1-1-2009

Thermal attributes of Chrysomya species

James F. Wallman
University of Wollongong, jwallman@uow.edu.au

Leigh Nelson
lan51@uow.edu.au

Mark P. Dowton
University of Wollongong, mdowton@uow.edu.au

Thermal attributes of Chrysomya species

Abstract

The correct identification of forensically important arthropods for post-mortem interval estimation is crucial, as the rate of larval development can vary substantially between species. The identification of forensically important blowflies of the genus Chrysomya (Diptera: Calliphoridae) may be hampered by their close morphological similarities, especially as immatures. The aim of this study was to establish whether genetically closely related blowfly species would share similar developmental profiles. This could permit the application of developmental data to a number of closely related species, including those for which thermodevelopmental studies are lacking. If Australian Chrysomya were found to share developmental profiles, identification of the blowfly specimen to a level beyond genus may not be necessary, or at least it may not be necessary to distinguish morphologically similar sister species. The three Chrysomya species studied were collected from the same geographical location (Cairns, Australia), reducing the effects of acclimation and population-level genetic variation. The experimental conditions in this study were virtually identical, which enabled direct comparisons to be made among the species. Blowfly larval lengths were obtained for 24-hourly intervals at constant temperatures of 25, 30, and 35 degrees C. The thermal preferences of newly-hatched feeding larvae were determined by their positions on a temperature gradient apparatus. This study established that all three species investigated differed significantly in their developmental profiles, despite the genetic closeness of the sister species Chrysomya megacephala (Fabricius) and Chrysomya saffranea (Bigot). Because of this, genetic distance was not considered to be a useful factor for predicting thermodevelopment profiles of closely related species within a genus, and highlights the necessity for correct species identification.

Keywords

species, attributes, chrysomya, thermal, CMMB

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

Publication Details

Wallman,J. F., Nelson, L. & Dowton, M. P. (2009). Thermal attributes of Chrysomya species. Entomologia Experimentalis et Applicata, 133(3), 260-275.

DOI: 10.1111/j.1570-7458.2009.00932.x

Thermal attributes of *Chrysomya* species

Leigh A. Nelson[1]*, M. Dowton[1] & J. F. Wallman[2]

[1]*Centre for Medical Bioscience, and* [2]*Institute for Conservation Biology and Environmental Management, School of Biological Sciences, University of Wollongong, New South Wales, Australia*

Accepted: 1 September 2009

Key words: Australia, blowflies, forensic entomology, development, thermogenesis, maggot mass, post-mortem interval, PMI, Diptera, Calliphoridae, genetic distance

Abstract

The correct identification of forensically important arthropods for post-mortem interval estimation is crucial, as the rate of larval development can vary substantially between species. The identification of forensically important blowflies of the genus *Chrysomya* (Diptera: Calliphoridae) may be hampered by their close morphological similarities, especially as immatures. The aim of this study was to establish whether genetically closely related blowfly species would share similar developmental profiles. This could permit the application of developmental data to a number of closely related species, including those for which thermodevelopmental studies are lacking. If Australian *Chrysomya* were found to share developmental profiles, identification of the blowfly specimen to a level beyond genus may not be necessary, or at least it may not be necessary to distinguish morphologically similar sister species. The three *Chrysomya* species studied were collected from the same geographical location (Cairns, Australia), reducing the effects of acclimation and population-level genetic variation. The experimental conditions in this study were virtually identical, which enabled direct comparisons to be made among the species. Blowfly larval lengths were obtained for 24-hourly intervals at constant temperatures of 25, 30, and 35 °C. The thermal preferences of newly-hatched feeding larvae were determined by their positions on a temperature gradient apparatus. This study established that all three species investigated differed significantly in their developmental profiles, despite the genetic closeness of the sister species *Chrysomya megacephala* (Fabricius) and *Chrysomya saffranea* (Bigot). Because of this, genetic distance was not considered to be a useful factor for predicting thermodevelopment profiles of closely related species within a genus, and highlights the necessity for correct species identification.

Introduction

The term 'forensic entomology' encompasses all areas of entomology as applied to the law (e.g., Benecke, 2001; Amendt et al., 2004). However, the term is most commonly associated with its involvement in cases of murder or suspicious death, where it can provide a minimum time since death, or post-mortem interval (PMI) (Catts & Goff, 1992; Anderson, 2001). The PMI can be estimated in two ways: from the predictable succession of arthropod colonisation of a corpse (the most important being the Diptera) and by the age of maggots, or fly larvae, present on a corpse (mostly of the family Calliphoridae; Schoenly et al., 1991; Anderson & VanLaerhoven, 1996; El-Kady, 1999). The latter is based on the application of existing developmental data for the species of blowfly present together with available temperature measurements (Sukontason et al., 2008). As the size and developmental stage of the blowfly larvae collected in a case provide important indicators for the estimation of the PMI, factors which affect these variables must be considered by the forensic entomologist (e.g., Byrd & Butler, 1997).

As ectotherms, the rates of most physiological processes in insects are highly dependent on environmental temperature (e.g., Higley & Haskell, 2001). Blowfly development is positively correlated with increasing temperatures (within developmental thresholds), producing a sigmoidal relationship (e.g., Sukontason et al., 2008). While temperature is the most important environmental variable influencing the rate of larval development (others include light duration and intensity, food availability, and competition), the duration of larval growth varies according to the

*Correspondence and present address: Leigh A. Nelson, CSIRO Entomology, GPO Box 1700, Australian Capital Territory, Australia 2601. E-mail: nelson.leigh@gmail.com

species, mainly on genetic bases (Campobasso et al., 2001).

Laboratory studies provide the forensic entomologist with a developmental time-scale for a particular species. Developmental stage and body length are the most common parameters for estimation of the age of immature blowflies (e.g., Byrd & Butler, 1996, 1997; Anderson, 2000; Byrd & Allen, 2001; Grassberger & Reiter, 2002a; Day & Wallman, 2006b). This approach is well supported by the literature and is admissible in court (Tarone & Foran, 2008). However, developmental data are limited and have typically been generated using non-standardised laboratory techniques, with different growth rates being reported for the same species (Kamal, 1958; Greenberg, 1991; Wells & Kurahashi, 1994; Anderson, 2000; Grassberger & Reiter, 2001). For instance, variable growth rates have been demonstrated for larvae raised under constant temperatures on different feeding substrates (e.g., Green et al., 2003; Kaneshrajah & Turner, 2004; Day & Wallman, 2006a). Larval density and competition have been found to influence developmental times and larval fecundity in some species (Goodbrod & Goff, 1990; Kheirallah et al., 2007). The application of larval length for PMI estimation is further complicated by these data not being available for all species that may be encountered in a forensic case.

Determination of the fly species and age are mandatory for the use of entomological evidence in forensic investigations (El-Kady, 1999; Song et al., 2007). The use of blowflies in PMI estimation is complicated by difficulties associated with correct species identification at all developmental stages (eggs, larvae, pupae, and adults) (e.g., Catts, 1992), particularly when only fragmented or degraded insect evidence is available for analysis (Stevens & Wall, 2001). Traditional morphology-based identification typically requires specialised taxonomic knowledge. While morphological identification is often possible, DNA-based identification may be required to separate morphologically indistinguishable species or specimens for which morphological characters are absent (Dawnay et al., 2007). Various DNA techniques have been proposed to identify forensically important blowflies (e.g., Malgorn & Coquoz, 1999; Wells & Sperling, 1999; Wallman & Donnellan, 2001; Wells et al., 2001; Chen et al., 2004; Zehner et al., 2004; Saigusa et al., 2005; Nelson et al., 2007, 2008). However, considerably little is known about the amount of genetic variation existing in blowfly populations, so this method is regarded as a complement to, rather than a substitute for, standard taxonomy-based identification of species (El-Kady, 1999; Nelson et al., 2007). The close morphological and genetic relationship of some species questions whether they differ sufficiently in their thermobiologies to warrant their discrimination for forensic purposes (Wallman & Donnellan, 2001). There has been a suggestion that developmental rates of some species could be deduced from their phylogenetic position relative to other species whose developments have been characterised (Wallman et al., 2005).

