Int J Legal Med (2014) 128:565–573
DOI 10.1007/s00414-013-0922-8

ORIGINAL ARTICLE

The analysis of temporal gene expression to estimate the age of forensically important blow fly pupae: results from three blind studies

Petra Boehme · Philipp Spahn · Jens Amendt · Richard Zehner

Received: 11 March 2013 / Accepted: 24 September 2013 / Published online: 1 November 2013

Abstract The estimation of the minimum postmortem interval can be implemented by age estimation of corpse-associated primary colonizers such as the blow flies (Diptera: Calliphoridae). In cases where pupae represent the oldest stages found on a body, their age must be added to the duration of prepupal development to estimate the minimum postmortem interval. Although methods to age blow fly larvae have been well established using morphology, length or weight and age estimation of pupae has proved challenging. In a previous work, we quantified the changes in mRNA levels of four differentially expressed genes during the metamorphosis of *Calliphora vicina* pupae, hence representing molecular markers for pupal age (i.e., time elapsed since pupariation). Here, we demonstrate how these data can be used to estimate pupal age with inverse prediction. We present three blind studies conducted under various conditions and show that age of *C. vicina* pupae can be well estimated based on gene expression data. As these data are quantitative and can be processed automatically, gene expression has the potential to outperform morphological analysis in age estimation of forensically relevant blow fly pupae.

Petra Boehme and Philipp Spahn contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00414-013-0922-8) contains supplementary material, which is available to authorized users.

P. Boehme (✉) · J. Amendt · R. Zehner
Institute of Forensic Medicine, Goethe University Frankfurt, Kennedyallee 104, 60596 Frankfurt am Main, Germany
e-mail: petraboehme@gmail.com

P. Boehme
Department of Aquatic Ecotoxicology, Goethe University Frankfurt, Max-von-Laue-Str. 3, 60438 Frankfurt am Main, Germany

P. Spahn
Division of Animal Genetics, University of Tuebingen, Auf der Morgenstelle 28, 72076 Tübingen, Germany

Keywords Forensic entomology · *Calliphora vicina* · Quantitative real-time PCR · Gene expression · Blow fly pupae

Introduction

The estimation of the minimum postmortem interval in death investigations using insects requires knowledge about the arthropod succession patterns or the age of the oldest specimens collected from the crime scene [1–5]. Calliphoridae (blow flies) are typically the first visitors of a corpse as they are able to detect and colonize a cadaver within minutes of death and are thus the focus of age estimation [3, 4, 6]. Throughout larval development, this can be performed accurately for various blow fly species as their morphology such as length, weight, and width reflects maturation. A measurement of these characters and subsequent comparison to species-specific developmental data is routinely used as a predictor of age in forensic entomological case work (e.g., [5, 7–9]). However, given a case where pupae represent the oldest specimen collected from the scene, a forensic entomologist needs to estimate the age of these pupae and add it to the estimated duration of prepupal development to obtain a minimum postmortem interval. Age estimation of blow fly pupae is challenging as the hard pupal case (puparium) prevents length increase and obscures morphological changes. Moreover, the color change of the puparium is only predictable within the first few hours of metamorphosis [10, 11]. Age estimation after dissection and visual analysis of developing adult structures is very laborious for the inexperienced and requires profound knowledge about pupal development [12, 13], exacerbating quick age estimation. Thus, it would be very beneficial to have an alternative procedure at hand which could be carried out by a standard forensic laboratory and would produce more standardized results. One promising method is the analysis of differentially expressed genes during

metamorphosis [14–18] as it relies on quantitative data and allows easy standardization.

During metamorphosis, a hierarchy of genes is differentially expressed to induce and regulate changes of the larval body structures (for instance [19–21]). In a previous work, we identified and monitored the differential temporal expression of four genes (*actin*, *arylphorin receptor* (*AR*), *15_2*, and *2014192*) during the metamorphosis of *Calliphora vicina* (Diptera: Calliphoridae) pupae which were reared at three constant temperatures (15, 20, 25 °C) [14]. Changes in mRNA levels (calculated as logarithmized fold changes (logFC) of gene expression) were recorded in 24-h intervals throughout metamorphosis and related to the onset of pupariation. All four genes showed significant accumulated degree hours (ADH)-dependent differential regulation, and consequently logFC values could be used to determine the pupal age. Here, based on the expression levels of the four investigated genes, we use standard least-squares regression and inverse prediction to obtain estimates of pupal age. Inverse prediction had been utilized previously in forensic entomology in order to estimate maggot age from weight [8] but little emphasis has been put on this method in recent years. Using a custom R script, we show that age inference based on gene expression data can be carried out automatically in a standardized manner and produces robust estimates of specimen age. We demonstrate feasibility and performance of this method by estimating pupal age of *C. vicina* in three blind studies.

Material and methods

Reference data

The gene expression data presented in [14] served as reference data for this study. The dataset spans expression of four genes (*15_2*, *2014192*, *actin*, *AR*) during pupal development of *C. vicina* reared at three constant temperatures (15, 20, and 25 °C). Onset of pupariation was determined as time span of 17 h after which approximately 100 post-feeding third instar larvae had unambiguously initialized pupariation.

For quantitation analysis of gene expression, five pupae were collected at 24-h intervals until adult flies were observed [14]. To quantify relative changes in gene expression at a certain time point, fold changes had been calculated in relation to the average expression at onset of pupariation. After log-2 transformation, these data were used as reference to carry out inverse prediction with a sample of unknown age.

