Forensic Science International: Synergy 1 (2019) 140–145

Contents lists available at ScienceDirect

Forensic Science International: Synergy

journal homepage: https://www.journals.elsevier.com/forensic-science-international-synergy/

Technical note: A rapid, non-invasive method for measuring live or preserved insect specimens using digital image analysis

Donald R. Bourne [a], Christopher J. Kyle [a, b, *], Helene N. LeBlanc [a, c], David Beresford [a, d]

[a] Environmental and Life Sciences Graduate Program, Trent University, Peterborough, Ontario, Canada
[b] Forensic Science Department, Trent University, Peterborough, Ontario, Canada
[c] Faculty of Science - Forensic Science, University of Ontario Institute of Technology, Oshawa, Ontario, Canada
[d] Biology Department, Trent University, Peterborough, Ontario, Canada

ARTICLE INFO

Article history:
Received 22 January 2019
Received in revised form
17 July 2019
Accepted 18 July 2019
Available online 19 July 2019

Keywords:
Forensic entomology
Digital image measurement
Morphometric
Insect size
Phormia regina
Forensic photography

ABSTRACT

The measurement of insects is an important component of many entomological applications, including forensic evidence, where larvae size is used as a proxy for developmental stage, and hence time since colonization/death. Current methods for measuring insects are confounded by varying preservation techniques, biased and non-standardized measurements, and often a lack of sample size given practical constraints. Towards enhanced accuracy and precision in measuring live insects to help avoid these variables, and that allows for different measurements to be analyzed, we developed a non-invasive, digital method using widely available free analytical software to measure live blow fly larvae. Using crime scene photographic equipment currently standard in investigation protocols, we measured the live length of 282 *Phormia regina* larvae. Repeated measurements of maggots, for all instars, were performed for several orientations and images. Most accurate measurements were obtained when maggots were oriented in their natural full extension. Killed specimens resulted in greater length measurements (Mean 1.79 ± 1.11 mm) when compared to live length. Herein, we report a technically simple, fast, and accurate measurement technique adapted for field and lab-based measurements, as well as, a simple linear equation for conversion of live length to standard killed length measurements. We propose this method be utilized for the standardization of forensic entomological evidence collection and development model creation.

1. Introduction

Insect evidence is primarily used to estimate an elapsed time since death at homicides. This is most often accomplished by relating size to developmental stage of immature Calliphoridae species. Development is predictable based on the close relationship between growth rate and temperature [1-5]. Current methods used to measure insect size can damage or kill insect specimens due to handling [6-8]. Additionally, methods for processing larval specimens require fixation and preservation of killed specimens. These methods are not standardized and may cause unpredictable phenotypic postmortem changes [3,9-15]. There is need for a standardized larval insect processing technique which negates the effects of killing. Furthermore, the creation of development models using killed specimens require replicates to infer growth, resources (e.g., climate chambers), and time. The use of live measurements would effectively track the actual growth of individuals or populations, while reducing processing time.

Current methods for measuring killed insects in forensic entomology include calipers, compound microscopes with eyepiece micrometers, and graticules. Villet reported the only live insect measurement technique within forensic entomology using a geometrical micrometer [8]. Although it is possible to perform live measurements with this method, it is limited in practice, as fast moving and/or delicate insects must remain in place parallel to the calibrated line. As such, the geographical micrometer technique has a greater potential for specimen damage during handling time, it is limited to linear measurements, and accuracy relies upon the researcher's ability to place and judge the specimens' margins. Further, measuring the length of live maggots is not recommended [10] as live maggots are continuously in motion and contract if

* Corresponding author. Environmental and Life Sciences Graduate Program, Trent University, Peterborough, Ontario, Canada.
E-mail address: christopherkyle@trentu.ca (C.J. Kyle).

https://doi.org/10.1016/j.fsisyn.2019.07.006

disturbed, altering their body length. With the availability of high resolution digital cameras, it is technically possible to measure moving specimens with minimal disruption and handling. Unlike investigators in other fields of entomology [16], forensic investigators have largely ignored newer methods for measuring and determining insect size, most likely due to the requirements of using the same processing procedures for collection of specimens as available species-specific development data [10,17]. Recent publications have used imaging for measuring insect morphometrics as a tool in species identification [18], yet no studies have been conducted that measure live insects to determine development stage.