Australian members of the genus *Chrysomya*, to which the species investigated in this study belong, have been characterised morphologically and molecularly (Wallman, 2001a; Wallman et al., 2005; Nelson et al., 2007, 2008). With the exception of *Chrysomya latifrons* (Malloch) and *Chrysomya incisuralis* (Macquart), the other seven Australian *Chrysomya* species have distributions beyond Australia, particularly within the Asian region (Kurahashi, 1989; Spradbery, 1991; Wells & Kurahashi, 1994; Sukontason et al., 2008). Although developmental characteristics have been reported for some members of this genus, no studies have been recorded in Australia. Several *Chrysomya* species in Australia display very similar morphologies, even as adults, and small genetic distances indicate recent divergences (Table 1) (Wallman et al., 2005). The two closest relatives, sister species *Chrysomya saffranea* (Bigot) and *Chrysomya megacephala* (Fabricius), were selected for study along with the hairy maggot blowfly, *Chrysomya rufifacies* (Macquart), a more distantly

Table 1 Mean percentage sequence divergences (Kimura two-parameter) by DNA region between sister species *Chrysomya megacephala* and *Ch. saffranea*, and their divergence from *Ch. rufifacies*

	Mean genetic distance (%)						
Relationship	COI barcode region[1]	COI[2]	COII[2]	ND4-ND4L[2]	Control region[3]	28S[3]	ITS2[4]
Ch. megacephala + *Ch. saffranea*	0.48	0.25	0.00	0.50	1.19	0.19	0.46
(*Ch. megacephala* + *Ch. saffranea*) + *Ch. rufifacies*	6.88	8.33	8.35	7.39	19.54	0.56	10.48

[1]Nelson et al. (2007); [2]Wallman et al. (2005); [3]M Dowton, unpubl.; [4]Nelson et al. (2008).
COI, cytochrome oxidase I subunit of mitochondrial DNA; COII, cytochrome oxidase II subunit of mitochondrial DNA; ND4-ND4L, NADH dehydrogenase subunit 4 and 4L of mitochondrial DNA; Control region, D-loop region of mitochondrial DNA; 28S, ribosomal DNA encoding the large ribosomal subunit; ITS2, second internal transcribed spacer of ribosomal DNA.

related member of this genus. These species were chosen on the basis of molecular analyses that revealed very low sequence divergences between *Ch. saffranea* and *Ch. megacephala*, and yet a relatively large genetic distance of these species from *Ch. rufifacies* (Table 1). These species could also be sourced from the same locality, which was favoured to avoid any local adaptation or acclimation that might exist for each species (Higley & Haskell, 2001; Donovan et al., 2006).

This study provides developmental data for three Australian *Chrysomya* species at three temperatures. These data are used to establish whether related species (as determined from genetic analyses) differ significantly in their growth rates. Of particular interest is whether one set of developmental data can be applied to a group of closely related flies. If two very closely related species display identical developmental profiles, their separation may not be necessary for the purposes of PMI estimation. If this is the case, efforts should be concentrated on determining the thermobiological preferences for forensically important species under standardised conditions with less emphasis placed on distinguishing related species by DNA identification. If larval development is shown to be species-specific, the need for correct species identification is crucial and warrants further examination.

Materials and methods

Choice of species

Chrysomya rufifacies, *Ch. megacephala*, and *Ch. saffranea* were selected for detailed study. Previous trapping had established their sympatry and abundance near Cairns, Qld, Australia (16°89′S, 145°75′E) (JF Wallman, pers. comm.). A comparison of the development of very closely related species (*Ch. saffranea* and *Ch. megacephala*) with that of a less closely related member of the same genus, *Ch. rufifacies*, would provide insight into the effect of genetic distance on developmental variation. These species have been recorded in crime scene samples in Australia (JF Wallman, pers. comm.; Levot, 2003). In addition, these species could be cultured in the laboratory successfully.

Fly cultures

Adult *Ch. rufifacies*, *Ch. megacephala*, and *Ch. saffranea* were collected near Cairns (16°89′S, 145°75′E). Flies were collected by sweep net from a piglet carcass used as an attractant. Females were identified in the field and placed in individual glass tubes containing a small amount of kangaroo mince [protein 21.6% (wt/wt), moisture 75.3% (wt/wt), fat 0.6% (wt/wt), ash 1.3% (wt/wt), cholesterol 23.2 mg 100 g^{-1}, sodium 41.7 mg 100 g^{-1}, potassium 308.4 mg 100 g^{-1}, and energy 409 kJ 100 g^{-1}; Luddenham Pet Meats, pers. comm.] and cotton wool. Cotton wool was previously found to encourage oviposition in these species. This may be due to tactile stimuli, similar to the fur of a carcass, which would provide a protected site in which to oviposit (Monzu, 1977). The adults were preserved in absolute ethanol for further identification in the laboratory, and are maintained at 4 °C in the Diptera collection in the School of Biological Sciences, University of Wollongong. Larvae were kept separately until the identity of their female parent was confirmed. Adult identifications were established morphologically according to Wallman (2001a). The identities of *Ch. saffranea* and *Ch. megacephala*, which share considerable morphological similarity, were confirmed further by analysis of the mitochondrial cytochrome oxidase I (COI) 'barcode' region, following the protocol of Nelson et al. (2007). For some specimens, the ribosomal second internal transcribed spacer (ITS2) was sequenced according to Nelson et al. (2008).

Once identified, the adults of the F_0 generation of each species were pooled and maintained in separate plastic cages (300 × 500 × 250 mm) with fly mesh lids. Cultures were kept in the rearing room of the Forensic Entomology Research and Analysis Laboratory, University of Wollongong, at 25 ± 3 °C with a photoperiod of L12:D12 that included a 15 min 'dusk' transition period of low light between each phase. Each culture was provided with granulated raw sugar and water ad libitum, and kangaroo mince for ovary maturation and oviposition.

Larval thermogenesis

Larvae feeding in a mass can elevate their growing temperatures considerably above ambient air temperature, leading to increased developmental rates compared to those expected from ambient temperatures alone (VanLaerhoven, 2008). The lower threshold of larval density needed to generate heat sufficient to override ambient fluctuations was not known for the species under investigation. Therefore, the number of larvae for each species that could be used per replicate without generating additional heat was investigated prior to the conduct of the temperature experiments. Numbers of larvae investigated were 0, 10, 20, 50, and 100. Freshly laid eggs were counted and placed on approximately 120 g of kangaroo mince. Each treatment was repeated in triplicate at a constant temperature of 25 ± 0.2 °C under a L12:D12 photocycle in an Axyos incubator (Brisbane, Qld, Australia) fitted with a Eurotherm 2604 temperature-control unit (Quantum Scientific, Murarrie, Qld, Australia) set at 60% relative humidity (r.h.). Each replicate contained three temperature loggers (iButtons; accuracy ±1.0 °C, resolution 0.5 °C; Maxim Integrated Products, Sunnyvale, CA, USA)

inserted into the feeding substrate to record temperatures experienced by the growing larvae. Additional temperature loggers were positioned within the incubator to record any variation present. Intermittent water spraying of the feeding substrates prevented desiccation. The replicates were left in the incubator until pupation had commenced. At the completion of each time point, the replicates were removed and the temperature data from the loggers downloaded.

Growth under different temperatures

The flies used in these experiments were between two and ten generations after being removed from their natural source populations. Inbreeding was not believed to affect the fecundity, longevity, and activity of the flies (Mackerras, 1933). Flies were allowed to oviposit on kangaroo mince (as above; Luddenham Pet Meats) placed in cages. Eggs were collected within 1 h of the first observation of oviposition. This was designated as the beginning of development (0 h), as it closely approximates the start of the biological clock used in PMI estimates (Catts & Goff, 1992; Sukontason et al., 2008). Clumps of approximately 50 eggs (clump size determined from prior experimentation) were separated and placed onto excess (ca. 120 g) kangaroo mince contained within 100 ml disposable polystyrene weigh boats. An initial study determined the number of maggots comprising each replicate, selected so as to negate temperature increases caused by a maggot mass effect. The weigh boats were placed into 1-l plastic rearing containers with ventilated fine mesh lids, on top of ca. 3 cm wheaten chaff, which provided a dry shelter for pupation. To ensure sufficient larval measurements could be made (while avoiding a maggot-mass effect), it was necessary to prepare separate replicates. Four replicates per species (at each 24-hourly time point) were placed randomly into a temperature-controlled cabinet at one of four temperature regimes (20, 25, 30, and 35 ± 0.2, °C) under a L12:D12 cycle. For the 25 °C temperature regime, the Axyos incubator (25 ± 1.0 °C) was used with 60 ± 5% r.h. For the remaining temperature regimes, a Thermoline incubator was used (Model TRI 396-1-SD; Thermoline Scientific, Smithfield, NSW, Australia). For this incubator, a beaker of water provided a r.h. of 60 ± 4%. Temperature and humidity within each cabinet were monitored with data loggers (iButtons and Tinyview TV-1500; Gemini Data Loggers, Chichester, UK).

Rearing containers were left undisturbed until an allocated time had elapsed. Time points were every 24 h from oviposition until at least 95% of each cohort had pupated. Pupation was characterised by failure of the larvae to elongate and move in response to being disturbed. Careful dissection of the feeding substrate ensured all larvae were recovered from the mince. Larvae were killed by immersion in just-boiled water, dried with paper towel, and preserved in 80% EtOH (Byrd & Castner, 2001; Day & Wallman, 2008). Larval lengths were measured to the nearest 0.1 μm using a Leica $MZ7_5$ dissecting microscope (Leica Microsystems, Wetzlar, Germany) and Motic Images Plus 2.0 ML software (Motic China Group Company, Xiamen, China). Previous research (AP Johnson & JF Wallman, unpubl.) had shown that there was no significant difference between measurements made with digital callipers and the Motic software. Body length was measured as the distance, viewed laterally, between the most distal parts of the head and the last abdominal segment. The proportions of first, second, and third instars (determined by examination of posterior spiracular slits under a dissecting microscope) and pupae were noted for each sample using accepted characters to separate the various blowfly development stages (e.g., Prins, 1982).

