Development of larvae of the Australian blowfly, *Calliphora augur* (Diptera: Calliphoridae), at constant temperatures

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Keywords: Forensic entomology, post-mortem interval, prediction interval

Abstract

Calliphora augur (Diptera: Calliphoridae) is a common carrion-breeding blowfly of forensic, medical and agricultural importance in eastern Australia. Despite this, detailed information on the developmental biology of *C. augur* is lacking. Here, we present the first comprehensive study on the development of all three larval instars of *C. augur*, fed on sheep's liver, at constant temperatures of 15, 20, 25, 30 and 35°C. We highlight decreasing variation in final larval length at sampling periods up to 30°C, although this variation increased again when larvae were grown at 35°C. 95% prediction intervals are provided for each constant temperature, enabling the age of larvae of *C. augur* to be estimated from their average length. These data will assist with the application of this species to forensic investigations.

Introduction

Forensic entomology is the study of insects as applied to legal issues. It may relate to medicolegal events, especially murders, for the purpose of uncovering information useful to an investigation (Catts & Goff, 1992). This is because decomposing vertebrate remains provide a temporary micro-habitat and food source that is attractive to a variety of insects and other invertebrates (Smith, 1986). Arthropods constitute a major element of this fauna, with insects, especially flies and their larvae, being the predominant group. Blowflies (Diptera: Calliphoridae) are ubiquitous and are typically the first insects to visit a dead body, often well before it is discovered by humans (Greenberg, 1991).

Data on the development of blowflies and other important carrion flies can help provide an estimate of the minimum time since death (or minimum post-mortem interval: mPMI), especially when combined with information on the succession patterns of insects on corpses (Sharma et al., 2015). The traditional approach adopted in developmental studies has been to generate growth data based on larval size or weight, and then use tables (Kamal, 1958; Nishida et al., 1986) or plots incorporating various models (Wells & LaMotte, 1995; Byrd & Butler, 1997; Grassberger & Reiter 2001; Donovan et al., 2006; Richards et al., 2009) to estimate the age of larvae collected from a corpse. This information would then be used to derive the time of egg or larval deposition and thus an estimate of the mPMI. However, many studies have neglected to provide mathematically explicit mPMI predictions, meaning that reliable predictions are still lacking for many calliphorid species. It is now generally suggested that studies of larval development provide mPMI predictions with a range of values rather than a single value, and that this range should be defined as a confidence set (Wells & LaMotte 2017).

Calliphora augur Fabricius is a common blowfly species found in eastern Australia (Wallman & Adams, 1997), the larvae of which are among those most commonly collected from forensic cases, including from inside buildings (Levot, 2003). It is ovoviviparous (Meier et al., 1999), and also an agent of myiasis in sheep and other animals (Fuller, 1932; Norris, 1959; Lee, 1968). While it has a preference for fresh carcasses (Monzu, 1977), it will also larviposit on putrid liver (Mackerras, 1933). While previous studies have considered the larval development of *C. augur* (Johnston & Hardy, 1923; Fuller, 1932; Mackerras, 1933; Mackerras & Freney, 1933; Levot et al., 1979; O'Flynn, 1983) none have made fine scale measurements across a range of constant temperatures, or have attempted to provide mathematically explicit mPMI predictions. This lack of accurate developmental data means that at present, *C. augur* cannot be used reliably in a forensic context. To ameliorate this, we present the first comprehensive analysis of larval growth at constant temperatures of this important species.

Our investigations are based on measurement of larval length throughout development, including observations on size overlap between larval instars. Most importantly, we calculate prediction intervals that can be used to mathematically infer larval age from size.

Materials and methods

Fly culture

A culture of *C. augur* was established from adults trapped at Wollongong, New South Wales (34° 25' S, 150°53' E). Experiments commenced using larvae of the F3 generation and were continued for 11 generations. The culture was refreshed regularly with wild-type (F_0) individuals from the same source population as above and maintained with mixed ages of females and males. The average fecundity of each female was estimated by separating 20 females and transferring them to 50 mL specimen pots containing approximately 10 g of sheep's liver. Larvae were counted the following day. Larvae and adults were kept in a temperature-controlled room at 25±3.5˚C and ambient humidity, with a 12:12 light:dark regime incorporating a 15 minute 'dusk' and 'dawn' transition period of low light. Adult flies were maintained in square plastic cages measuring 330 mm long, 220 mm wide and 250 mm high (external dimensions). The flies in each cage were provided with water and sugar *ad libitum*, and chopped sheep's liver in a small plastic weigh boat for larviposition as required. Larvae were reared in square white 2 L plastic containers, measuring 170 mm wide along each side and 90 mm deep. Most of the centre of the lid of each container was cut away and replaced with fine mesh to permit ventilation. In each container, larvae were sustained on sheep's liver in the weigh boats placed atop a layer of wheaten chaff approximately 20 mm deep to enable pupariation. After pupariation, pupae were manually sorted from the chaff and transferred to a cage. Sheep's liver was provided just prior to eclosion to provide females with a constant source of protein for ovary maturation (Mackerras, 1933).