Blowfly larvae are soft bodied and difficult to measure accurately whether alive or dead. Movement occurs by contraction of oblique muscles while forward movement is a series of regular longitudinal lengthening and shortening [19,20]. The head is thrust forward to establish an anchor point then posterior contraction is initiated. It is the period of establishing an anchor point where the maggot is at full extension. It should be noted that maggot crawling is not altered by substrate texture [21]. Given these complicating and potentially confounding factors influencing larval length, in this paper we propose a novel and practical application which allows for multiple live insect measurements in a rapid and non-invasive application. Through high resolution digital image analysis, the accurate measurement of insect morphometrics can be accomplished. Assessment of the technique was conducted using the forensically important species, *Phormia regina* (Diptera: Calliphoridae) [2].

2. Materials and methods

2.1. Digital image measurement

We used a high-resolution D-SLR Camera (12 MP or greater) outfitted with an appropriate lens, external flash, scale/ruler, and hot-shoe bubble level. To achieve sufficient image resolution, a minimum of a 12 megapixels camera was required. We used a standard 18 mm–55mm lens for larger (approx. > 8 mm) specimens, and a 100 mm macro lens for the smaller first and second instar specimens. The camera was placed on a tripod (Fig. 1). A stage micrometer was used to test the accuracy of a standard steel ruler with 0.5 mm graduations. This ruler was included in each photo. In use, a live maggot (larva) was placed in a Petri dish on a flat white background beside the graduated scale. The camera was then positioned so that the sensor was level and parallel to the shooting surface. An image was taken once the specimen and scale were both in focus with the insect in the proper orientation for measurement. Multiple images were taken in rapid succession. Image analysis was completed through use of ImageJ [22,23] an open source imaging program (http://rsbweb.nih.gov/ij/).

2.2. Phormia regina rearing

Adult *P. regina* were netted over a pig carcass, and then housed in 400 mm x 400 mm rearing cages (sugar-water and milk powder *ab libitum*) to obtain eggs from wild caught specimens. Subsequent larvae were raised on fresh beef liver, in containers housed in an environmental chamber with the temperature set to 25 °C ± 2.5 °C.

2.3. Photographic comparisons

Total body length of *P. regina* larvae was measured using four differing methods: killed specimen using eyepiece micrometer, killed specimen using digital calipers, killed specimen using image technique, and live specimen using image technique. Live maggots were photographed and measured. These maggots were then killed by hot water immersion (80-90 °C for 30-60 s) [9] and then stored in 1.5 ml micro centrifuge tubes containing 80% ethanol with individual labels. Total body length was measured for 282 live specimens, and these measurements were compared to the measurements of the killed/preserved specimens. Killed photo documented larval length was measured with image technique, digital calipers, and eyepiece micrometer. All image measurements were completed using ImageJ [22,23].