Temperature preferences

Larvae can regulate their own temperatures by behavioural positioning within the maggot mass. Developmental rates of blowflies at their preferred temperature can be used to calculate accelerated larval development caused by maggot-mass formation (Byrd & Butler, 1996). To determine the temperature preferences of newly-emerged feeding larvae of the three species, an experiment was set up utilising an existing temperature gradient apparatus similar to that described by Byrd & Butler (1997). The apparatus consisted of a copper tray, one end of which was attached to a heating element fixed at a constant temperature of 60 °C, while the other end was connected to an insulated box containing ice at 0 °C. The copper tray was filled with a 50:50 (vol/vol) mixture of kangaroo mince and hydrated water saving crystals (Yates® Waterwise Water Storing Crystals; Orica, Silverwater, NSW, Australia) to a depth of 1 cm, which provided a feeding substrate for the larvae that would not desiccate at the high temperatures experienced at the heated end of the gradient. The tray was encased by a transparent Perspex tube with foam plugs at either end, for insulation and to prevent the escape of larvae. Pluggable access holes, spaced at 100 mm intervals along the length of the tube, facilitated temperature measurements and the insertion of larvae. Prior to the addition of any larvae, the kangaroo mince mixture was allowed to equilibrate for 1 h to the opposing temperatures at each end of the gradient, resulting in a gradient ranging from approximately 16–57 °C.

Eggs were obtained on kangaroo mince from individual cultures and monitored hourly until hatching. For each experiment, 30 newly-hatched first instars were randomly selected and distributed evenly along the length of the

copper tray at 100 mm intervals. Larvae were left for 24 h, after which time their positions were recorded. Temperatures were measured at 100 mm intervals along the copper tray using a digital temperature probe (Model HI 93510N; Hanna Instruments, Keysborough, Vic, Australia). The temperature preferences of the larvae were then inferred. All larvae were recovered from the copper tray at the conclusion of each experiment, which was repeated in triplicate for each species. An additional control experiment was conducted for each species, in which all experimental conditions were identical to those described, except that no temperature differences were applied to either end of the copper tray (i.e., the experiment was conducted at room temperature, at an average of 21 °C).

Larval survivorship

Early studies established that kangaroo mince had a tendency to desiccate readily at the heated end of the copper tray, changing the composition of the feeding substrate markedly. The addition of the water storage crystals counteracted this problem, but their effect on the larvae was unknown. A separate experiment was therefore conducted to assess the effect of the water storage crystals on larval survivorship. For each species, 30 newly-hatched larvae were placed on either 120 g kangaroo mince or 120 g of a 50:50 (vol/vol) mixture of kangaroo mince and water storage crystals. Four replicates per species were produced and left for 24 h at 20.2 ± 2 °C, after which all maggots were killed as previously described. In this experiment, survivorship was calculated as the number of larvae that survived from egg to approximately first instar on the growth medium – the approximate time frame investigated for the temperature preference experiments.

Data and statistical analysis

Data from iButtons were downloaded with iButton Viewer software (iButton-TMEX Runtime Environment version 3.12, Maxim Integrated Products, Sunnyvale, CA, USA); data from the Tinyview TV-1500 were downloaded with Tinytag Explorer (version 4.4.183; Gemini Data Loggers). These data were imported into Excel® (Microsoft Corporation, Redmond, WA, USA).

Statistical analyses were performed using JMP (Version 5.1; SAS Institute, Cary, NC, USA). In all tests, a 5% level of significance was used. Survivorship was assessed using the Student's t-test, comparing numbers of surviving larvae grown on kangaroo mince alone with those grown on kangaroo mince containing water storage crystals. These comparisons were repeated for each species. While there was not a linear relationship between temperature and distance along the temperature gradient, a plot of the inverse of temperature (°C) vs. distance along the gradient showed a strong linear relationship. The line of best fit relating inverse of temperature to distance was used to predict the temperature at a certain distance. Larval temperature preferences were calculated by inference from this standard curve. For larval growth experiments at three temperatures, the mean larval lengths of different species at the same time point and temperature were compared with one-way analysis of variance (ANOVA). Tukey-Kramer HSD was used to determine which species differed.

Results

Species identification

The specimens were identified by adding their COI DNA sequences to an existing barcode database for this genus (Nelson et al., 2007). This analysis confirmed morphological identifications in all but two instances: two *Ch. saffranea* specimens were resolved paraphyletic to a clade comprising *Ch. megacephala* and *Ch. saffranea*. The two specimens showed a COI divergence of 0.15%, with a mean divergence of 0.40 and 0.54%; from all other *Ch. saffranea* and *Ch. megacephala* specimens, respectively. This is far higher than the intraspecific divergence noted previously for *Ch. saffranea* of 0.043% (range 0.00–0.152%; Nelson et al., 2007). To investigate this further, the ITS2 regions were sequenced from these specimens. Both ITS2 sequences were identical. Sequence analysis revealed ITS2 divergences of 0.29 and 0.47% from *Ch. saffranea* and *Ch. megacephala* specimens, respectively. While the specimens showed the lowest divergence from *Ch. saffranea*, their larvae were nonetheless excluded from the fly cultures.

Larval thermogenesis

The 0, 10, 20, 50, and 100 larvae treatments displayed no detectable deviations from the temperatures recorded from the control (data not shown). To remain cautious, 50 larvae per replicate were considered a suitable number for the temperature development studies that followed.

Growth under different temperatures

The four replicates for a single 24-hourly time point, temperature, and species were pooled as no significant difference was observed among these ($P>0.05$). Eggs occluded (hatched) between 6 and 24 h after oviposition at all temperatures except 20 °C, where eggs occluded between 24 and 48 h. Considerable difficulties were experienced when rearing larvae of all species, particularly *Ch. saffranea*, at 20 °C. High mortalities, considerably stunted larvae and high variability in larval lengths were produced; therefore this temperature was believed to be too cool for adequate

growth to take place. Consequently, data collected at 20 °C were not considered reliable and were excluded from further analyses.

As expected, the growth profiles showed a decrease in larval length from third instar until pupation (Figure 1). After the maximum length was achieved at each temperature, there was a distinct decline in the numbers of larvae available for analysis due to the progression from third instar to the pupal stage. Because of this, data from later time points were not suitable for statistical comparisons of larval lengths. Maximum mean lengths at 25 °C were 15 086 µm (*Ch. saffranea:* range = 12 071–18 406 µm, n = 187), 14 922 µm (*Ch. megacephala:* range = 11 622–17 130 µm, n = 189), and 11 961 µm (*Ch. rufifacies:* range = 8 560–14 255 µm, n = 250). Maximum mean lengths at 30 °C were 15 215 µm (*Ch. saffranea:* range = 8 253–17 839 µm, n = 211), 13 434 µm (*Ch. megacephala:* range = 1 389–20 442 µm, n = 297), and 12 882 µm (*Ch. rufifacies:* range = 8 717–15 267 µm, n = 160). Maximum mean lengths at 35 °C were 13 221 µm (*Ch. saffranea:* range = 10 454–15 861 µm, n = 194), 13 754 µm (*Ch. megacephala:* range = 10 993–15 861 µm, n = 175), and 14 683 µm (*Ch. rufifacies:* range = 12 452–17 461 µm, n = 174). There was no distinct trend in the differences between the species; analysis revealed significant differences in larval lengths for each species at the majority of time points for each temperature. This was particularly the case for 30 °C, where all measurements were significantly different. There were a few cases where significant differences in length were not detected between two species, but where the two species in question together differed significantly from the third. This occurred for 96 h at 25 °C where *Ch. megacephala* and *Ch. rufifacies* differed significantly from *Ch. saffranea*, and for 120 h where *Ch. megacephala* and *Ch. saffranea* differed significantly from *Ch. rufifacies*. This was also observed for 24 h at 35 °C where *Ch. megacephala* and *Ch. saffranea* differed significantly from *Ch. rufifacies*. There was no obvious trend in the size order for each species for each temperature examined, although *Ch. saffranea* appeared to be larger than the other species at the majority of time points compared.

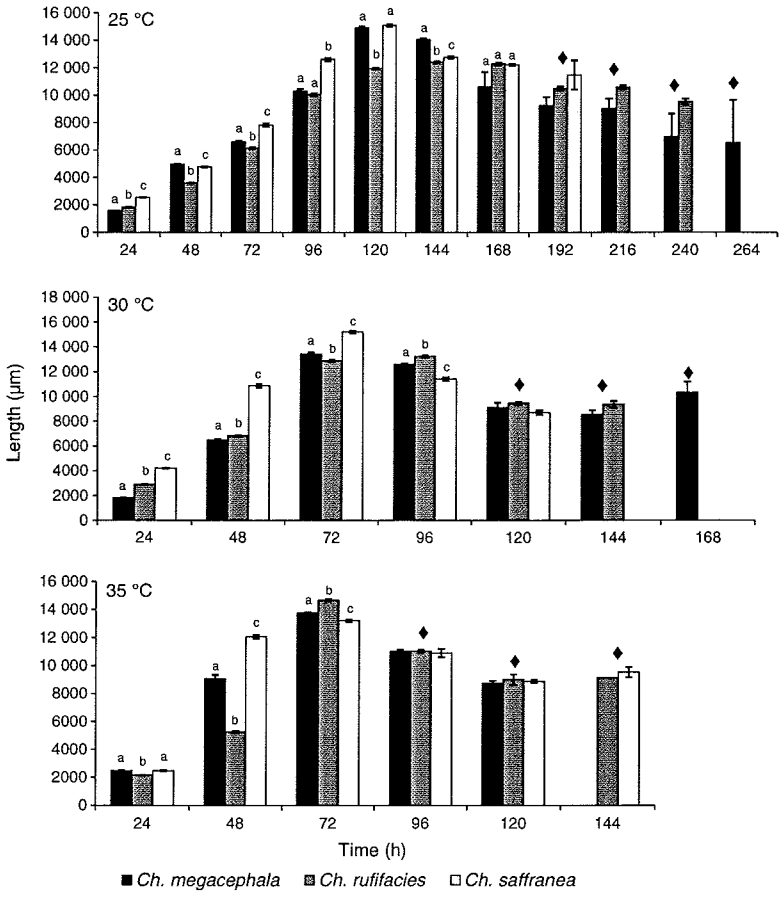

Figure 1 Mean (± SE) larval lengths of *Chrysomya megacephala*, *Ch. rufifacies*, and *Ch. saffranea* measured at 24-hourly intervals, growing at constant temperatures of 25, 30, and 35 °C under a photoperiod of L12:D12. The same letter connecting species at a given time point and temperature denote non-significant differences in mean lengths (t-test: P>0.05); ♦ = insufficient larvae in sample for valid statistical comparison to be made.