Blind study experiments

Blind study #1 and #2 were run at constant 20 or 25 °C temperatures, respectively, in an incubator (MKKL 600/2 Lintek). Study #3 was run outdoors in a private area at the Institute of Forensic Medicine in Frankfurt am Main, Germany. The same *C. vicina* colony was used for all experiments and originated from wild caught flies collected in Frankfurt am Main. Temperatures were recorded half-hourly using data loggers (DS1922L Temperature Logger iButtons—Maxim/Dallas).

Adult *C. vicina* were allowed to oviposit on fresh beef liver over a 24-h time period. Liver containing eggs and larvae were transferred into 300-mL plastic cups and put into rearing chambers at a constant 20 °C (study #1) and 25 °C (study #2) or left outdoors under ambient conditions (15.9 °C on average) (study #3). Ground meat (half beef, half pork) was supplied ad libitum until larvae entered the post-feeding stage. Post-feeding maggots were able to leave the food source by crawling out of the 300-mL cup and into a sawdust-filled 5-L plastic container [14]. When larvae was found to unambiguously have entered pupariation (within a 17-h range for study #1, 2.5 h for study #2, and 18 h for study #3), they were separated from remaining post-feeding larvae and this time point, here defined as "onset of pupariation," was noted.

Samples were collected randomly (i.e., number of individuals, time and day) throughout metamorphosis in experiment #1 and #2 until eclosion. For study #3, five individuals were collected every 24 h.

Pupae were immediately homogenized in 1-mL TRIzol® Reagent (Invitrogen) after collection and stored at −20 °C until qRT-PCR analysis. Collection parameters were unknown to the researcher who carried out the subsequent molecular analysis. A total of 20 pupae in study #1, 18 in study #2, and 90 in study #3 were analyzed.

Molecular analysis and analysis of qRT-PCR data

RNA extraction, cDNA synthesis, and qRT-PCR set-up were performed as described in [14].

Least-squares regression and inverse prediction

Standard least-squares regression was used to fit a fourth-order polynomial to each of the four-gene expression datasets. The intercept term was set to zero since logFC values must be zero at onset of pupariation and omission of this restraint did not achieve substantial improvements in model fit nor alterations of results (data not shown). Depending on the nature of the reference data, one might regress gene expression against age or against ADH, the units of thermal energy accumulated during development. In the latter case, all subsequent calculations would first yield ADH estimations which would then have to be transformed back into sample age considering the thermal environment in which the sample had been exposed to [22]. Prediction bounds around the regression curves were

calculated according to standard procedures. For an expression measurement y of a single gene from a sample of unknown age, the set of ages for which y lies between the 1-α prediction bounds constitutes a 1-α confidence set on sample age (see Appendix; α=0.05 was used in this study). With expression measurements y_1, y_2, y_3, and y_4 of four genes from a sample of unknown age, a 1-α confidence interval on sample age is achieved by taking the intersection of the $(1-\alpha)^{1/4}$ confidence sets deduced for each individual gene (see Fig. 1 and Appendix). Typically, this set is single interval, but the intersection may also produce multiple disjoint intervals or an empty set which would correspond to ambiguous or failed age estimation, respectively.

If the reference data contains ADH, rather than age, the obtained confidence interval on ADH is transformed into sample age by considering the thermal history of the sample. In cases of constant ambient temperature, as in blind studies #1 and #2, ADH values were simply divided by the effective temperature (i.e., the difference between ambient temperature and the species-specific lower developmental threshold [22]). In cases of fluctuating ambient temperature, as in blind study #3, the study was either treated as having been run at the total average ambient temperature ("total average reconstruction") or the recordings of the average ambient temperature taken in 12-h intervals were considered. In this case, one moves back in time until the area under the temperature curve (12-h averages) equals the ADH value inferred for the specimen ("12-h average reconstruction").

All computational processing was automated using our custom script blow fly age calculator (BLOWFLAC), written in R [23]. BLOWFLAC carries out inverse prediction from logFC data from any developmental stage (appropriate reference data can be provided as input file). The script is available from the authors upon request.