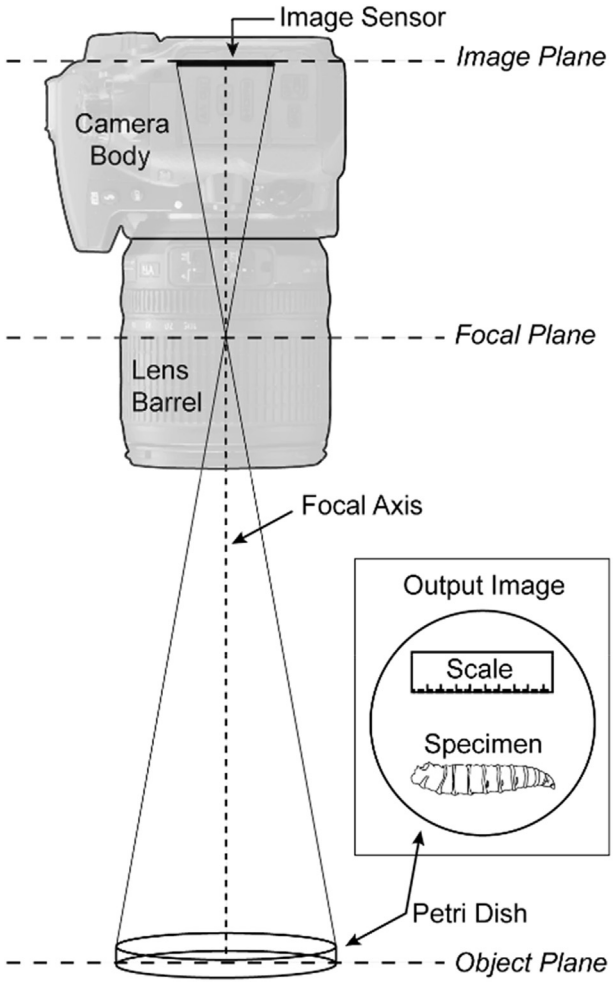

Fig. 1. Diagram of the insect measurement method set-up and components used for the study.

2.4. Assessment of agreement between techniques

Digital photo measurements using ImageJ were assessed for agreement to currently used specimen measurement methods by means of the Bland-Altman approach [24]. The values for each technique using the three different morphological features measured were plotted against each other and compared against Y = X (the line of equality). The difference between measurement techniques is depicted against their means to observe variation along a horizontal plane.

3. Results

3.1. Assessing measurement error

The reproducibility (precision) and amount of measurement error (accuracy) that arose from the use of the digital imaging program was estimated. For 1st, 2nd, and 3rd instar larvae, using images in all orientations resulted in an average of 2.29 mm ± 0.065 length with up to 6.64% variability, 6.12 mm ± 0.05 length with up to 2.2% variability, and 14.37 mm ± 0.2 length with up to 3.51% variability, respectively. In contrast, straight 1st instar orientation, were found to have up to 1.05% (Std. dev. 0.008), 2nd 0.53% (Std. dev. 0.013), and 3rd 0.36% (Std. dev. 0.02) difference in repeated measurements and less variation overall. Measurements taken of naturally extended larvae resulted in the greatest lengths (gray box Fig. 2) where other orientations were shorter. Accuracy of within image scale measurement was tested to determine the most precise method to set scale. Firstly, measured a single 0.5 mm increment 10 times, to determine the variation using the digital method, and then measured 10 alternate 0.5 mm increments, to determine the variation along the scale bar. This same procedure was repeated using 2 mm increments. The 10 digital measurements of the single 0.5 mm scale produced a mean of 0.5054 mm (SD = 0.0046); and for the 10 digital measurements of the single 2 mm increment, a mean of 2.0049 mm (SD = 0.0094). For the different 0.5 mm and 2 mm scale increments, the means were 0.5282 mm (SD = 0.0156, n = 10) and 2.0172 mm (SD = 0.0154, n = 10), respectively. These results demonstrate that measurement error can be reduced by using larger increments (2 mm) to calibrate an image. Minor errors between 0.5 mm increments are spread out across the 2 mm distance reducing the amount of error in the measurement.

Fig. 3. 2nd instar *Phormia regina* larva killed specimen 30s HWK in 80% ethanol vs live same specimen at full natural extension.

3.2. Live vs killed maggots

Killing and preserving specimens resulted in specimens being longer and narrower than when these were alive (Fig. 3). Killed image measured length on average 1.79 mm greater than alive image method (Fig. 4A). Killed digital caliper measured length on average 1.86 mm greater than alive image method (Fig. 4B). Killed eyepiece micrometer measured length on average 1.4 mm greater than alive image method (Fig. 4C). A gradual increase in difference in length in killed specimens is observed until the upper lengths of maggots are reached. Maggots ~>13 mm are observed to differ between live image length to killed methods with a range of 0-4 mm greater. An over estimation in total body length up to 49.7% in killed image, 54.5% in killed micrometer, and 47.1% in killed digital caliper is observed when compared to live measurements. This is likely due to the variable change in size during the killing and preservation process [9,13]. A plot of live vs killed lengths using the image method shows that a linear line of best fit (Y = 1.1351x + 0.576) gives an R-squared of 0.9692 (Fig. 5).