Development at constant temperatures

Each replicate consisted of freshly laid larvae randomly collected from the main culture. Preliminary studies on larval density indicated that some endogenous heat generation occurred with as few as 25 larvae growing on 50 g of sheep's liver. To ensure that the larvae developed at the ambient temperature being examined, each replicate (a plastic weigh boat containing larvae and meat) was limited to 10 larvae. These replicates were grown at specific constant temperatures of 15, 20, 25, 30 and 35˚C, and 60±5% humidity, in an Axyos (Brisbane, Queensland, Australia) environmental cabinet. Since only one cabinet was available, growth at each temperature was studied in turn. Larvae were left

undisturbed in darkness in the environmental cabinet until collection, because disturbance can delay pupariation (Mackerras, 1933; O'Flynn, 1983). To account for growth rates increasing with temperature, we sampled more frequently at higher temperatures.

The growth period of each replicate of 10 larvae at a particular temperature was chosen by lottery. For the first three to six days, six-hourly sampling per sample was done at 15°C, while four-hourly sampling was conducted at 20, 25, 30 and 35°C. Six replicates were used for the first sample period, and every 48 hours thereafter, and three replicates were used for every other time interval (either 12- or 24-hourly, depending on temperature). The choice of these specific sampling frequencies was guided by the growth curves of O'Flynn (1980; 1983), and frequencies were kept relatively high to minimise error in the calculation of the prediction intervals.

Sample collection, handling and preservation

At collection, all larvae were killed and fixed immediately by immersion in boiling water, dried with paper towel and preserved in 80% EtOH. Larvae were placed into glass vials posteriad to prevent head-curling (Day & Wallman, 2006) and length and instar were recorded.

Measurement and statistics

The body lengths of larvae were measured with the aid of a dissecting microscope and Mitutoyo Absolute digimatic digital callipers (Kawasaki, Japan) after the larvae had been in preservative for a minimum of ten days (Day & Wallman, 2008). Body length was measured as the distance, viewed laterally, between the most distal parts of the head and the last abdominal segment. The lengths of pupae were also measured with callipers. The ambient temperature within the temperature-controlled cabinet was monitored with data loggers (iButtons: Maxim Integrated Products Inc., Sunnyvale, USA).

The dataset consists of the measurements of the lengths of larvae grown at temperatures of 15ºC, 20ºC, 25ºC, 30ºC, and 35ºC. Since no larvae were measured more than once, the length data for the various time intervals should be regarded as independent. As such, the variable of interest for this study was the variation in growth rate, as measured by length, at the different temperature treatments.

The instar of each larva was also recorded when it was measured as either 1st, 2nd, or 3rd. As the dataset is rather large (more than 3000 datapoints), presenting it in its entirety would be impractical, and we therefore display the data in scatterplots for each of the temperatures (the small number of intermediate instars were removed from these graphs in order to aid readability). Note that the intervals

differ for the various temperatures; the intervals were chosen to accurately capture the growth of the larvae, with the higher temperatures naturally necessitating more frequent sampling: the larvae at 15°C were measured every 24 hours, those at 20°C every 6 hours, and the rest every 4 hours.

Results

The average numbers of eggs laid were 58±14 per gravid female. First-instar larvae were largest on average when grown at 25°C. Similarly, second instars were also largest when grown at 25°C, but they also displayed a much smaller variation at this temperature compared with the other temperatures examined. Third-instar larvae were largest on average when grown at 20°C, but were above the overall mean (for third-instars) when growing at 15, 20, 25 and 30°C. The only temperature at which the average length of third instars dropped below their overall mean was at 35°C. Post-feeding third instars were largest on average when grown at 20°C.

Utilizing the dataset

Our dataset can provide potentially valuable information to an investigator by simply comparing the ratios of the various instars found at a crime scene with those obtained in our experiment. To this end, we present graphs showing the association between size and time for each of the temperatures measured (Figure 1).

For instance, supposing that a batch of larvae is recovered from a body from a location where the average temperature has been 15°C, and these larvae are roughly evenly split between instars 2 and 3, then consulting Figure 1 would give a range of the mPMI between 72 and 120 hours, with 96 hours being the best estimate. On the other hand, if only larvae in instar 1 are recovered from a body at an average temperature of 35°C, then the mPMI is confidently less than 12 hours, and so forth. This should help to establish the mPMI in certain circumstances, in particular when the percentages of the various instars are specific to a relatively small time interval, but is clearly not good enough in other instances, most notably those corresponding to longer mPMIs in which one is likely to find only larvae in instar 3. These cases require a more refined analysis, which we now provide.

It is assumed that an investigator is able to procure at least five larvae from a crime scene and is able to correctly record their average length, as we refer to in the ‘measurement and statistics’ section. We will provide 95% prediction intervals for the average length of the larvae for each combination of time, instar, and temperature; this is analogous to but different than giving confidence intervals, since we

are predicting the mean value of a sample rather than the population mean. A detailed explanation of the process can be found in Geisser and Johnson (2006), but for the benefit of the reader we briefly review the theory behind the method. Those unfamiliar with some of the standard distributions and facts from statistics theory used below are advised to consult almost any appropriate statistics textbook or online resource.