Inspection of the data at all three temperatures reveals what appears to be a rightward shift of the growth patterns for *Ch. megacephala* and *Ch. rufifacies* relative to the pattern for *Ch. saffranea*. At 30 °C, *Ch. saffranea* changes in length from being the largest of all three species at 72 h to the smallest at 96 h. This suggests that *Ch. saffranea* reached its maximum size ahead of the other two species and is already starting to shrink at the following time point. The data for 30 and 35 °C are somewhat limited due to the rapid development of the species at these temperatures, rendering relatively few larval length time points for comparison. It is apparent that more frequent sampling could enhance these data. For instance, at 35 °C, it is quite feasible that *Ch. saffranea* attains its maximum length some time between 48 and 72 h, but that the present sampling regime was unable to reveal this.

As sampling was conducted at 24-hourly intervals, large changes in the proportions of first, second, and third instars and pupae were observed for each species at each time point (Figures 2–4). For instance, for *Ch. megacephala* at 25 °C, 100% of the 24 h sample comprised first instars, and 100% of the 48 h sample comprised second instars. The transition between these stages could be revealed by more frequent sampling during this period. The transition from first to second instars at 25 °C was slowest for *Ch. rufifacies*, with 38% of the 48 h sample still consisting of first instars. First and second instars were both observed from 24 h for all species at 30 and 35 °C. The third instar lasted the longest in

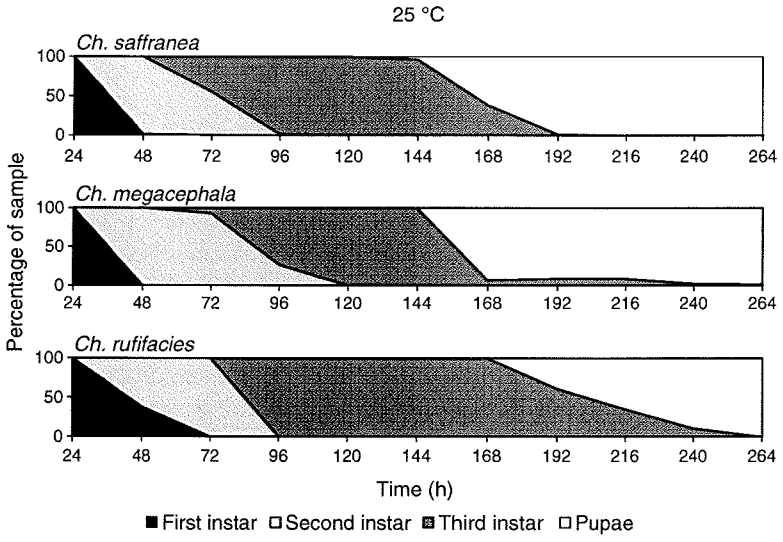

Figure 2 Percentages of *Chrysomya megacephala*, *Ch. rufifacies*, and *Ch. saffranea* immatures (first, second, and third instars and pupae) observed per time point (hours since egg laying) when grown at 25 °C.

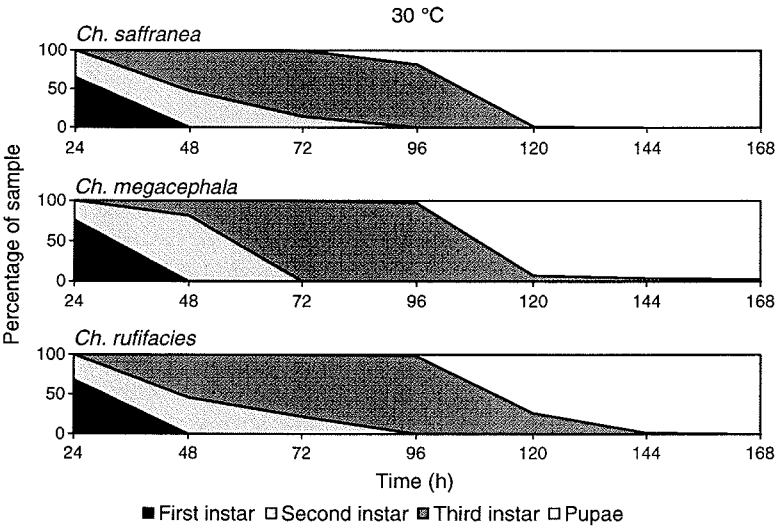

Figure 3 Percentages of *Chrysomya megacephala*, *Ch. rufifacies*, and *Ch. saffranea* immatures (first, second, and third instars and pupae) observed per time point (hours since egg laying) when grown at 30 °C.

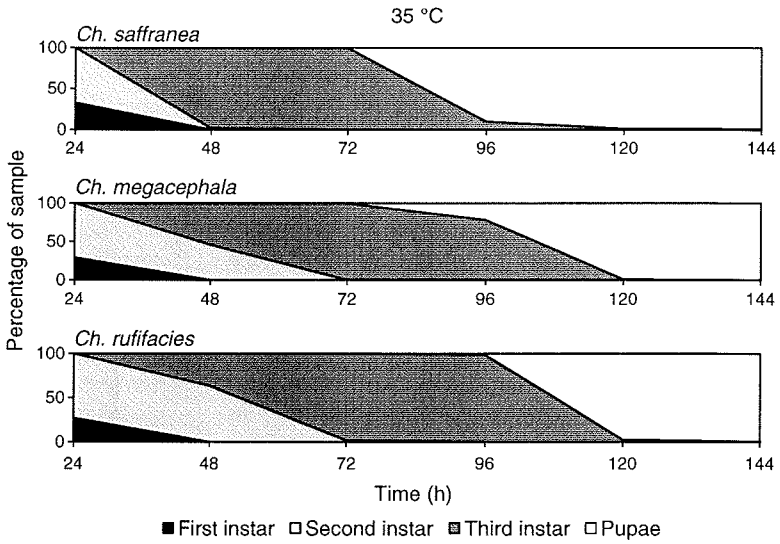

Figure 4 Percentages of *Chrysomya megacephala*, *Ch. rufifacies*, and *Ch. saffranea* immatures (first, second, and third instars and pupae) observed per time point (hours since egg laying) when grown at 35 °C.

all species. At 25 °C, third instars were first recorded at 72 h for *Ch. megacephala* and *Ch. saffranea*, and at 96 h for *Ch. rufifacies*. This stage lasted several days after the first observations of pupation for these species at 25 °C. At 30 and 35 °C, third instars were observed from 48 h. A significant difference in the proportions of the larval stages was observed at this time ($P<0.05$). This is particularly notable for *Ch. saffranea* at 35 °C, where the sample consisted wholly of third instars, whereas the other species displayed a large proportion of second instars. The transition from third instars to pupae for *Ch. rufifacies* at 25 and 30 °C appeared to be slower than the other two species, with a much larger overlap of the two stages during this period. A significant difference in the larval stages was seen at 168 h at 25 °C ($P<0.05$), with *Ch. rufifacies* third instars comprising 100% of the sample, whereas the majority of larvae for the other two species had pupated. A similar difference was observed at 96 h at 35 °C ($P<0.05$), where almost all *Ch. rufifacies* comprised third instars and the majority of *Ch. saffranea* larvae had pupated. At 25 °C, pupation was first observed at 144 h (*Ch. saffranea*), 168 h (*Ch. megacephala*), and 192 h (*Ch. rufifacies*). At 30 and 35 °C, pupation was first observed at 96 h for all species. The 5–10 °C increase in temperature from 25 °C sped up the onset of pupation by 48, 72, and 96 h for *Ch. saffranea*, *Ch. megacephala*, and *Ch. rufifacies*, respectively. The decrease in time to pupation with increase in temperature was expected. Many *Ch. rufifacies* larvae were observed pupating within or very close to the feeding substrate, while larvae of the other two species preferentially chose the chaff as their pupation site.

As a whole, these results do not support the hypothesis that more closely related species share more similar growth rates, as *Ch. megacephala* and *Ch. saffranea* showed no significant similarity for each temperature investigated, compared with their similarity with *Ch. rufifacies*. Growth rates appear to be species-specific and influenced by factors other than genetic closeness. While most of the mean lengths differed significantly, examination of data for the three species at each temperature revealed overlapping lengths at each time point. For instance, the largest difference in mean lengths was seen at 35 °C at 48 h; examination of the spread of the lengths at this point reveals considerable overlap in the sizes of larvae among the species (Figure 5). This overlap in larval sizes is even more apparent at time points for which the difference in the means is lower, for example at 25 °C at 72 h (Figure 5).