3.3. Agreement between measuring techniques

Killed specimen (Image) measured length on average 0.37 mm

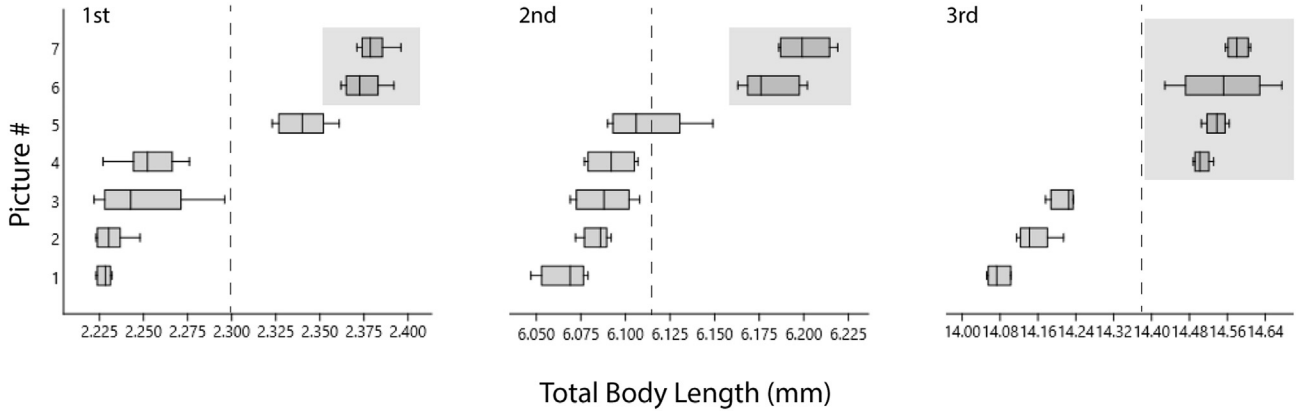

Fig. 2. Representative specimens for 1st, 2nd, and 3rd instar *P. regina* larvae measured 5 times for each of 7 photographs. Dotted vertical line represents mean of image measurements for respective instar. Gray box highlights specimens in ideal orientations.