Suppose that, for a given time, instar, and temperature, the length of a maggot is normally distributed with mean μ and variance σ^2. We regard our experimental data as a sample from this population of size N, and wish to test the hypothesis that a sample procured from a crime scene by an investigator was taken from the same population. The crime scene sample is regarded as independent from the experimental sample, and let m be the number of larvae taken from the time scene. Let us label the sample mean from our experiment as $\bar{x}_{\text{exp}}$ and from the crime scene as $\bar{x}_{\text{cri}}$.

As is well known, $\bar{x}_{\text{exp}}$ and $\bar{x}_{\text{cri}}$ will be normally distributed random variables, each with mean μ but with variances σ^2/N and σ^2/m respectively. Since $\bar{x}_{\text{exp}}$ and $\bar{x}_{\text{cri}}$ are independent random variables, their difference $\bar{x}_{\text{exp}} - \bar{x}_{\text{cri}}$ will also be normally distributed, but now with mean 0 and variance $\sigma^2/N + \sigma^2/m$. It follows that $Z = (\bar{x}_{\text{exp}} - \bar{x}_{\text{cri}})/\sqrt{\sigma^2(\frac{1}{N}+\frac{1}{m})}$ is a standard normal (mean 0, variance 1). If we knew the population variance σ^2, we could use a standard normal table to create 95% prediction intervals, but since we do not (as is typical) we will use the sample variance s^2 from our experiment in its place. Now, $\frac{(N-1)s^2}{\sigma^2}$ will have a $\chi^2(N\text{-}1)$ distribution, and will be independent of Z (it is independent of $\bar{x}_{\text{cri}}$ by assumption, and of $\bar{x}_{\text{exp}}$ by the standard independence of sample mean and variance for normal random variables). Thus, $\frac{Z}{\sqrt{s^2/\sigma^2}}$ will follow a t_{n-1} distribution. Replacing Z by the expression that defined it and cancelling the σ^2 in the numerator and denominator, we see that $(\bar{x}_{\text{exp}} - \bar{x}_{\text{cri}})/\sqrt{s^2(\frac{1}{N}+\frac{1}{m})} \sim t_{n-1}$. Our 95% prediction interval for $\bar{x}_{\text{cri}}$ is therefore $\bar{x}_{\text{exp}} \pm t_{.025,\text{N-1}}\sqrt{s^2(\frac{1}{N}+\frac{1}{m})}$.

A few comments on the normality assumption made above are in order. It is commonly accepted that measurements such as the size of organisms are generally modelled well by normal distributions; indeed, we ran the standard Kolmogorov-Smirnov and Shapiro-Wilk normality tests on the data, and found that most of the data subsets for each time, instar, and temperature are consistent with an underlying normal distribution, with a small number of exceptions. Furthermore, even in the case of

the exceptions, the *t*-test is known to be highly robust against non-normality, for the reason that even when the population does not follow a normal distribution the sample means $\bar{x}_{\text{exp}}$ and $\bar{x}_{\text{cri}}$ will still be approximately normally distributed (Lumley et al 2002). This assures us that the method described above is general and not dependent upon the normality assumption.

We present the prediction intervals in table form below (Table 1), with a separate section for each combination of temperature and instar. Note that we have not provided tables for the intermediate stages (first- to second-instar, or second- to third-instar) since we feel that they do not appear in sufficient numbers to provide valuable prediction intervals; we have also removed from Table 1 the times at which there fewer than five larvae of a particular instar present, for the same reason. The prediction intervals are given in the form (a,b), which is an equivalent notation to $a \leq \bar{x}_{\text{cri}} \leq b$. An investigator should make use of these tables by calculating the mean length of their sample of larvae, and then comparing this with the prediction intervals given in the table corresponding to the correct instar and average temperature; if the mean of the sample lies within the prediction interval for a given time, then that time should be regarded as a possible age for the larvae. We have provided prediction intervals for crime scene sample sizes $m = 5$ and $m = 30$; the $m = 5$ interval should be used for sample sizes between 5 and 29, and the $m = 30$ interval for any m over 30.

