at the same rate as those fed on porcine liver, were comparable sizes throughout the life cycle, and a similar number of adult flies survived to eclosion in both treatments. In comparison, those reared on a pre-prepared BA diet developed more slowly and were reduced in size and fecundity compared to those fed on the HBYA and liver diets. Therefore, the HBYA diet can be used to rear sterile larvae for antimicrobial studies, whilst a BA diet—although commonly used to support bacterial growth—is detrimental to fly development rate, size and survival, and should be used with caution in joint insect–microbial studies. In addition, bacteria such as *Staphylococcus aureus* can grow rapidly on the media-rich HBYA diet, which is useful for in vivo studies investigating interactions between microbes and insect (Barnes and Gennard 2011). It is proposed that this diet should be used as a standard for maggot debridement studies in the future.

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