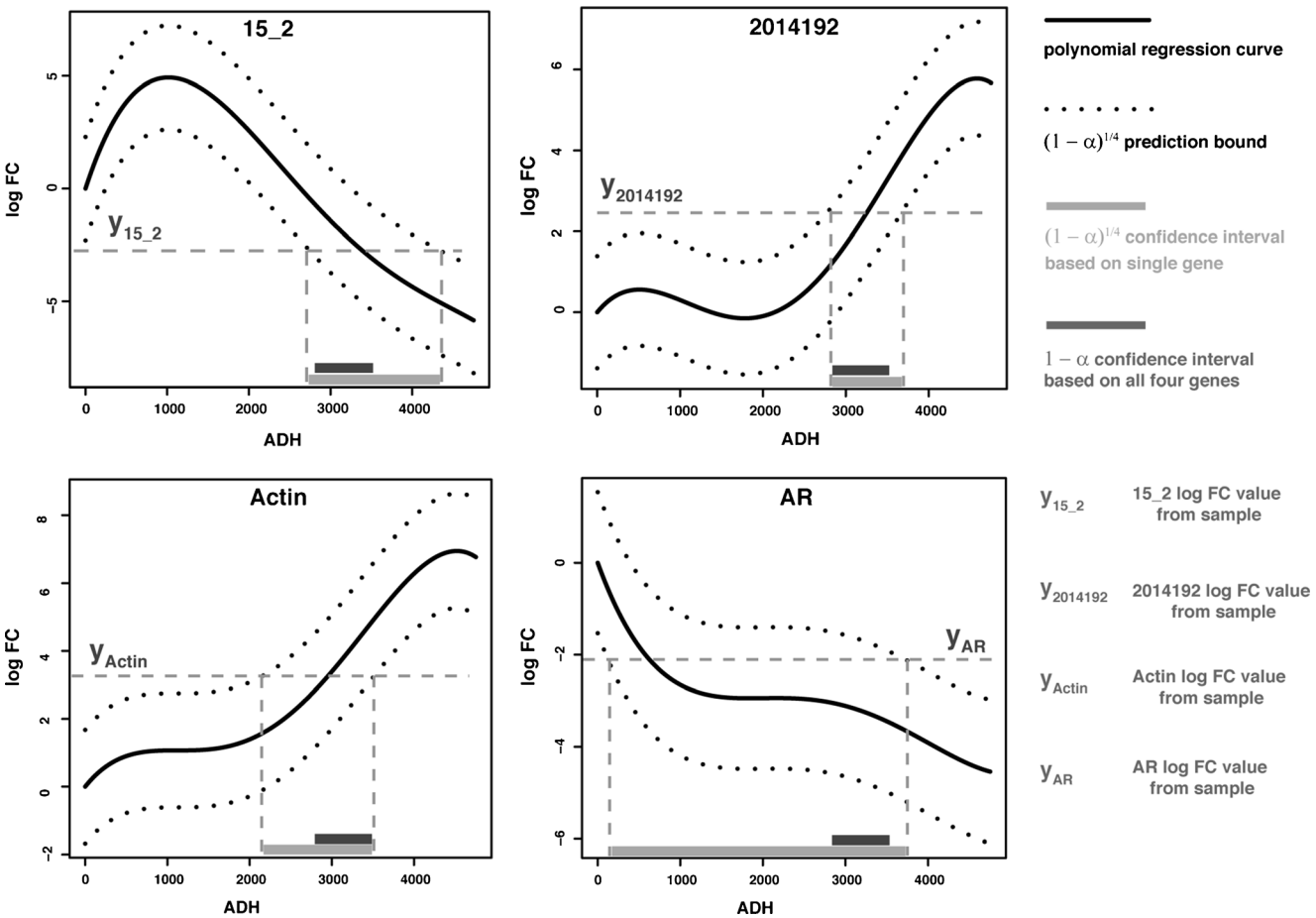

Fig. 1 Inverse prediction from expression data of multiple genes. Scheme showing expression profiles (regression curves with $(1-\alpha)^{1/4}$ prediction bounds) of the four genes used as molecular predictors of pupal age. *ADH* accumulated degree hours measures the thermal energy acquired up to a certain time point in development. ADH=0 corresponds to the onset of pupariation. For logFC measurements obtained from a pupa sample of unknown age ($y_{15\_2}, y_{2014192}, y_{Actin}, y_{AR}$), an ADH estimation ($(1-\alpha)^{1/4}$ confidence interval) is first calculated separately for each gene (*light gray intervals*). Graphically, this is carried out by intersecting the *horizontal line* through the logFC value with the prediction bounds around the regression curve. The intersection of these four intervals constitutes the ADH estimation (1-α confidence interval) based on all four genes (*dark gray interval*). Finally, ADH is transformed into age by considering the temperature history the sample had been exposed to

Results

In this study, we investigated whether gene expression would prove useful to estimate pupal age (i.e., time since pupariation), specifically, to estimate a lower bound for the pupal age since minimum age is the primary concern for estimation of a minimum postmortem interval. Gene expression data described in [14] served as reference data for inverse prediction.

Blind study #1 (constant 20 °C)

In blind study #1, a total of 20 specimens were collected at 12 different days. Gene expression data from the reference study performed at 20 °C was used as reference dataset for age estimation. Two samples resulted in an empty confidence interval and one sample resulted in an ambiguous estimation (i.e., two disjoint intervals). In the remaining samples, a single estimated age interval was achieved which gave a correct lower bound on pupal age in all cases (Fig. 2a). On average, the estimated minimum age approximated the true minimum age by 1.2±0.5 days (median ± s.d.), and the estimated maximum age approximated the true maximum age by 0.4± 0.8 days (median ± s.d.) (Fig. 2a). A lower developmental threshold of 0 °C was assumed. Other thresholds yielded identical estimations (data not shown).

Blind study #2 (constant 25 °C)

In blind study #2, a total of 18 specimens collected at nine different days were processed. Gene expression data from the reference study performed at 25 °C was used as a reference dataset for age estimation. Four samples produced ambiguous estimations. In the remaining samples, a single estimated age interval was achieved which gave a correct lower bound on pupal age in 11 out of 14 cases (79 %) (Fig. 2b). On average, the estimated minimum age approximated the true minimum age by 0.8±1.0 days (median ± s.d.), and the estimated maximum age approximated the true maximum age by 0.3± 0.3 days (median ± s.d.) (Fig. 2b). A lower developmental threshold of 0 °C was assumed. Other thresholds yielded identical results (data not shown).