Temperature preferences and larval survivorship

Initial observation of the survivorship of larvae fed on kangaroo mince alone vs. a 50:50 (vol/vol) mixture of kangaroo mince and water storage crystals appeared to suggest a slightly higher survival on kangaroo mince without crystals. However, closer inspection of the data revealed the difference to be an average of less than one larva per treatment. For all species, there was no statistically significant difference in survivorship on kangaroo mince alone or on a 50:50 (vol/vol) mixture of kangaroo mince and water storage crystals ($P>0.05$). Consequently, these data were combined to give mean survival rates of 65.4, 67.1, and 70% for *Ch. megacephala*, *Ch. saffranea*, and *Ch. rufifacies*, respectively. There was no significant difference between survival rates among the species ($P>0.05$). These data

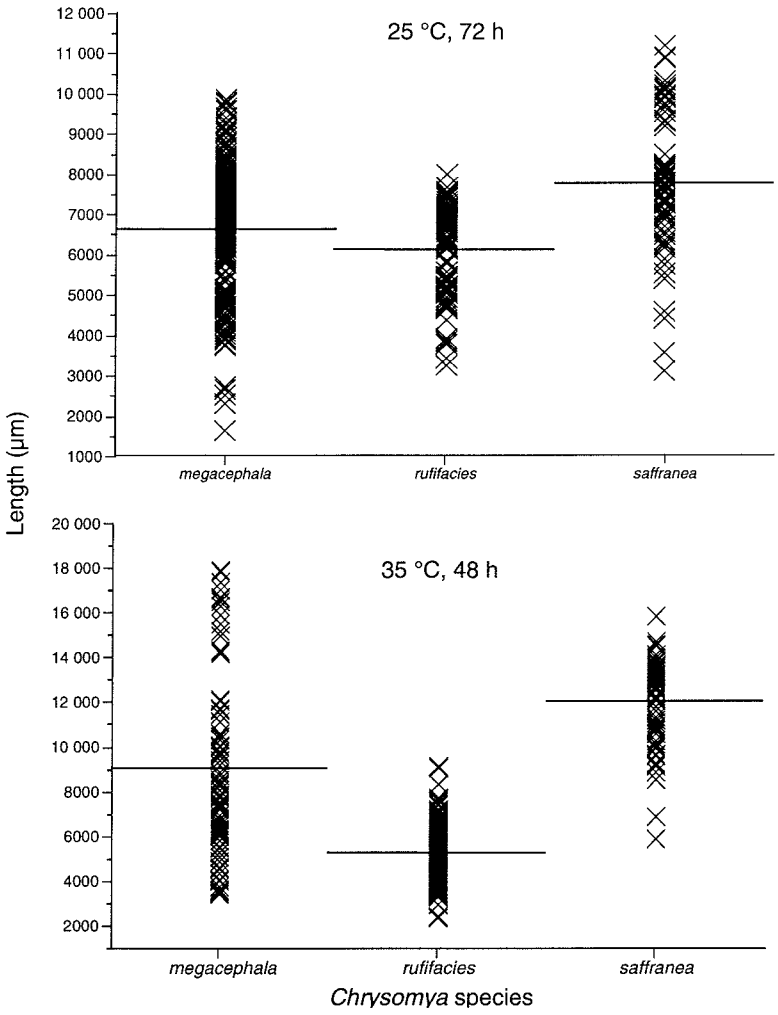

Figure 5 Examples of the variation in larval lengths of *Chrysomya megacephala*, *Ch. rufifacies*, and *Ch. saffranea* larvae at two times and temperatures, where means (horizontal lines) were significantly different (t-test: $P<0.05$).

indicated the expected percentage of larvae that would be recovered from the copper tray at the conclusion of the temperature preference experiments.

The mean survival for each species from the temperature gradient experiments was calculated as the percentage of larvae that were recovered from the feeding substrate at the conclusion of the experiment. The mean survivorship for each species was 51.1, 71.1, and 81.1% for *Ch. megacephala*, *Ch. saffranea*, and *Ch. rufifacies*, respectively. The only significant difference in survivorship on the temperature gradient was that between *Ch. megacephala* and *Ch. rufifacies* ($P = 0.022$). However, the survivorships noted here for each species were not significantly different to those predicted by the earlier study, where no gradient temperature was applied ($P>0.05$).

The mean temperatures (± SE) preferred by each species were 38.9 ± 0.44, 39.2 ± 0.26, and 34.1 ± 0.16 °C for *Ch. megacephala*, *Ch. saffranea*, and *Ch. rufifacies*, respectively (Figure 6). There was no significant difference between the temperature preferences of *Ch. megacephala* and *Ch. saffranea* ($P = 0.39$). However, these temperatures were both significantly different to the mean temperature preferred by *Ch. rufifacies* larvae ($P<0.0001$). The control experiment indicated that, with a lack of heating or cooling applied to either end of the gradient, maggots dispersed randomly along the length of the copper tray.

Discussion

Species identifications

The identities of the *Chrysomya* specimens collected in the field were confirmed by molecular analyses, with the exception of the two 'mystery' specimens for which conclusive identifications were not possible. Because the DNA

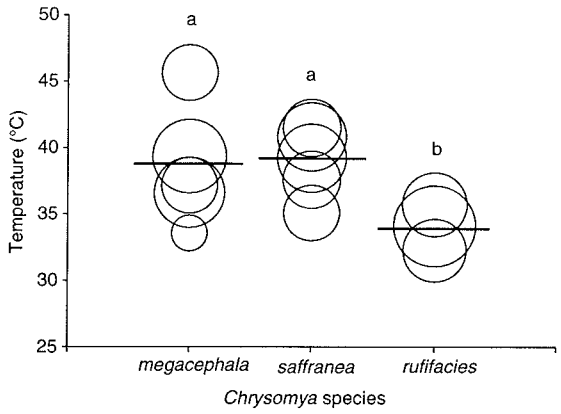

Figure 6 Distributions of *Chrysomya megacephala*, *Ch. rufifacies*, and *Ch. saffranea* larvae after 24 h feeding on a temperature gradient (approximately 16–57 °C) comprising a 50:50 (vol/vol) mixture of kangaroo mince with water storage crystals. Circles represent groups of larvae at a particular temperature. The temperature is indicated by the centre of each circle, and the area is proportional to the number of larvae present. Mean temperatures are indicated by bars; identical letters above measurements indicate non-significant differences between species (t-test: $P<0.05$).

sequences of these two specimens differed substantially from both *Ch. megacephala* and *Ch. saffranea* (and indeed all other *Chrysomya* species contained within the databases used), their larvae were not included in the cultures that were used for developmental experiments. This was necessary, as one of the principal aims of this study was to measure the effect, if any, of genetic distance on developmental profiles. Further sequence analysis could reveal the true identities of these specimens. Indeed, it may be the case that the extent of intraspecific variation existing in *Ch. megacephala* and *Ch. saffranea* has not been encompassed by the COI 'barcode' and ITS2 databases employed for these identifications (Wells & Williams, 2007). Very little is known about the population ecology of these two species in Australia. The low interspecific genetic differences noted between *Ch. megacephala* and *Ch. saffranea* suggest that these species have only recently diverged (Wallman et al., 2005). The close relationship of these species was reinforced further by the identification of a suspected *Ch. megacephala/Ch. saffranea* hybrid caught in Cairns (Nelson et al., 2007). The extent and abundance of such hybridisation is not known, but its occurrence led to questions regarding the status of these two species as separate entities.

Larval densities

It has been shown that larval population density is related to the increase in heat generated relative to ambient temperatures (Marchenko, 2001; Slone & Gruner, 2007). In many studies, the ambient temperatures (e.g., the set-points of temperature-controlled cabinets) are reported, but the temperatures actually experienced by the growing larvae are neglected (e.g., Byrd & Butler, 1997). In order to compare growth of all three species at constant temperatures, it was necessary to conduct an initial study investigating larval densities to ensure that larvae were actually growing at the temperatures to which they were being exposed. The larval numbers (of all three species) investigated in the preliminary study did not raise the substrate temperature above ambient, suggesting that an excess of 100 larvae per 120 g kangaroo mince would be required to achieve this. This is consistent with the findings of Kheirallah et al. (2007) who showed that 170 larvae per 120 g of substrate were required to raise the temperature of the feeding substrate above ambient. The conservative use of 50 larvae per 120 g of feeding substrate was decided upon for this study. While this is an unrealistic situation in comparison with that likely to exist in an actual carcass or dead body, it was necessary to minimise the uncontrolled variables for the sake of this study (O'Flynn, 1983). Indeed, approximately 200 larvae can arise from just one oviparous blowfly (Mackerras, 1933).

Although the aim of this study was to eliminate any maggot-mass effect, the literature suggests that it is vital to consider this when estimating the PMI based on larval development. While insufficient larval numbers have been shown to be detrimental to larval survival, increased larval density can decrease the developmental time due to higher temperatures, the accumulation of digestive enzymes, and the physical mixing and perforation of the food source which facilitates feeding (Turner & Howard, 1992). This is of particular importance given that air temperatures (obtained from the scene or nearby weather stations) are typically employed for PMI estimates (Archer, 2004), which rarely take into account the effect of increased temperatures experienced by the growing larvae (e.g., Cianci & Sheldon, 1990).