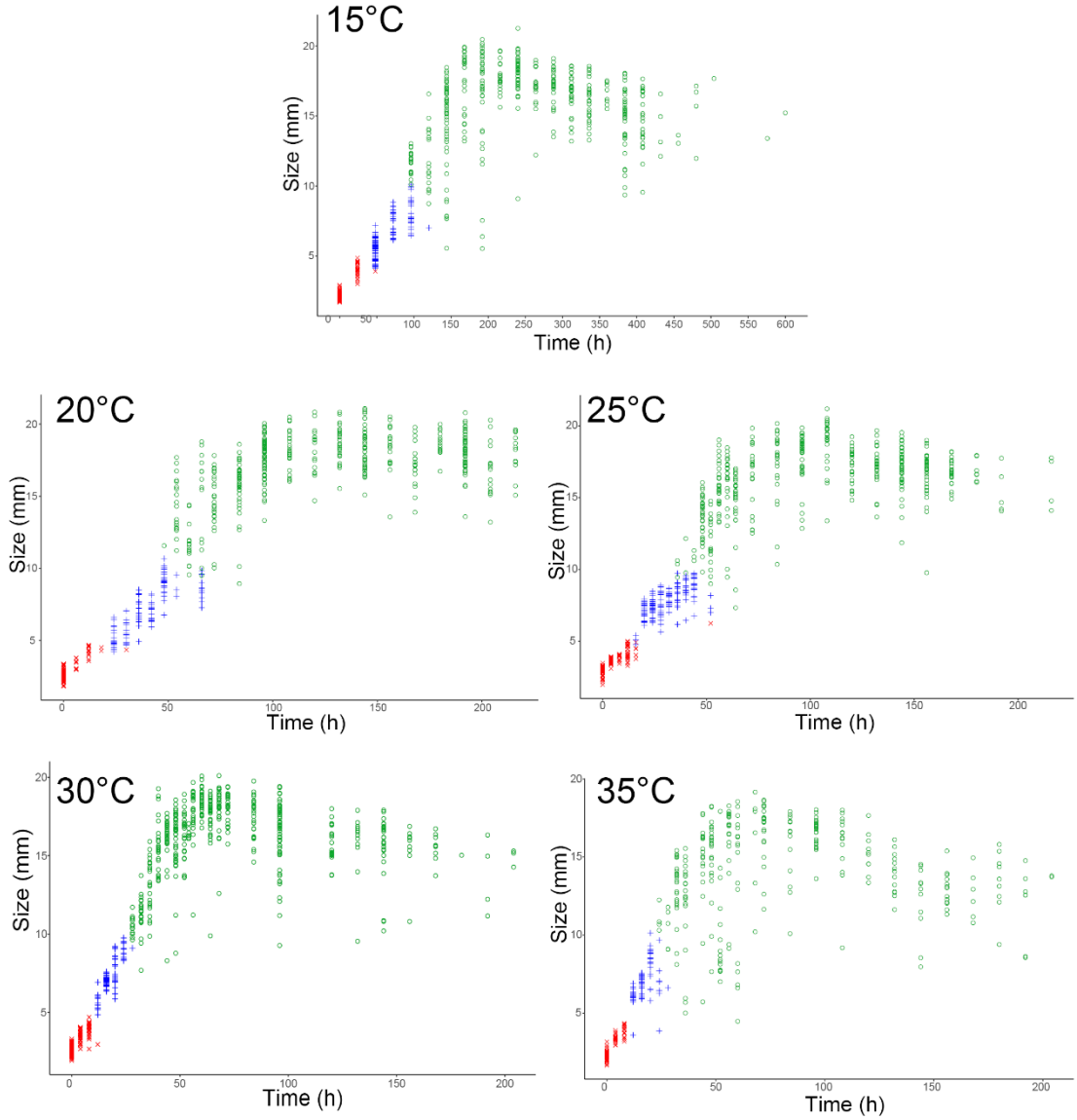

Figure 1. Growth curves of *Calliphora augur* larvae at constant temperatures. Data points represent individual larvae at each treatment time. First-instar larvae are represented by the red ×, second-instar by the blue +, and third-instar by the green o. The first plot (15°C) has a different scale on the x-axis.

Table 1. The 95% prediction intervals based on the developmental curves of *Calliphora augur* larvae at five different constant temperatures (15, 20, 25, 30, 35°C). n = sample number, $\bar{x}_{exp}$ = sample mean, s^2 = sample variance. $m = 5$ interval is the prediction interval to be used for sample sizes between 5 and 29 larvae, $m = 30$ interval is the prediction interval to be used for sample sizes larger than 30 larvae.

Time (hours)	***N***	$\bar{x}_{exp}$	s^2	***m* = *5* interval**	***m* = *30* interval**
Instar 1, Temp 15 ºC					
0	59	2.1725	0.072	(1.989,2.356)	(2.052,2.293)
24	25	4.0148	0.240	(3.636,4.393)	(3.741,4.289)
Instar 2, Temp 15 ºC					