Blind study #3 (fluctuating temperature)

Study #3 was executed outdoors under fluctuating temperature with a 15.9 °C average (±3.4 °C), and yielded a total of 90 specimens on 19 different days. To mimic the situation in true cases where reference studies that would perfectly match the conditions at a crime scene are usually not available, we tested the feasibility of age estimation based on gene expression if only approximate reference data were at hand. Therefore, a dataset with expression data at constant 15 °C [14] was used for reference. In contrast to studies #1 and #2, the transformation of ADH into age is also more tedious due to the non-constant ambient temperature. We tested both the "total average" and the "12-h average" reconstruction approach (see Materials and methods) as a forensic entomologist will be forced to apply different degrees of approximation depending on how well the temperature history of the scene can be traced back. Four samples led to an empty confidence set, and one sample produced ambiguous age estimation. In the remaining samples, a single estimated age interval was obtained. Using the total average approach, a correct lower bound on pupal age was achieved in 76 out of 85 cases (89 %) (Fig. 3a). On average, the estimated minimum age approximated the true minimum age by 1.6±1.7 days (median ± s.d.), and the estimated maximum age approximated the true maximum age by 1.1±1.6 days (median ± s.d.) (Fig. 3a). Using the 12-h average approach, a correct lower bound was achieved in 78 out of 85 cases (92 %) (Fig. 3b), and on average, the estimated minimum age approximated the true minimum age by 1.4± 1.7 days (median ± s.d.), and the estimated maximum age approximated the true maximum age by 1.0±1.6 days (median ± s.d.) (Fig. 3b). The 12-h average reconstruction noticeably increased the accuracy of estimation, especially in old pupae (Fig. 3b, arrow). However, the age of the oldest pupae appeared to be systematically underestimated (Fig. 3a) indicating that ADH requirements to complete pupal development may vary significantly in *C. vicina* depending on ambient temperature (see Discussion). A lower developmental threshold of 0 °C was assumed (Fig. 3). Age reconstruction varied slightly if other thresholds were assumed, but changes in correct lower bound percentage or distribution of estimation error proved to be negligible (Fig. 4).

Discussion

Gene expression data has been previously reported as a predictor of age [15, 17, 24, 25], but most genes displayed limited variation during pupal development, thus not ideal for inverse prediction. In previous work [14], we identified genes with significant differential expression over the period of metamorphosis providing a solid basis for age estimation, and these genes, indeed, proved sufficient to allow age estimation using inverse prediction from a standard least-squares regression. Although age estimation of pupae only provides a minimum time interval after pupariation, this information will be critical to give an estimate of the minimum postmortem interval if duration of the prepupal stages can be reliably estimated, for instance, at an indoor scene with constant ambient temperature. For the direct estimation of total pupal age (i.e., elapsed time since oviposition, not pupariation) suitable genes remain to be identified which would exhibit a high degree of temporal variation over all stages of development in order to achieve