Another important factor that needs to be considered is the effect of inter-species competition on larval development (Williams & Richardson, 1984; Kheirallah et al., 2007). There is substantial evidence that blowfly larvae are negatively influenced by competition for food, leading, for example, to a reduction in size, increased mortality and undersized adults (Ireland & Turner, 2006). There is an added dynamic of competition from predatory and cannibalistic *Ch. rufifacies* larvae (Goodbrod & Goff, 1990). It is highly likely that more than one blowfly species will be recovered from a body, therefore the effects of inter-species competition need to be taken into account (Levot, 2003).

Larval development

The length of time from oviposition to larval hatching (6–24 h) corresponded to that reported previously for *Chrysomya* species (Sukontason et al., 2008). The changes in length measurements over time, recorded in this study, were expected (Levot et al., 1979). The exponential increase in larval size in just a few days is necessary to accommodate the rapidly expanding crop and intestine, which is facilitated by the plasticity of the cuticle and the two moults during this time (Greenberg, 1991). The third instar was the longest stage in duration for all species, indicating that it is the larval form most likely to be encountered in forensic cases (Greenberg, 1991; Wallman & Donnellan, 2001b). The latter portion of the larval development profiles, during which the larvae have finished feeding, shows the reduction in length typical of this stage, where growth has stopped (Williams & Richardson, 1984; Greenberg, 1991). At this point, larval size is no longer a useful criterion for age due to the reversal of body size and larger variance in weight and length (Wells & Kurahashi, 1994; Tarone & Foran, 2008). There is also the probability of high variability in the post-feeding stage, and blowfly larvae can delay pupation if conditions are sub-optimal (Wells & Kurahashi, 1994; Byrd & Butler, 1997). For these reasons, it is evident that attention should be focused on understanding length variation in the earlier portions of larval growth, where it is a more useful measure of larval age.

The influence of temperature on the development of these species was exemplified by comparing the proportions of immature stages comprising each sample. The onset of pupation was brought forward considerably by raising the temperature from 25 to 30 °C. A similar reduction in the time to pupation was not seen when the temperature was raised from 30 to 35 °C, indicating that these temperatures are close to the thermal optima for these species. The reduction in time required to complete development gives an obvious competitive advantage to blowfly larvae in utilising the finite resource of a carcass (Heinrich, 1996). The responses of these species to temperature demonstrate their adaptive success as carrion breeders in consuming this transient food source efficiently and rapidly (Levot et al., 1979). The tendency of *Ch. rufifacies* to pupate on or close to the food substrate, observed in this study, has been noted previously (Norris, 1965; Greenberg, 1991; Baumgartner, 1993). This behavioural trait is believed to be a survival mechanism, made possible by the spines which are characteristic of the 'hairy maggot', and are present on the surface of the puparium (Sukontason et al., 2008). This textured surface renders the puparium somewhat like a plant burr, which cannot be removed easily by predators from the hair and other remains of the dead animal in which the larvae were developing.

The high mortalities and general poor performance of larvae at 20 °C was not expected. Cairns (the region from which the fly populations originated) is tropical, experiencing fairly uniform temperatures throughout the year (Australian Bureau of Meteorology, 2008). Midsummer temperatures range from 23 to 31 °C, while midwinter temperatures range from 18 to 26 °C. Interestingly, the average minimum temperature only falls below 20 °C between May and September, with the lowest mean minimum of 17 °C being recorded in July. During these months, it is possible that the activity of *Chrysomya* species present in this region declines or that the species disappears altogether, as happens in southern Australia. It has been reported that members of this genus have temperature tolerances that correspond with their tropical distribution (Baumgartner, 1993). However, it is expected that larval development remains possible during the cooler months due to the maggot-mass effect in infested carrion in the field. This would maintain high temperatures, overcoming unfavourable weather conditions and extending the breeding season (Greenberg & Tantawi, 1993; Slone & Gruner, 2007). Indeed, maggot-masses have been observed to still be active when air temperatures are as low as −4 °C (Greenberg, 1991). The maggot-mass effect was deliberately avoided in the present study, but may be required for adequate development of larvae at this temperature.

The exact experimental conditions adopted by other researchers are unknown, thereby making differences between other studies and the present one difficult to assess. The mean sizes of *Ch. rufifacies* larvae at 25 °C were considerably smaller than those reported by Byrd & Butler (1997). This discrepancy could be related to the different experimental conditions utilised by these authors, including continuous light, a pork food medium, and flies sourced from another continent (North America). The mean sizes of *Ch. megacephala* larvae at 25, 30, and 35 °C were considerably smaller overall than those reported by Nishida et al. (1986), working with a Japanese population, although the experimental conditions of this study are unknown. However, the development times of *Ch. rufifacies*, *Ch. megacephala*, and *Ch. saffranea* correspond with those reported by O'Flynn (1983) for Australian populations.

Although the mean lengths per species differed significantly for the majority of times and temperatures examined in this study, there was a considerable spread of larval lengths (at a specific time and temperature). A common practice in forensic cases is to select the largest larvae from the sample to measure (e.g., Byrd & Butler, 1998; Higley & Haskell, 2001). The basis for this approach is that the

largest larvae represent the oldest individuals present in a sample, and are most likely to have originated from the first egg laying episode. Some studies have employed the same methodology for the generation of growth curve data in developmental studies (e.g., Byrd & Butler, 1996, 1997, 1998; Grassberger & Reiter, 2001; Greenberg & Kunich, 2002; Sukontason et al., 2008). This practice has been criticised due to the high variability in larval lengths for a given age (e.g., Donovan et al., 2006), a phenomenon confirmed by this study. The natural variation in larval size for each age clearly warrants further investigation as to whether selecting only the largest individuals is a valid procedure for the generation of growth curves to be used for PMI estimations.

Standardised larval killing and preservation methods were employed throughout this study, which allowed direct comparisons of larval lengths for each species, temperature, and time point. However, the killing and preservation method used has important implications for PMI estimation, given that different protocols have been shown to affect the measurable larval length (Tantawi & Greenberg, 1993; Day & Wallman, 2008). For the purposes of PMI estimation, it is therefore important that larvae be killed and preserved by the same method as those from which developmental growth curves were generated. Alternatively, the possibility of length alteration by another killing and preservation technique needs to be taken into account when making age inferences from existing developmental data. In this study, kangaroo mince was utilised as it was found to be reliable in terms of its consistency and quality; it was easily measured, and the *Chrysomya* maggots were previously found to respond poorly to liver (ovine and bovine) as a feeding substrate (LA Nelson, pers. obs.), which is often employed in larval development studies (Levot et al., 1979; Williams & Richardson, 1984; Wall et al., 1992; Wells & Kurahashi, 1994; Anderson, 2000; Grassberger & Reiter, 2001, 2002b; Ames & Turner, 2003). The components of kangaroo mince were also believed to be more representative of an entire body, comprising more than one tissue type.

Larval temperature preferences

The larval temperature preference experiments showed *Ch. rufifacies* to prefer a temperature approximately 4.8 °C lower than *Ch. megacephala* and 5.1 °C lower than *Ch. saffranea*. This was an interesting result given that *Ch. rufifacies* has previously been shown to tolerate higher temperatures, and has even been implicated in elevating them (Williams & Richardson, 1984). However, this corresponded closely to the preferred temperature of 35.1 ± 0.4 °C for this species shown by Byrd & Butler (1997) after 24 h on a very similar gradient device. Further investigation into the temperature preferences of these species, and the preferences of other forensically important genera, is warranted.

The survivorships for all three species on the temperature gradient were low, but not significantly different to those predicted by the initial study for both food substrates. It is possible that larvae deposited at the two ends of the temperature extremes (approximately 16 and 57 °C) died before they could reposition themselves at a more biologically tolerable temperature along the gradient. The *Chrysomya* maggots formed distinct masses on the temperature gradient, which was different to the more sparsely distributed pattern observed for two *Calliphora* species that have been investigated on the temperature gradient under the same conditions previously (AP Johnson & JF Wallman, unpubl.). Thermal optima, such as those established by these gradient experiments, reveal important information regarding the temperature preferences of growing larvae. Because larvae possess the ability to thermoregulate within the maggot-mass, experiments such as this one indicate the likely temperatures experienced by larvae collected from a mass on a corpse at a death scene. Such data enhance the accuracy of the PMI estimate by enabling adjustments to be made for the presence of maggot-masses.

Comparing growth of *Chrysomya* species

The production of growth curves is typical of studies that provide thermodevelopmental data for the purpose of PMI estimation (e.g., Grassberger & Reiter, 2002a; Sukontason et al., 2008). The growth data generated here were required only to establish whether the lengths of species under investigation differed significantly at various time points throughout their development. While it may be tempting to fit growth curves to these data, any curve-fitting approach involves its own assumptions and arbitrary modelling choices (Wells & LaMotte, 2001). It was not the purpose of this study to investigate methods to model larval development, which continue to be described (von Zuben et al., 1998; Richards & Villet, 2008; Tarone & Foran, 2008), as do methods to compare growth curves (e.g., Wang, 2000). For these reasons, curves were not fitted to the data.

The experimental design employed in this study sets it apart (to date) from other published larval development studies. The developments of such closely related blowfly species have not previously been compared. As the species were collected from the same geographical location, the effects of acclimation and population-level genetic variation were not variables in this study. The experimental conditions in this study were virtually identical, which enabled direct comparisons to be made among the species.