48	65	5.4798	0.530	(4.986,5.974)	(5.159,5.801)
72	26	7.3438	0.782	(6.666,8.022)	(6.856,7.832)
96	21	8.2752	1.286	(7.366,9.184)	(7.602,8.948)
Instar 3, Temp 15 ºC					
96	21	11.7024	0.632	(11.065,12.340)	(11.231,12.174)
120	17	11.9724	4.935	(10.096,13.849)	(10.543,13.402)
144	51	14.3841	10.767	(12.105,16.663)	(12.868,15.901)
168	25	17.3384	5.108	(15.593,19.084)	(16.075,18.602)
192	42	16.7124	13.344	(14.117,19.308)	(14.949,18.476)
216	19	17.7626	1.108	(16.899,18.627)	(17.114,18.411)
240	43	17.9709	3.062	(16.731,19.211)	(17.131,18.811)
264	20	17.149	2.339	(15.909,18.389)	(16.225,18.073)
288	31	17.0787	1.668	(16.119,18.038)	(16.403,17.754)
312	32	16.5694	1.934	(15.542,17.597)	(15.849,17.290)
336	29	16.0203	2.029	(14.950,17.090)	(15.260,16.780)
360	8	16.5700	0.607	(15.696,17.444)	(15.837,17.303)
384	41	14.7044	4.616	(13.173,16.236)	(13.661,15.748)
408	25	14.8204	4.069	(13.263,16.378)	(13.693,15.948)
Instar 1, Temp 20 ºC					
0	45	2.5538	0.164	(2.268,2.839)	(2.361,2.746)
6	11	3.3409	0.104	(3.027,3.655)	(3.088,3.594)
12	13	4.2562	0.135	(3.919,4.593)	(3.990,4.522)
Instar 2, Temp 20 ºC					
24	18	5.1522	0.519	(4.553,5.752)	(4.699,5.605)
30	13	5.5415	0.538	(4.869,6.214)	(5.011,6.072)
36	20	7.0295	0.865	(6.276,7.783)	(6.468,7.591)
42	19	7.1032	0.404	(6.581,7.625)	(6.712,7.495)
48	22	9.0773	0.772	(8.380,9.774)	(8.564,9.590)
66	10	8.5380	0.643	(7.727,9.349)	(7.876,9.200)
Instar 3, Temp 20 ºC					
54	18	14.4444	3.65	(12.855,16.034)	(13.243,15.646)
60	15	11.6647	1.906	(10.456,12.874)	(10.728,12.601)
66	14	14.8379	10.474	(11.943,17.733)	(12.575,17.101)
72	27	14.6637	3.818	(13.177,16.151)	(13.598,15.729)
84	38	15.6639	3.861	(14.249,17.079)	(14.692,16.636)
96	64	17.7798	2.149	(16.784,18.776)	(17.132,18.428)
108	24	18.245	1.689	(17.233,19.257)	(17.509,18.981)
120	18	18.1456	2.534	(16.821,19.470)	(17.144,19.147)
132	27	18.6196	1.475	(17.695,19.544)	(17.957,19.282)
144	44	18.2527	2.302	(17.181,19.325)	(17.528,18.977)

156	19	18.3826	2.562	(17.069,19.696)	(17.397,19.369)
168	18	17.2128	2.393	(15.926,18.500)	(16.240,18.186)
180	16	18.6719	0.627	(17.992,19.352)	(18.149,19.194)
192	54	18.1091	1.673	(17.216,19.002)	(17.518,18.700)
204	16	16.8313	3.503	(15.223,18.439)	(15.596,18.066)
216	14	18.0257	2.066	(16.740,19.311)	(17.021,19.031)
Instar 1, Temp 25 ºC					
0	48	2.9033	0.121	(2.660,3.147)	(2.740,3.066)
4	27	3.6081	0.037	(3.462,3.754)	(3.503,3.713)
8	11	3.81	0.048	(3.597,4.023)	(3.638,3.982)
12	30	4.268	0.214	(3.923,4.613)	(4.024,4.512)
16	6	4.2917	0.178	(3.732,4.852)	(3.807,4.777)
Instar 2, Temp 25 ºC					
20	23	7.143	0.291	(6.719,7.567)	(6.833,7.453)
24	28	7.31	0.352	(6.862,7.758)	(6.990,7.630)
28	21	7.5662	0.864	(6.821,8.311)	(7.015,8.118)
32	18	7.9783	0.203	(7.603,8.353)	(7.695,8.262)
36	16	8.275	0.712	(7.550,9.000)	(7.718,8.832)
40	16	8.6106	0.564	(7.965,9.256)	(8.115,9.106)
44	17	8.7971	0.609	(8.138,9.456)	(8.295,9.299)
Instar 3, Temp 25 ºC					
48	34	13.6715	2.144	(12.600,14.743)	(12.925,14.418)
52	19	12.1405	2.881	(10.747,13.534)	(11.095,13.186)
56	29	15.8128	2.342	(14.663,16.962)	(14.996,16.629)
60	27	16.0007	4.955	(14.307,17.695)	(14.787,17.214)
64	27	14.7056	5.339	(12.947,16.464)	(13.446,15.966)
72	21	16.5886	5.495	(14.710,18.467)	(15.197,17.980)
84	24	17.3142	5.99	(15.409,19.220)	(15.928,18.701)
96	39	17.7841	2.745	(16.595,18.973)	(16.970,18.599)
108	24	18.8704	3.099	(17.500,20.241)	(17.873,19.868)
120	27	17.2167	1.342	(16.335,18.098)	(16.585,17.848)
132	28	17.1954	1.806	(16.180,18.211)	(16.471,17.920)
144	45	17.0431	2.249	(15.986,18.100)	(16.331,17.755)
156	33	16.5845	2.917	(15.329,17.840)	(15.707,17.462)
168	21	16.7257	0.818	(16.001,17.451)	(16.189,17.262)
180	7	16.8843	0.592	(15.956,17.812)	(16.094,17.675)
192	6	15.6000	2.257	(13.606,17.594)	(13.873,17.327)
Instar 1, Temp 30 ºC					
0	60	2.5583	0.104	(2.338,2.779)	(2.414,2.703)
4	28	3.5518	0.153	(3.256,3.847)	(3.341,3.763)