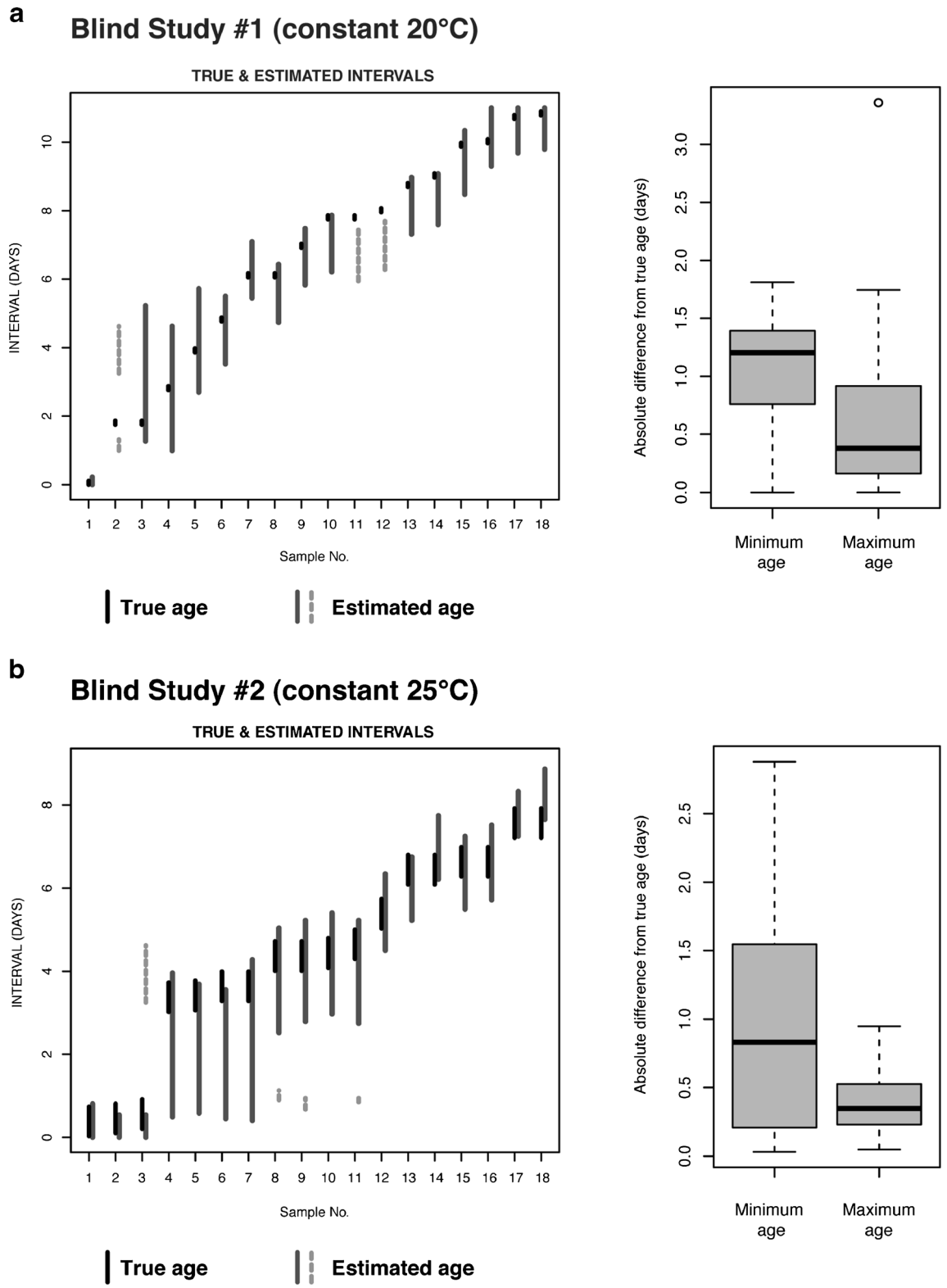

Fig. 2 Blind studies #1 and #2 (20 and 25 °C). Results from two blind studies executed at constant 20 (**a**) and 25 °C (**b**). Two studies, run at constant 20 or 25 °C, respectively, served as references. Interval diagrams (*left*) show true age intervals (*black*) and estimated age for each blind sample (*dashed*, if the estimated age does not overlap the true age; *solid grey* otherwise). *Multiple bars* at the same sample represent disjoint estimated age intervals. Samples with empty estimated age intervals were not plotted. *Boxplots* (*right*) show the distribution of the estimation error (absolute difference between estimated minimum/maximum age and true minimum/maximum age). Whisker length is 1.5 the interquartile range and *circles* represent outliers. Samples with failed or ambiguous age estimations were excluded from the boxplots. Assumed lower developmental threshold 0 °C