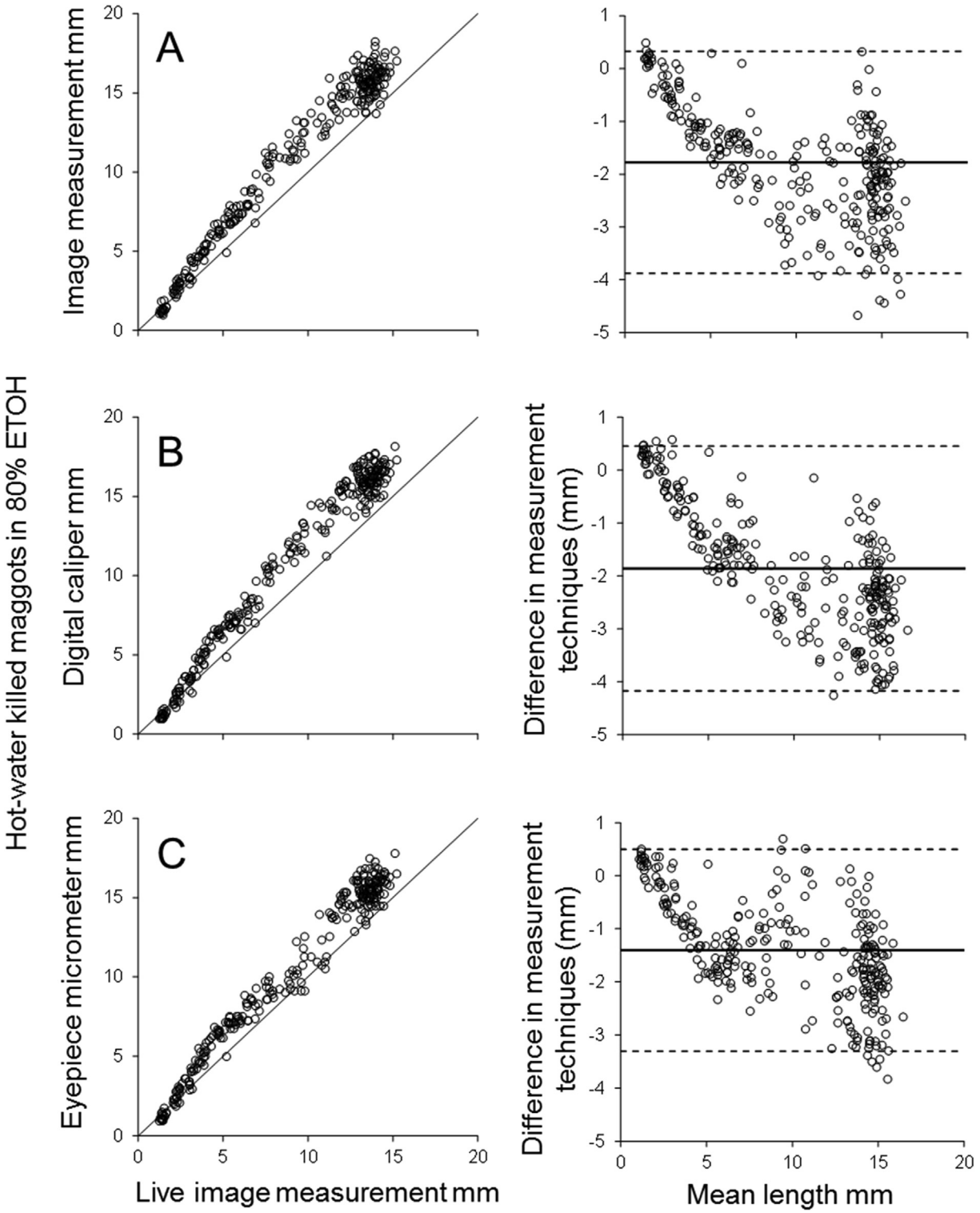

Fig. 4. Measurements of live maggots. Graphs to the left: solid line is the x = y line. Graphs to the right: the solid line is the mean and the upper and lower dashed lines are the mean + - 1.96 SD respectively.

greater than the killed eyepiece micrometer method. Digital caliper and killed image showed the least deviation in measurements through all maggot instars. Killed specimen (Image) measured length on average 0.09 mm greater than the digital caliper method. Killed specimen (Digital Caliper) measured length on average 0.46 mm greater than the eyepiece micrometer method. Eyepiece micrometer displays a lesser length measurement for specimens within the 9-14 mm length range.

4. Discussion

The purpose of this research was to report and assess the

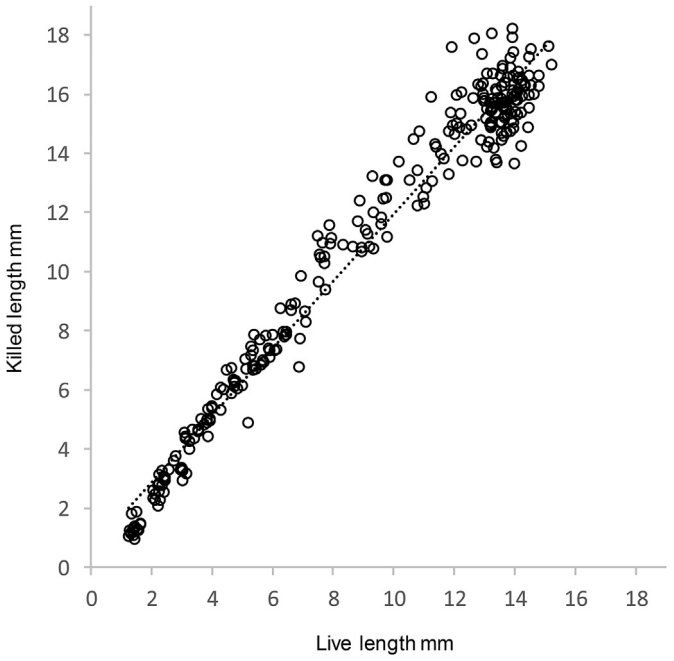

Fig. 5. Simple linear regression of imaged live measured length and imaged killed length. $Y = 1.1351x + 0.576$, $R^2 = 0.9692$.