8	29	3.9228	0.168	(3.615,4.231)	(3.704,4.141)
Instar 2, Temp 30 ºC					
12	14	5.6279	0.278	(5.156,6.100)	(5.259,5.997)
16	29	7.0290	0.106	(6.784,7.274)	(6.855,7.203)
20	21	7.6262	0.946	(6.847,8.406)	(7.049,8.203)
24	13	9.0131	0.288	(8.521,9.505)	(8.625,9.401)
Instar 3, Temp 30 ºC					
28	13	10.5392	0.349	(9.998,11.081)	(10.112,10.967)
32	23	11.2465	1.399	(10.317,12.176)	(10.567,11.926)
36	25	13.1956	2.810	(11.901,14.490)	(12.259,14.132)
40	25	15.5992	2.760	(14.316,16.882)	(14.671,16.528)
44	30	16.1283	2.987	(14.838,17.419)	(15.216,17.041)
48	38	16.0895	3.748	(14.695,17.484)	(15.131,17.048)
52	27	16.4419	2.032	(15.357,17.527)	(15.665,17.219)
54	6	16.4567	0.141	(15.958,16.955)	(16.025,16.888)
56	25	17.7292	2.825	(16.431,19.027)	(16.790,18.669)
60	28	18.4364	0.625	(17.839,19.034)	(18.010,18.863)
64	34	17.4650	2.535	(16.300,18.630)	(16.654,18.276)
68	28	18.0279	1.991	(16.961,19.094)	(17.267,18.789)
72	23	18.0283	0.908	(17.280,18.777)	(17.481,18.576)
84	28	17.4275	1.600	(16.471,18.384)	(16.746,18.109)
96	53	16.3130	4.688	(14.815,17.811)	(15.320,17.306)
120	22	15.7395	1.246	(14.854,16.625)	(15.088,16.391)
132	12	15.7417	4.745	(13.689,17.795)	(14.104,17.379)
144	28	15.6121	3.940	(14.112,17.112)	(14.542,16.682)
156	10	15.3620	2.889	(13.642,17.082)	(13.958,16.766)
168	9	15.5289	0.800	(14.581,16.477)	(14.745,16.313)
Instar 1, Temp 35 ºC					
0	63	2.2735	0.082	(2.079,2.468)	(2.147,2.400)
4	16	3.3800	0.074	(3.146,3.614)	(3.201,3.559)
8	19	3.8411	0.129	(3.546,4.136)	(3.62,4.062)
Instar 2, Temp 35 ºC					
12	18	6.0122	0.462	(5.447,6.578)	(5.585,6.440)
16	27	6.8485	0.261	(6.460,7.237)	(6.570,7.127)
20	21	8.3519	0.920	(7.583,9.121)	(7.783,8.921)
24	8	6.8000	2.611	(4.988,8.612)	(5.280,8.320)
Instar 3, Temp 35 ºC					
28	5	10.4360	1.655	(8.480,12.392)	(8.711,12.161)
32	25	13.0576	3.477	(11.618,14.498)	(12.015,14.100)
36	20	11.9110	9.849	(9.367,14.455)	(10.015,13.807)

44	19	14.5695	8.228	(12.215,16.924)	(12.803,16.336)
48	27	15.2704	5.556	(13.477,17.064)	(13.985,16.556)
52	15	10.0413	9.612	(7.327,12.756)	(7.939,12.144)
56	20	16.1190	6.689	(14.022,18.216)	(14.556,17.682)
60	17	13.4012	22.225	(9.418,17.384)	(10.367,16.435)
68	6	15.8800	11.792	(11.322,20.438)	(11.932,19.828)
72	22	16.7327	3.414	(15.267,18.198)	(15.654,17.811)
84	14	15.7243	5.512	(13.624,17.824)	(14.083,17.366)
96	28	16.5311	0.826	(15.844,17.218)	(16.041,17.021)
108	14	15.7743	5.267	(13.721,17.827)	(14.170,17.379)
120	10	15.2280	1.652	(13.928,16.528)	(14.166,16.290)
132	13	13.9485	1.780	(12.726,15.171)	(12.983,14.914)
144	11	12.2182	5.073	(10.025,14.411)	(10.449,13.987)
156	13	12.9208	1.246	(11.898,13.944)	(12.113,13.728)
168	6	12.6067	2.463	(10.523,14.69)	(10.803,14.411)
180	9	13.4256	3.840	(11.349,15.502)	(11.708,15.143)
192	7	11.9214	5.784	(9.021,14.821)	(9.451,14.392)