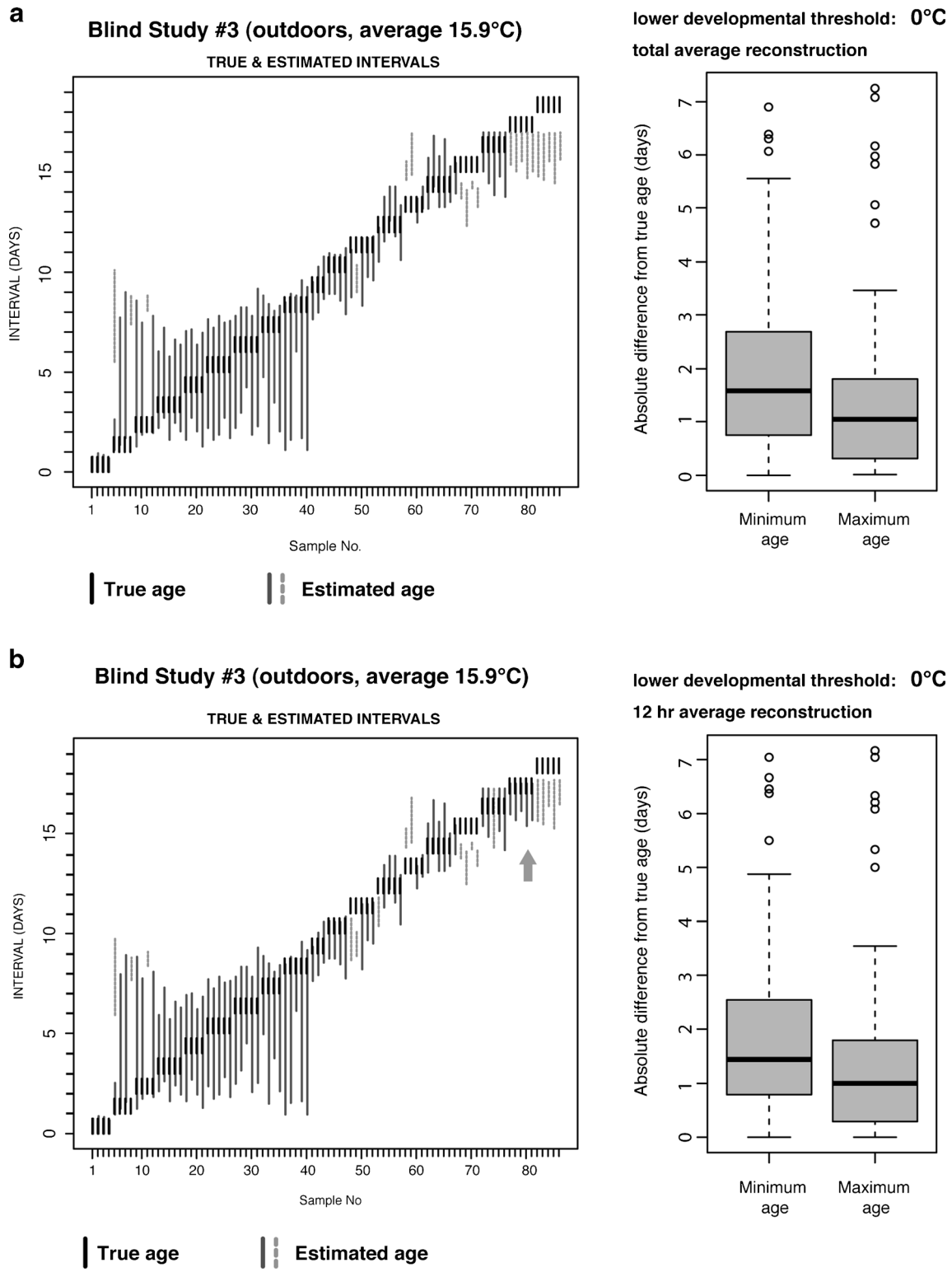

Fig. 3 Blind study #3 (fluctuating temperature, assumed lower dev. threshold 0 °C). Results from the blind study executed outdoors at fluctuating temperature obtained through total average age reconstruction (**a**) or 12-h average age reconstruction (**b**) (see Materials and methods). A study run at constant 15 °C served as reference. Interval diagrams (*left*) show true age intervals (*black*) and estimated age for each blind sample (*dashed*, if the estimated age does not overlap the true age; *solid grey* if otherwise. Samples with empty estimated age intervals were not plotted. *Multiple bars* at the same sample represent disjoint estimated age intervals). *Boxplots* (*right*) show the distribution of estimation error. Whisker length is 1.5 the interquartile range. *Circles* represent outliers. Samples with failed or ambiguous age estimations were excluded from the boxplots. Assumed lower developmental threshold 0 °C