viability of imaging and measuring the length of live insects, particularly *P. regina* larvae. We found that live *P. regina* larvae, at all stages, could be accurately measured. This finding somewhat contrasts with studies of *L. sericata* where 3rd instars were found to have weaker correlation of length to developmental stage [25]. We also found in-focus larvae in improper orientation (not naturally extended with mouthparts anchored), could still effectively be measured, however, other orientations resulted in slightly underestimated size and a greater percent variation.

Artificial elongation and lateral flattening/narrowing width was observed in killed and preserved maggots when compared to measurements from maggots using the image measurements (Figs. 3 and 4). The length of preserved maggots is known to change due to initial killing and preservation [9,13]. From our research, it is possible to convert from live length to approximate killed length, at least for *P. regina*, using a simple linear equation ($Y = 1.1351x + 0.576$) with 96.9% accuracy, a finding similar to the strong correlation of live to frozen maggots in a storage buffer [26] for *L. sericata*. Estimation of killed larval length from live allows the use of existing development models for the estimation of age, however, we suggest that new development models are produced based on live maggot measurements to negate the unpredictable effects of different preservation methods [3,9-15].

5. Technical advantages and contributions

Current procedures for the collection of insect evidence are to preserve a sample of immature insects obtained at a corpse while rearing an alternative sample to adults for identification [13]. Preserved specimens are measured to determine their size and predict their age based on temperature dependent development models. Preservation of immature insects has been demonstrated to alter their length, to different degrees depending on the species, duration in preservation, preservation temperature, killing method, and type of preservation [9,13,26]. Damage to specimens from handling (especially 1st and 2nd instars) and difficulty in measuring parameters other than length, limit the current methods available to measure larval insects. Measuring live maggots using image analysis required little to no handling of the specimens maintaining their natural shape. With the emergence of high resolution digital cameras, forensic photography has become a significant method to preserve crime scene data, and is regarded as the most accurate means for documenting evidence, autopsies, and crime scenes [27]. Additionally, processing logs should be used for analyzed images in order to be admissible in court [28]. ImageJ is capable of documenting alterations via plugins (metadata and audit trail log) [29].

CONVENIENCE: utilizes equipment available at most crime scenes and does not require hot water for immediate blanching of maggots.

SKILLSET: highly trained forensic photographers have the ability to capture high quality images.

BEST PRACTICES: simple integration into standard operating procedures and management of image evidence (SWIGIT/SWGDE).

NON-DESTRUCTIVE: a digital record of insect size at the time of image will not degrade (avoids damage to evidence). Killing/preservation of specimens is not required (Non-destructive sampling).

ACCURACY: live measurements negate the effects of preservation. The higher the resolution image and scale, the greater the measurement accuracy.

IMAGE PROCESSING: freely available open source software and intuitive GUI.

LIMITED SPECIMENS: young maggots can be measured live then reared to adults for identification.

DEVELOPMENT: the true growth of individual larval insects, populations, or samples can be measured with minimal handling and resources (less replicates).

MODELLING: technique greatly reduces replicates required and allows all individuals within a population or cohort to be measured, which may account for mean, median, mode increase accuracy and precision of model [4,30].

MEASUREMENT PARAMETERS: multiple morphological features can be measured, potentially features with less variability than length and a better indicator of overall age.

Conflicts of interest

There are no conflicts of interest with this publication nor in the selection of reviewers.

Declarations of interest

None.

Acknowledgements

Funding via Canadian Police Research Council (CPRC) to CJK and DB; and Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant to CJK. Thank you also to the members of the Beresford and Kyle labs for their support throughout this work.