The investigator may also be interested in forming prediction intervals for time, temperature, and instar combinations that do not appear above. The table below (Table 2) may be of some use if the combination appears there, but there are still some combinations (for example, temperature 35°, instar 3, time 76 hours) for which we have no data. As a simple rule of thumb, in this case, if the nearest times for which there are data (here 72 and 84 hours) include $\bar{x}_{\text{cri}}$ then the time in question should be regarded as possible. If the investigator requires a precise prediction interval then we recommend interpolating the means and variances linearly from the nearest points in time to estimate the mean and variance at the point in question, and taking the degrees of freedom to be equal to the minimum of the degrees of freedom at the nearest points (this is a slight simplification of the method used in Wells & Lamotte, 1995). For example, in our case of temperature 35°C, instar 3, time 76 hours, we look at the closest time points for the given temperature and instar, which are at 72 and 84 hours. The means are at 16.733 and 15.724, respectively, and the variances are 3.414 and 5.512. We therefore estimate the mean and variance at 76 hours to be $\left(\frac{2}{3}\right)16.733 + \left(\frac{1}{3}\right)15.724 = 16.397$ and $\left(\frac{2}{3}\right)3.414 + \left(\frac{1}{3}\right)5.512 = 4.113$; note that the values at 72 hours are weighted double those at 84 hours, because 76 is twice as far from 84 as from 72. There are 22 measurements at 72 hours and 14 measurements at 84 hours, so we take as our degrees of freedom *min* (22-1,14-1) = 13. We therefore obtain prediction intervals by the same method as before of (14.497,18.297) for m = 5 and (14.979,17.815) for m = 30.

For completeness, Table 2 provides the sample means and variances (when they exist) for the cases for which we had between one and five measurements (we have not formulated prediction intervals in these cases). Any remaining time, temperature, and instar combinations that do not appear in either the above or below tables are ones for which we have no measurements, and for these the interpolation method described above should be used to form prediction intervals, if necessary.

Table 2. The sample means and variances of the growth of *Calliphora augur* larvae for cases which included between one and five measurements. Dots represent variances that could not be calculated due to there being only one sample.

Instar	**Temp (ºC)**	**Time**	***n***	$\bar{x}_{exp}$	s^2
1	**15**	48	1	3.910	.
2	-	120	1	7.020	.
3	-	432	4	14.203	3.890
-	-	456	2	13.340	0.168
-	-	480	4	15.385	5.512
-	-	504	1	17.680	.
-	-	576	1	13.400	.
-	-	600	1	15.230	.
1	**20**	18	2	4.395	0.026
-	-	30	1	4.350	.
2	-	54	3	8.707	0.584
3	-	48	1	11.570	.
1	**25**	52	1	6.250	.
2	-	16	3	5.093	0.099
-	-	52	3	7.490	0.381
3	-	36	3	10.150	0.373
-	-	40	2	10.955	2.856
-	-	44	3	10.940	0.125
-	-	216	4	16.050	3.518
1	**30**	12	1	2.960	.
2	-	28	1	9.100	.
3	-	180	1	15.030	.
-	-	192	4	13.663	5.704
-	-	204	4	15.003	0.241
2	**35**	28	1	6.61 0	.
3	-	24	4	11.023	0.723
-	-	204	2	13.760	0.003

Discussion

This study provides the first detailed dataset of the development of the Australian carrion-breeding blowfly *Calliphora augur*. The results we provide here reinforce the previous work done on this species but go substantially further by providing a level of statistical confidence in the application of developmental data in forensic investigations.

Fecundity

Robust and highly active first-instar larvae of *C. augur* hatch promptly after deposition, giving this species an ecological advantage over oviparous species for exploiting small, quickly perishable carcasses (Norris, 1959). Johnston & Tiegs (1922) reported that *C. augur* is capable of depositing eggs or larvae, with the eggs usually hatching within six hours. In the present study, we noted that *C. augur* will normally deposit living larvae but will also lay soft infertile eggs for some days before normal larviposition occurs – similar to the observations of Mackerras (1933) and O'Flynn (1980). The average number of larvae per female has previously been noted as 50 (Mackerras, 1933) or 58 larvae (Norris, 1959). Callinan (1980) recorded a range of between 22.5 and 98.3 larvae per female in a controlled environment and a range of between 0.3 and 45.2 larvae per female in a field environment. The average numbers of eggs laid in the present study were 58±14 per gravid female, which is consistent with these previous data.

Development at constant temperatures

The outcomes of some other developmental studies have been limited by a less thorough approach than taken here. For example, Byrd and Butler (1998) noted that their sampling method selected for the fastest growing larvae, which often indicates a shorter period than could otherwise be deduced from normal collection techniques at a crime scene. The influence of size extremes upon variance has also been considered by Wells & LaMotte (1995). In the present study, we included larvae of all sizes to demonstrate the full range that may be encountered in crime scene samples. Variation in larval body length appeared to decrease steadily from 15 to 30°C and then increased again dramatically at 35°C. The decrease in variation with increasing temperature may relate to a more even growth rate at higher temperatures. However, the subsequent increase in variation at 35°C likely reflects the fact that the larvae are reaching their thermal limit, at which point some larvae may begin to suffer from increased heat and oxidative stress, hindering their growth and increasing the perceived variation (Rivers et al. 2011). The variation in overlap of the growth stages, particularly the transitional forms between instars, emphasises the importance of assessing crime scene samples for instar and/or transitional forms, and

collecting an adequate number of specimens. While there has been comment on the value of transitional forms (Catts, 1991), few published papers include this information and some workers disregard their influence on size ranges (e.g. Wells & LaMotte, 1995).