After examining the developmental profiles for the three *Chrysomya* species investigated, questions remained regarding the practical application of growth data for PMI estimation. The hypothesis was that members of a genus would share similar enough developmental profiles to allow inferences to be made for a species of the same genus for which detailed developmental studies were lacking. Although all three sets of data were found, on the whole, to differ significantly, a large spread (overlap) of larval lengths was seen for each species at each time point and temperature.

To put the *Chrysomya* growth data in perspective with another genus, it seemed logical to compare them with data for another forensically significant species, *C. augur*. In New South Wales, the genera *Calliphora* and *Chrysomya* are reported in 66 and 44% of forensic entomology cases, respectively (Levot, 2003). There are many reported cases where a *Calliphora* species occurred together with a *Chrysomya* species, and 52% of the time this was *Ch. rufifacies* with *C. augur* (Levot, 2003). A comparison was made of the growth data of the *Chrysomya* species at three temperatures with equivalent data from Day (2006) (Figure 7). This comparison revealed a downward shift of the *Chrysomya* species length data relative to those of *C. augur*, which were all significantly different at comparable times since oviposition ($P<0.05$). One difference which could contribute to the more rapid growth and larger sizes of *C. augur* larvae is that these flies are ovoviviparous, depositing live larvae directly onto the food source. This competitive advantage eliminates the extra time required by egg-laying species for occlusion, enabling larval growth immediately. Despite this, considerable differences can be seen between the *Chrysomya* and *Calliphora* data. When viewed in this context, it would be realistic to expect that, with more detailed data, confidence limits could delineate a more general *Chrysomya* growth curve that could be used to infer the ages of these species compared with those of *Calliphora*. Such a concept could have important applications given that these two genera are often found together in forensic cases, where the ages of the larvae of both species would be required for PMI estimation. The extent to which this would be of any practical use would need to be tested, as would the effect of competition between these species.

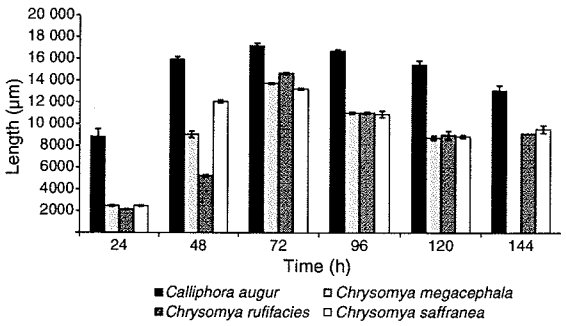

Figure 7 Comparison of *Chrysomya* larval lengths (± SE) plotted with corresponding data for *Calliphora augur* (Day, 2006) at 35 °C. *Calliphora augur* lengths differed significantly from *Chrysomya* lengths at each time point investigated (t-test: $P<0.05$). Comparisons at 25 and 30 °C not shown.

Conclusions

Blowfly evolution shows a strong selection for rapid location and consumption of the transient food sources that comprise their main breeding medium (Greenberg, 1991; Kheirallah et al., 2007). Several factors have been identified that could influence variations in fly development observed in the literature, including larval densities, photoperiod (constant vs. alternating), temperature regime (constant vs. fluctuating), relative humidity, food source, and geographic origin of the population (e.g., Wigglesworth, 1965; Goodbrod & Goff, 1990; Williams & Richardson, 1984; Sukontason et al., 2008). It is likely that population-specific factors influence the growth rates of species. This is highlighted by the variation in growth rates that have been noted for geographical races of the same species (El-Kady, 1999). Concerns have been expressed regarding the application of development data collected for one population of flies when applied to another population of the same species (Erzinçlioglu, 1983). It is therefore imperative to investigate growth rates of species from different regions in order to evaluate the reliability of such data (El-Kady, 1999).

It is apparent that genetic closeness does not afford the forensic entomologist the luxury of inferring a developmental profile for a species from that of a close relative. It is for this reason that correct species identification and application of the corresponding developmental data are imperative for an accurate PMI estimation. Future research required in this area includes replicated growth rate data under a variety of variables and temperatures, conducted under prescribed, standardised conditions to enable direct comparisons among populations and species. There is a need for laboratory experiments to be supplemented with data collected in the field and from casework, which would enable the evaluation of laboratory-collected data in terms of its practical applicability for the estimation of the PMI.

Acknowledgements

The authors are grateful to the Forensic Services Group of the New South Wales Police Force for financial support of a portion of this work.

References

Amendt J, Krettek R & Zehner R (2004) Forensic entomology. Naturwissenschaften 91: 51–65.

Ames C & Turner B (2003) Low temperature episodes in development of blowflies: implications for postmortem interval estimation. Medical and Veterinary Entomology 17: 178–186.

Anderson GS (2000) Minimum and maximum development rates of some forensically important Calliphoridae (Diptera). Journal of Forensic Sciences 45: 824–832.

Anderson GS (2001) Insect succession on carrion and its relationship to determining time of death. Forensic Entomology: The Utility of Arthropods in Legal Investigations (ed. by JH Byrd & JL Castner), pp. 143–176. CRC Press, Boca Raton, FL, USA.

Anderson GS & VanLaerhoven SL (1996) Initial studies on insect succession on carrion in southwestern British Columbia. Journal of Forensic Sciences 41: 617–625.

Archer MS (2004) The effect of time after body discovery on the accuracy of retrospective weather station ambient temperature corrections in forensic entomology. Journal of Forensic Sciences 49: 553–559.

Australian Bureau of Meteorology (2008) Climate statistics for Australian locations, summary statistics. CAIRNS AERO: http://www.bom.gov.au/climate/averages/ (accessed September 2008).

Baumgartner DL (1993) Review of *Chrysomya rufifacies* (Diptera: Calliphoridae). Journal of Medical Entomology 30: 338–352.

Benecke M (2001) A brief history of forensic entomology. Forensic Science International 120: 2–14.

Byrd JH & Allen JC (2001) The development of the black blow fly, *Phormia regina* (Meigen). Forensic Science International 120: 79–88.

Byrd JH & Butler JF (1996) Effects of temperature on *Cochliomyia macellaria* (Diptera: Calliphoridae) development. Journal of Medical Entomology 33: 901–905.

Byrd JH & Butler JF (1997) Effects of temperature on *Chrysomya rufifacies* (Diptera: Calliphoridae) development. Journal of Medical Entomology 34: 353–358.

Byrd JH & Butler JF (1998) Effects of temperature on *Sarcophaga haemorrhoidalis* (Diptera: Sarcophagidae) development. Journal of Medical Entomology 35: 694–698.

Byrd JH & Castner JL (2001) Forensic Entomology: The Utility of Arthropods in Legal Investigations. CRC Press, Boca Raton, FL, USA.

Campobasso CP, Vella GD & Introna F (2001) Factors affecting decomposition and Diptera colonization. Forensic Science International 120: 18–27.

Catts EP (1992) Problems in estimating the postmortem interval in death investigations. Journal of Agricultural Entomology 9: 245–255.

Catts EP & Goff ML (1992) Forensic entomology in criminal investigations. Annual Review of Entomology 37: 253–272.

Chen W-Y, Hung T-H & Shiao S-F (2004) Molecular identification of forensically-important blow fly species (Diptera: Calliphoridae) in Taiwan. Journal of Medical Entomology 41: 47–57.

Cianci TJ & Sheldon JK (1990) Endothermic generation by blow fly larvae *Phormia regina* developing in pig carcasses. Bulletin of the Society of Vector Ecology 15: 33–40.

Dawnay N, Ogden R, McEwing R, Carvalho GR & Thorpe RS (2007) Validation of the barcoding gene COI for use in forensic genetic species identification. Forensic Science International 173: 1–6.

Day DM (2006) Development of Immature Blowflies and their Application to Forensic Science. Masters Thesis. School of Biological Sciences, University of Wollongong, NSW, Australia.

Day DM & Wallman JF (2006a) Influence of substrate tissue type on larval growth in *Calliphora augur* and *Lucilia cuprina*. Journal of Forensic Sciences 51: 657–663.

Day DM & Wallman JF (2006b) Width as an alternative measurement to length for post-mortem interval estimations using *Calliphora augur* (Diptera: Calliphoridae). Forensic Science International 159: 158–167.

Day DM & Wallman JF (2008) Effect of preservative solutions on preservation of *Calliphora augur* and *Lucilia cuprina* larvae (Diptera: Calliphoridae) with implications for post-mortem interval estimates. Forensic Science International 179: 1–10.

Donovan SE, Hall MJR, Turner BD & Moncrieff CB (2006) Larval growth rates of the blowfly, *Calliphora vicina*, over a range of temperatures. Medical and Veterinary Entomology 20: 106–114.

El-Kady EM (1999) Problems facing application of forensic entomology. Pakistan Journal of Biological Sciences 2: 280–289.

Erzinçlioglu Z (1983) The application of entomology to forensic medicine. Medicine, Science and the Law 23: 57–63.

Goodbrod JR & Goff ML (1990) Effects of larval population density on rates of development and interactions between two species of *Chrysomya* (Diptera: Calliphoridae) in laboratory culture. Journal of Medical Entomology 27: 338–343.

Grassberger M & Reiter C (2001) Effects of temperature on *Lucilia sericata* (Diptera: Calliphoridae) development with special reference to the isomegalen- and isomorphen-diagram. Forensic Science International 120: 32–36.

Grassberger M & Reiter C (2002a) Effect of temperature on development of *Liopygia* (=*Sarcophaga*) *argyrostoma* (Robineau-Desvoidy) (Diptera: Sarcophagidae) and its forensic implications. Journal of Forensic Sciences 47: 1–5.