References

[1] J.H. Byrd, J.C. Allen, The development of the black blow fly, Phormia regina (Meigen), Forensic Sci. Int. 120 (2001) 79–88, https://doi.org/10.1016/S0379-0738(01)00431-5.

[2] P.D. Nabity, L.G. Higley, T.M. Heng-Moss, Effects of temperature on development of Phormia regina (Diptera: Calliphoridae) and use of developmental data in determining time intervals in forensic entomology, J. Med. Entomol. 43 (2006) 1276–1286, https://doi.org/10.1603/0022-2585(2006)43[1276:EOTODO]2.0.CO;2.

[3] J. Amendt, C.P. Campobasso, E. Gaudry, C. Reiter, H.N. LeBlanc, M.J.R. Hall, Best practice in forensic entomology–standards and guidelines, Int. J. Leg. Med. 121 (2007) 90–104, https://doi.org/10.1007/s00414-006-0086-x.

[4] C.S. Richards, M.H. Villet, Factors affecting accuracy and precision of thermal summation models of insect development used to estimate post-mortem intervals, Int. J. Leg. Med. 122 (2008) 401–408, https://doi.org/10.1007/s00414-008-0243-5.
[5] S. Matuszewski, M. Szafałowicz, Temperature-dependent appearance of forensically useful beetles on carcasses, Forensic Sci. Int. 229 (2013) 92–99, https://doi.org/10.1016/j.forsciint.2013.03.034.
[6] J.T. Smiley, C.S. Wisdom, Photographic estimation of weight of insect larvae, Ann. Entomol. Soc. Am. 75 (1982) 616–618, https://doi.org/10.1093/aesa/75.6.616.
[7] S.D. Porter, Fast, accurate method of measuring ant head widths, Ann. Entomol. Soc. Am. 76 (1983) 866–867, https://doi.org/10.1093/aesa/76.5.866.
[8] M.H. Villet, An inexpensive geometrical micrometer for measuring small, live insects quickly without harming them: technical note, Entomol. Exp. Appl. 122 (2007) 279–280, https://doi.org/10.1111/j.1570-7458.2006.00520.x.
[9] T.I. Tantawi, B. Greenberg, The effect of killing and preservative solutions on estimates of maggot age in forensic cases, J. Forensic Sci. 38 (1993) 702–707, https://doi.org/10.1520/JFS13458J.
[10] Z.J.O. Adams, M.J.R. Hall, Methods used for the killing and preservation of blowfly larvae, and their effect on post-mortem larval length, Forensic Sci. Int. 138 (2003) 50–61, https://doi.org/10.1016/j.forsciint.2003.08.010.
[11] D.M. Day, J.F. Wallman, Effect of preservative solutions on preservation of Calliphora augur and Lucilia cuprina larvae (Diptera: Calliphoridae) with implications for post-mortem interval estimates, Forensic Sci. Int. 179 (2008) 1–10, https://doi.org/10.1016/j.forsciint.2008.04.006.
[12] J. Amendt, C.S. Richards, C.P. Campobasso, R. Zehner, M.J.R. Hall, Forensic entomology: applications and limitations, Forensic Sci. Med. Pathol. 7 (2011) 379–392, https://doi.org/10.1007/s12024-010-9209-2.
[13] C.S. Richards, C.C. Rowlinson, M.J.R. Hall, Effects of storage temperature on the change in size of Calliphora vicina larvae during preservation in 80% ethanol, Int. J. Leg. Med. 127 (2013) 231–241, https://doi.org/10.1007/s00414-012-0683-9.
[14] R. Rosilawati, O. Baharudin, R.A. Syamsa, H.L. Lee, W.A. Nazni, Effects of preservatives and killing methods on morphological features of a forensic fly, Chrysomya megacephala (Fabricius, 1794) larva, Trop. Biomed. 31 (2014) 785–791.