The reader will note an interesting feature of the plots, namely that the measured lengths increase linearly with time to a certain point, but then begin to decrease after that. This late-stage reduction in larval length is a result of post-feeding shrinkage that calliphorid larvae undergo after leaving the carcass, prior to pupariation. This post-feeding shrinkage can cause some difficulty in interpreting the age of larvae, because the smaller length of post-feeding larvae makes them appear younger. However, this can be solved if investigators record where larvae are collected, as post-feeding larvae will depart the carcass and move into the surrounding substrate. If larvae are collected away from the carcass, it can be presumed that they are in the post-feeding stage and as such, post-feeding confidence intervals should be used to infer their age. Furthermore, post-feeding larvae will rapidly evacuate the crop prior to pupariation, and thus crop dissection can be performed to check for the presence of food particulates. If no food particulates are found, then it can be assumed that the larvae are post-feeding. For a more thorough discussion of this technique see Archer et al. (2017).

While some other previous studies have included data on the development of *C. augur* (Johnston & Hardy, 1923; Fuller, 1932; Mackerras, 1933; Mackerras & Freney, 1933; Levot et al., 1979; O'Flynn, 1983), they were usually conducted as parts of larger projects and provide insufficient detail for inferring an mPMI. For example, Fuller (1932) only reported the average length of the maggot when full grown (18 mm), with which our findings concur. Mackerras (1933) referred only to complete development, with 21-22 d recorded for growth in summer conditions in an insectary and 18-20 d for development in a room at 20°C. Levot et al. (1979) examined weights of *C. augur* larvae at 27-28°C and found the time to reach maximum larval weight to be 65.5 hours. While direct comparisons cannot be drawn with this, we observed that the time to reach maximum larval length at 30°C was 72 h. The techniques of Levot et al. (1979) were very different to ours, with a large mass of larvae feeding on excess liver and each sampling event removing some individuals. It is possible that the larvae in their experiment were actually exposed to higher temperatures than the ambient of 27-28°C via massing, and that the feeding mass was better able to liquefy the substrate, and therefore grow more quickly, than the larvae in the current study. Comprehensive work by O'Flynn (1980; 1983) examined larval growth at temperatures between 5 and 45°C but did not produce sufficient data for explicit mPMI predictions. At 5°C, the minimum period from larviposition to commencement of wandering was 54 d; some larvae survived for 110 d but none pupated.

Larval temperature preference

Archer and Elgar (2003) studied blowfly larval feeding strategies in carrion and found that site preference changes with the length of time the carcass was exposed to the elements. Examination of instar size and temperature in our study (Figure 1) indicates that larvae also appear to have temperature preferences that relate to their growth stage. The only temperature at which the average length of third instars dropped below their overall mean was at 35°C. This drop appears to be further evidence that this is around the upper thermal limit for this species. Post-feeding third instars were largest on average when grown at 20°C. These results indicate that the influence of temperature on larval growth is more complex than it appears. Detailed studies are needed to investigate movement and temperature preferences of individual larvae growing in a mass.

Substrate and larval growth

Importantly, it is well established that the growth and survival of blowfly larvae is dependent upon their food source. For example, studies on *Calliphora vicina* and *Calliphora vomitoria* have shown substantial differences in larval growth between mixed minced meats and beef liver (Neideregger et al. 2013) and there are almost certainly differences in larval growth between livestock and human tissue (Wallman and Archer 2020). While the prediction intervals we provide are based on *C. augur* fed on sheep's liver, these data are assumed to apply to the equivalent growth in human bodies – an assumption that holds true for comparisons between *C. vicina* larvae fed on porcine and human tissue (Bernhardt et al. 2017). Nevertheless, this assumption may not hold true for all *Calliphora* species, and as such there remains a pressing need for studies that compare the growth rates and fitness of blowflies reared on tissues from different animals.

Conclusions

Other workers in forensic entomology have explored various models to infer larval age, including techniques based on weight (Wells & LaMotte, 1995; von Zuben et al., 1998). Such approaches have obvious limitations, as it is difficult to control for the moisture content of the larvae after their collection. Previous studies based on larval size have predominantly used plots based upon models involving isomorphen or isomegalen diagrams (Grassberger & Reiter, 2001) or thermal summation (ADH) (Donovan et al., 2006; Higley & Haskell, 2010). Unlike many former approaches, the technique we outline here allows a clear statement of the statistical rigour underlying a maggot age estimate, while avoiding difficulties inherent in thermal summation, especially the reliance on the linear portion of the development curve and the estimation of the minimum developmental threshold (Nabity et al., 2006). Although inferences of larval age from our data must be based on the specific temperatures

used in our study, the predicted average developmental temperature in forensic cases will, nonetheless, often be no more precise than the nearest 5°C because of uncertainty about the amount of heat generated by the feeding larvae. Overall, the 95% prediction intervals we provide for each constant temperature, enable the age of larvae of *C. augur* to be estimated from their average length, for the first time enabling the reliable application of this species to forensic investigations.

Acknowledgements

We thank the University of Wollongong for financial assistance with this study.

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