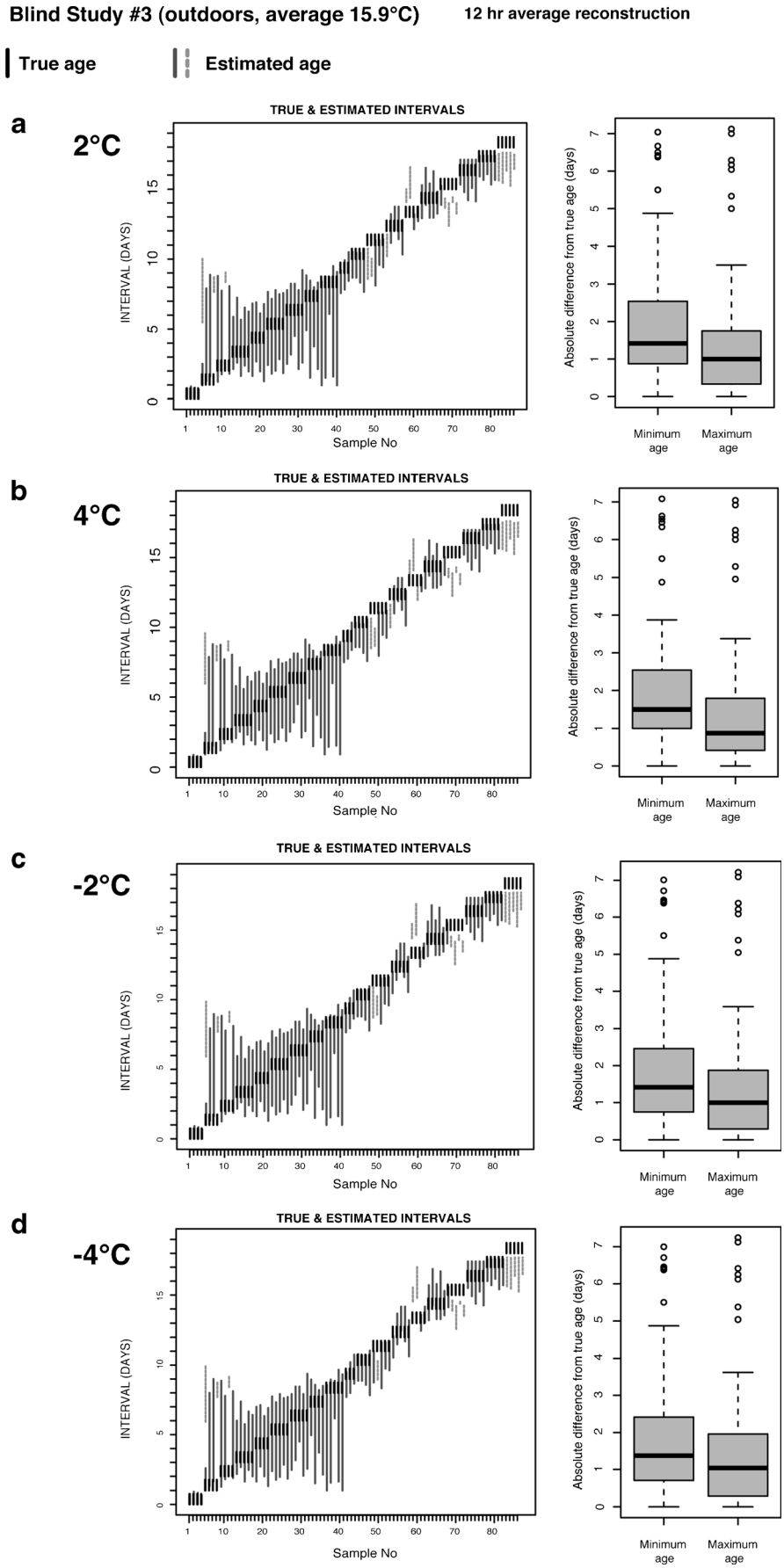

Fig. 4 Blind study #3 (fluctuating temperature, alternative assumed lower developmental thresholds). Results from the blind study executed outdoors at fluctuating temperature obtained through 12-h average age reconstruction assuming alternative lower developmental thresholds. Nearly identical results were achieved with respect to correct lower bound and distribution of estimation error. The problem of systematic underestimation of the oldest pupae persisted, independent of the assumed threshold

sufficient predictive power. Ideally, these candidates show a monotonous increase or decrease in expression (at least over the time span of a specific developmental stage) since otherwise inverse prediction may easily result in ambiguous age estimations (see profile of *15_2* in our dataset, Fig. 1). With steadily increasing knowledge about gene regulation during metamorphosis, as well as the recent assembly of the first Calliphorid transcriptome [26], a rich genetic data resource will become available to find appropriate markers meeting these criteria. It is important to note, though, that utilization of multiple genes for inverse prediction relies on the assumption of independent expression which restricts the marker choice somewhat as certain combinations (such as a transcription factor and one of its targets) are more unlikely to fulfill this requirement. In addition, it is, of course, advisable to process a sufficiently large number of pupae collected at the scene to ensure that the oldest individual found represents a good estimate of the time since the first third instar had pupated.

In this study, we used a reference dataset comprising ADH since this represents a commonly used concept to model development of immature entomological specimens [12, 27–29]. For convenience, it is often assumed in forensic entomology that completion of a developmental period occurs after passing a species-specific ADH constant, an approximation that relies on an assumed linear relationship between surrounding temperature and developmental rate [22]. Blind studies #1 and #2 are equivalent to a model of development vs. age rather than ADH since they were run under constant temperatures and since the reference data perfectly matched the conditions in the study. As demonstrated in blind study #3, however, application of ADH reference data taken at temperatures not matching the scene entails the risk of systematic age estimation error as the 15 °C reference study only spanned to a maximum of 6,480 ADH (0 °C threshold) until completion of metamorphosis while the pupae in study #3 accumulated substantially more degree hours for eclosion (approx. 7,800 ADH, 0 °C threshold). Thus, the reference data did not cover the full range of ADH taken in the blind study leading to systematic underestimation. These results question whether the notion of ADH being a species-specific constant represents a feasible assumption for use in forensic entomology. In fact, recent studies clearly show that the amounts of ADH required to complete stages of development do vary significantly among populations of the same species as well as among different temperatures [9, 16, 30–32]. Blind study #3 represents a typical situation encountered in investigations where the entomologist is confronted with a fluctuating temperature history at the scene but has reference data from studies under constant temperatures. Under these circumstances, it is noteworthy that gene expression data still allowed for accurate age estimation, but it is clear that there is a need to investigate the degree by which ADH requirements of immature blow flies vary with temperature or among populations. Also, establishment of a large-scale publically available database of gene expression reference data including a broad range of species, temperatures, and geographic regions would represent a fruitful long-term goal to further promote the utility of gene expression based age estimation in forensic entomology.

Although considerable progress has been made concerning morphology-based age determination of blow fly pupae [12, 33], the quantitative nature of gene expression data holds potential for its utilization in legal investigations because it allows a statistical framework which will enable a forensic entomologist to present probability-based statements regarding age estimation. For this, however, it is imperative to overcome current limitations in the generation of appropriate reference data, such as uncertainty in setting the onset of pupariation and the unresolved dependency of ADH data on ambient temperature. Nonetheless, the execution of inverse prediction using a fully automated computational procedure with custom scripts as in this study, represents a substantial improvement in methodology since it allows one to process reference data from any developmental stage (if available) and, more importantly, facilitates reproducibility and comparability among different datasets and research groups. Thus, this approach makes an important step towards standardization of methods in forensic entomology [9].

Acknowledgments The authors thank the anonymous reviewers and Prof. Christine Picard for her very helpful suggestions. Thanks to Dr. Yvonne Kendel and Michèle Baqué for their support in the blind study experiments. This project was financially supported by the Deutsche Forschungsgemeinschaft (project number: ZE 501/2-1).

References

1. VanLaerhoven SL, Anderson GS (1999) Insect succession on buried carrion in two biogeoclimatic zones of British Columbia. J For Sci 44(1):32–43
2. Wells JD, LaMotte LR (2009) Estimating the postmortem interval. In: Castner JL, Byrd JH (eds) Forensic entomology - The utility of arthropods in legal investigations, 2nd edn. CRC Press, New York
3. Amendt J, Richards CS, Campobasso CP, Zehner R, Hall MJ (2011) Forensic entomology: applications and limitations. Forensic Sci Med Pathol 7(4):379–392
4. Smith KGV (1986) A manual of forensic entomology. London and Cornell University Press, London
5. Grassberger M, Reiter C (2001) Effect of temperature on *Lucilia sericata* (Diptera: Calliphoridae) development with special reference to the isomegalen- and isomorphen-diagram. For Sci Int 120(1–2):32–36
6. Villet M, Amendt J (2011) Advances in entomological methods for death time estimation. In: Turk EE (ed) Forensic Pathology Reviews. Humana Press, Heidelberg
7. Richards CS, Crous KL, Villet M (2009) Models of development for blowfly sister species *Chrysomya chloropyga* and *Chrysomya putoria*. Med Vet Entomol 23(1):56–61
8. Wells JD, LaMotte LR (1995) Estimating maggot age from weight using inverse prediction. J For Sci 40(4):585–590
9. Amendt J, Campobasso CP, Gaudry E, Reiter C, LeBlanc HN, Hall MJ, Entomology. EAfF (2007) Best practice in forensic