[15] V. Bugelli, C. Pietro Campobasso, M.A. Verhoff, J. Amendt, Effects of different storage and measuring methods on larval length values for the blow flies (Diptera: Calliphoridae) Lucilia sericata and Calliphora vicina, Sci. Justice 57 (2017) 159–164, https://doi.org/10.1016/j.scijus.2016.10.008.
[16] H.V. Daly, Insect morphometrics, Annu. Rev. Entomol. 30 (1985) 415–438, https://doi.org/10.1146/annurev.en.30.010185.002215.
[17] J. Byrd, J.C.-B. y C, F. Entomology, U. 2010, Insects of forensic importance, in: J.H. Byrd, J.L. Castner (Eds.), Researchgate.Net, CRC Press, Boca Raton, 2010. https://www.researchgate.net/file.PostFileLoader.html?id=5808b62b615e2736f60c96a5&assetKey=AS%3A419239644155905%401476965928884#page=62. (Accessed 27 June 2018).
[18] N. Sontigun, K.L. Sukontason, B.K. Zajac, R. Zehner, K. Sukontason, A. Wannasan, J. Amendt, Wing Morphometrics as a Tool in Species Identification of Forensically Important Blow Flies of Thailand, Parasites Vectors (2017) 10, https://doi.org/10.1186/s13071-017-2163-z.
[19] R. Chapman, Legs and locomotion, in: S. Stephen J, D. Angela E (Eds.), Insects Struct. Funct., fifth ed., Cambridge University Press, Cambridge, 2013.
[20] D. Berrigan, D.J. Pepin, How maggots move: allometry and kinematics of crawling in larval Diptera, J. Insect Physiol. 41 (1995) 329–337, https://doi.org/10.1016/0022-1910(94)00113-U.
[21] D. Berrigan, J.R. Lighton, Bioenergetic and kinematic consequences of limblessness in larval Diptera, J. Exp. Biol. 179 (1993) 245–259.
[22] W.S. Rasband, ImageJ, U. S. National Institutes of Health, 1997-2018. Bethesda, Maryland, USA, https://imagej.nih.gov/ij/.
[23] M.D. Abramoff, P.J. Magelhaes, S.J. Ram, Image processing with imaje, J, Biophotonics Int. 11 (2004) 36–42.
[24] J. Martin Bland, D. Altman, Statistical methods for assessing agreement between two methods of clinical measurement, Lancet 327 (1986) 307–310, https://doi.org/10.1016/S0140-6736(86)90837-8.
[25] A.M. Tarone, D.R. Foran, Generalized additive models and lucila sericata growth: assessing confidence intervals and error rates in forensic entomology, J. Forensic Sci. 53 (2008), https://doi.org/10.1111/j.1556-4029.2008.00744.x.
[26] A.M. Tarone, D.R. Foran, Gene expression during blow fly development: improving the precision of age estimates in forensic entomology, J. Forensic Sci. 56 (2011), https://doi.org/10.1111/j.1556-4029.2010.01632.x.
[27] H. Singh, P. Kumar, R. Nanra, A.H. Kumar Citation Singh, A. Kumar, Why is the crime scene photographed? .... There is not a single answer!, Internet J. Forensic Sci. 5 (2012), https://doi.org/10.5580/2a6a.
[28] Scientific Working Group on Imaging Technology (SWGIT) Guidelines, Use of Imaging Technology in the Criminal Justice System, 2010. Version 3.3, https://www.swgit.org/pdf/Section%201%20Overview%20of%20SWGIT%20and%20the%20Use%20of%20Imaging%20Technology%20in%20the%20Criminal%20Justice%20System?docID=35 (accessed March 19, 2019).
[29] K. Miles, Integrity of Science Image Data Issues and Emerging Standards, P.I. Outcomes, in: http://www.indiana.edu/~lmic/Workshops/Image%20Processing%20Workshop%20copy/IU-Bloomington2013.pdf (accessed March 19, 2019).
[30] J.D. Wells, M.C. Lecheta, M.O. Moura, L.R. LaMotte, An evaluation of sampling methods used to produce insect growth models for postmortem interval estimation, Int. J. Leg. Med. 129 (2015) 405–410, https://doi.org/10.1007/s00414-014-1029-6.