Grassberger M & Reiter C (2002b) Effect of temperature on development of the forensically important holarctic blow fly *Protophormia terraenovae* (Robineau-Desvoidy) (Diptera: Calliphoridae). Forensic Science International 128: 177–182.

Green PWC, Simmonds MSJ & Blaney WM (2003) Diet nutriment and rearing density affect the growth of black blowfly larvae, *Phormia regina* (Diptera: Calliphoridae). European Journal of Entomology 100: 39–42.

Greenberg B (1991) Flies as forensic indicators. Journal of Medical Entomology 28: 565–577.

Greenberg B & Kunich JC (2002) Entomology and The Law – Flies as Forensic Indicators. Cambridge University Press, Cambridge, MA, USA.

Greenberg B & Tantawi TI (1993) Different developmental strategies in two boreal blow flies (Diptera: Calliphoridae). Journal of Medical Entomology 30: 481–484.

Heinrich B (1996) The Thermal Warriors: Strategies of Insect Survival. Harvard University Press, Cambridge.

Higley LG & Haskell NH (2001) Insect development and forensic entomology. Forensic Entomology: The Utility of Arthropods in Legal Investigations (ed. by JH Byrd & JL Castner), pp. 43–80. CRC Press, Boca Raton, FL, USA.

Ireland S & Turner B (2006) The effects of larval crowding and food type on the size and development of the blowfly, *Calliphora vomitoria*. Forensic Science International 159: 175–181.

Kamal AS (1958) Comparative study of thirteen species of sarcosaprophagous Calliphoridae and Sarcophagidae (Diptera). I. Bionomics. Annals of the Entomological Society of America 51: 261–271.

Kaneshrajah G & Turner B (2004) *Calliphora vicina* larvae grow at different rates on different body tissues. International Journal of Legal Medicine 118: 242–244.

Kheirallah A, Tantawi TI, Aly A & El-Moaty Z (2007) Competitive interaction between larvae of *Lucila sericata* (Meigen) and *Chrysomya albiceps* (Weidemann) (Diptera: Calliphoridae). Pakistan Journal of Biological Sciences 10: 1001–1010.

Kurahashi H (1989) Family Calliphoridae. Catalog of the Diptera of the Australasian and Oceanian Region (ed. by NL Evenhuis), pp. 702–718. Bishop Museum Press, Honolulu, HI, USA.

Levot G (2003) Insect fauna used to estimate the post-mortem interval of deceased persons. General and Applied Entomology 32: 31–39.

Levot G, Brown K & Shipp E (1979) Larval growth of some calliphorid and sarcophagid Diptera. Bulletin of Entomological Research 69: 469–475.

Mackerras MJ (1933) Observations of the life-histories, nutritional requirements and fecundity of blowflies. Bulletin of Entomological Research 24: 353–362.

Malgorn Y & Coquoz R (1999) DNA typing for identification of some species of Calliphoridae – an interest in forensic entomology. Forensic Science International 102: 111–119.

Marchenko MI (2001) Medicolegal relevance of cadaver entomofauna for the determination of the time of death. Forensic Science International 120: 89–109.

Monzu N (1977) Coexistence of Carrion Breeding Calliphoridae (Diptera) in Western Australia. PhD dissertation. University of Western Australia, Perth, WA, Australia.

Nelson LA, Wallman JF & Dowton M (2007) Using COI barcodes to identify forensically and medically important blowflies. Medical and Veterinary Entomology 21: 44–52.

Nelson LA, Wallman JF & Dowton M (2008) Identification of forensically important *Chrysomya* (Diptera: Calliphoridae) species using the second ribosomal internal transcribed spacer (ITS2). Forensic Science International 177: 238–247.

Nishida K, Shinonaga S & Kano R (1986) Growth tables of fly larvae for the estimation of post mortem interval. Ochanomizu Igaku Zasshi 34: 157–172.

Norris KR (1965) The bionomics of blowflies. Annual Review of Entomology 10: 47–68.

O'Flynn M (1983) The succession and rate of development of blowflies in carrion in southern Queensland and the application of these data to forensic entomology. Journal of the Australian Entomological Society 33: 137–148.

Prins AJ (1982) Morphological and biological notes on six South African blow-flies (Diptera, Calliphoridae) and their immature stages. Annals of the South African Museum 90: 201–217.

Richards CS & Villet MH (2008) Factors affecting accuracy and precision of thermal summation models of insect development used to estimate post-mortem intervals. International Journal of Legal Medicine 122: 401–408.

Saigusa K, Takamiya M & Aoki Y (2005) Species identification of the forensically important flies in Iwate prefecture, Japan based on mitochondrial cytochrome oxidase gene subunit I (*COI*) sequences. Legal Medicine 7: 175–178.

Schoenly K, Griest K & Rhine S (1991) An experimental field protocol for investigating the postmortem interval using multidisciplinary indicators. Journal of Forensic Sciences 36: 1395–1415.

Slone D & Gruner S (2007) Thermoregulation in larval aggregations of carrion-feeding blow flies (Diptera: Calliphoridae). Journal of Medical Entomology 44: 516–523.

Song Z, Wang X & Liang G (2007) Species identification of some common necrophagous flies in Guangdong province, southern China based on the rDNA internal transcribed spacer 2 (ITS2). Forensic Science International 175: 17–22.

Spradbery JP (1991) A Manual for the Diagnosis of Screw-worm Fly. Australian Government Publishing Service, Canberra, ACT, Australia.

Stevens JR & Wall R (2001) Genetic relationships between blowflies (Calliphoridae) of forensic importance. Forensic Science International 120: 116–123.

Sukontason K, Piangjai S, Siriwattanarungsee S & Sukontason KL (2008) Morphology and developmental rate of blowflies *Chrysomya megacephala* and *Chrysomya rufifacies* in Thailand: application in forensic entomology. Parasitology Research 102: 1207–1216.

Tantawi TI & Greenberg B (1993) The effect of killing and preservative solutions on estimates of maggot age in forensic cases. Journal of Forensic Sciences 38: 702–707.

Tarone AM & Foran DR (2008) Generalized additive models and *Lucilia sericata* growth: assessing confidence intervals and error rates in forensic entomology. Journal of Forensic Sciences 53: 942–948.

Turner B & Howard T (1992) Metabolic heat generation in dipteran larval aggregations: a consideration for forensic entomology. Medical and Veterinary Entomology 6: 179–181.

VanLaerhoven SL (2008) Blind validation of postmortem interval estimates using developmental rates of blowflies. Forensic Science International 180: 76–80.

Wall R, French N & Morgan KL (1992) Effects of temperature on the development of the sheep blowfly *Lucilia sericata* (Diptera: Calliphoridae). Bulletin of Entomological Research 82: 125–131.

Wallman JF (2001a) A key to the adults of species of blowflies in southern Australia known or suspected to breed in carrion. Medical and Veterinary Entomology 15: 433–437.

Wallman JF (2001b) Third-instar larvae of common carrion-breeding blowflies of the genus *Calliphora* (Diptera: Calliphoridae) in South Australia. Invertebrate Taxonomy 15: 37–51.

Wallman JF & Donnellan SC (2001) The utility of mitochondrial DNA sequences for the identification of forensically important blowflies (Diptera: Calliphoridae) in southeastern Australia. Forensic Science International 120: 60–67.

Wallman JF, Leys R & Hogendoorn K (2005) Molecular systematics of Australian carrion-breeding blowflies (Diptera: Calliphoridae) based on mitochondrial DNA. Invertebrate Systematics 19: 1–15.

Wang Y-G (2000) On comparison of growth curves: how do we test whether growth rates differ? Fisheries Bulletin 98: 874–880.

Wells JD & Kurahashi H (1994) *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) development: rate, variation and the implications for forensic entomology. Japanese Journal of Sanitary Zoology 45: 303–309.

Wells JD & LaMotte LR (2001) Estimating the post-mortem interval. Forensic Entomology: The Utility of Arthropods in Legal Investigations (ed. by JH Byrd & JL Castner), pp. 259–281. CRC Press, Boca Raton, FL, USA.

Wells JD & Sperling FAH (1999) Molecular phylogeny of *Chrysomya albiceps* and *C. rufifacies* (Diptera: Calliphoridae). Journal of Medical Entomology 36: 222–226.

Wells JD & Williams DW (2007) Validation of a DNA-based method for identifying Chrysomyinae (Diptera: Calliphoridae) used in a death investigation. International Journal of Legal Medicine 121: 1–8.

Wells JD, Pape T & Sperling FAH (2001) DNA-based identification and molecular systematics of forensically important Sarcophagidae (Diptera). Journal of Forensic Sciences 46: 1098–1102.

Wigglesworth VB (1965) The Principles of Insect Physiology. Methuen & Co., London, UK.

Williams H & Richardson A (1984) Growth energetics in relation to temperature for larvae of four species of necrophagous flies (Diptera: Calliphoridae). Australian Journal of Ecology 9: 141–152.

Zehner R, Amendt J, Schütt S, Sauer J, Krettek R & Povolný D (2004) Genetic identification of forensically important flesh flies (Diptera: Sarcophagidae). International Journal of Legal Medicine 118: 245–247.

von Zuben CJ, Bassanezi RC & von Zuben FJ (1998) Theoretical approaches to forensic entomology: II. Mathematical model of larval development. Journal of Applied Entomology 122: 275–278.