entomology—standards and guidelines. Int J Legal Med 121(2):90–104
10. Bainbridge SP, Bownes M (1981) Staging the metamorphosis of *Drosophila melanogaster*. J Embryol Exp Morphol 66:57–80
11. Robertson CW (1936) The metamorphosis of *Drosophila melanogaster*, including an accurately timed account of the principal morphological changes. J Morphol 59:351–399
12. Davies K, Harvey ML (2013) Internal morphological analysis for age estimation of blow fly pupae (Diptera: Calliphoridae) in postmortem interval estimation. J For Sci 58(1):79–84
13. Zajac BK (2011) Morphologische und histologische Methoden zur Bestimmung des Alters forensisch relevanter Fliegenpuppen. Goethe University, Frankfurt a.M
14. Boehme P, Spahn P, Amendt J, Zehner R (2013) Differential gene expression during metamorphosis: a promising approach for age estimation of forensically important *Calliphora vicina* pupae (Diptera: Calliphoridae). Int J Legal Med 127(1):243–249
15. Tarone AM, Jennings KC, Foran DR (2007) Aging blow fly eggs using gene expression: a feasibility study. J For Sci 52(6):1350–1354
16. Tarone AM, Picard CJ, Spiegelman C, Foran DR (2011) Population and temperature effects on *Lucilia sericata* (Diptera: Calliphoridae) body size and minimum development time. J Med Entomol 48(5):1062–1068
17. Ames C, Turner B, Daniel B (2006) Estimating the post-mortem interval (II): The use of differential temporal gene expression to determine the age of blowfly pupae. Int Congr Ser 1288:795–797
18. Zehner R, Mösch S, Amendt J (2006) Estimating the postmortem interval by determining the age of fly pupae: are there any molecular tools? Prog For Gen 11:619–621
19. Buszczak M, Segraves WA (2000) Insect metamorphosis: out with the old, in with the new. Curr Biol 10(22):R830–R833
20. White KP, Rifkin SA, Hurban P, Hogness DS (1999) Microarray analysis of *Drosophila* development during metamorphosis. Science 286(5447):2179–2184
21. Bowen ID, Mullarkey K, Morgan SM (1996) Programmed cell death during metamorphosis in the blow-fly *Calliphora vomitoria*. Micros Res Tech 34(3):202–217
22. Higley LG, Haskell NH (2009) Insect development and forensic entomology. In: Byrd JH, Castner JL (eds) Forensic entomology - The utility of arthropods in legal investigations, 2nd edn. CRC Press, New York
23. R Devlopment Core Team (2010) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna
24. Tarone AM, Foran DR (2011) Gene expression during blowfly development: improving the precision of age estimates in forensic entomology. J For Sci 56(Suppl 1):S112–S122
25. Mösch SA (2005) Molekularbiologische Altersbestimmung an Puppen der forensisch relevanten Schmeißfliege *Lucilia sericata*. Diploma Thesis, Fachhochschule Aachen
26. Sze SH, Dunham JP, Carey B, Chang PL, Li F, Edman RM, Fjeldsted C, Scott MJ, Nuzhdin SV, Tarone AM (2012) A de novo transcriptome assembly of *Lucilia sericata* (Diptera: Calliphoridae) with predicted alternative splices, single nucleotide polymorphisms and transcript expression estimates. Insect Mol Biol 21(2):205–221
27. Donovan SE, Hall MJ, Turner BD, Moncrieff CB (2006) Larval growth rates of the blowfly, *Calliphora vicina*, over a range of temperatures. Med Vet Entomol 20(1):106–114
28. Arnott S, Turner B (2008) Post-feeding larval behaviour in the blowfly, *Calliphora vicina*: effect on post-mortem interval estimates. For Sci Int 177(2–3):162–167
29. Michaud J-P, Moreau G (2011) A statistical approach based on accumulated degree-days to predict decomposition-related processes in forensic studies. J For Sci 56(1):229–232
30. Ames C, Turner BD (2003) Low temperature episodes in development of blowflies: implications for postmortem interval estimation. Med Vet Entomol 17(2):178–186
31. Anderson GS (2000) Minimum and maximum development rates of some forensically important Calliphoridae (Diptera). J For Sci 45(4): 824–832
32. Reibe S, Doetinchem PV, Madea B (2010) A new simulation-based model for calculating post-mortem intervals using developmental data for *Lucilia sericata* (Dipt.: Calliphoridae). Parasitol Res 107(1):9–16
33. Brown K, Thorne A, Harvey M (2012) Preservation of *Calliphora vicina* (Diptera: Calliphoridae) pupae for use in post-mortem interval estimation. For Sci Int 223:176–183