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PII: S0379-0738(24)00053-7

DOI: https://doi.org/10.1016/j.forsciint.2024.111972

Reference: FSI111972

To appear in: *Forensic Science International*

Received date: 20 December 2023
Revised date: 21 February 2024
Accepted date: 27 February 2024

Please cite this article as: Luise Thümmel, Colin Degoutrie, Alicia Fonseca-Muñoz and Jens Amendt, Developmental differences in spatially distinct populations of the forensically relevant blow fly *Lucilia sericata* – About the comparability of developmental studies (and case work application), *Forensic Science International,* (2024) doi:https://doi.org/10.1016/j.forsciint.2024.111972

Developmental differences in spatially distinct populations of the forensically relevant blow fly *Lucilia sericata* – About the comparability of developmental studies (and case work application)

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ABSTRACT

The cosmopolitan blow fly *Lucilia sericata* is often used in forensic case work for estimating the minimum post-mortem interval (PMI_{min}). For this, the age of immature specimens developing on the dead body is calculated by measuring the time taken to reach the sampled developmental stage at a given temperature. To test whether regional developmental data of *L. sericata* is valid on a global scale, the time taken to reach different developmental stages was compared between a population from Mexico and one from Germany at two different constant temperatures.

The German population of *L. sericata* was collected in Frankfurt/Main, while the Mexican population originated near Oaxaca de Juarez and was transported to Germany in the larval stage. Only the F1 generation was used to avoid adaption of the Mexican flies. Eggs were immediately placed at 20 °C and 30 °C. Five times 30 freshly eclosed larvae per replicate (n=5) were then transferred to a cup of minced meat in separate containers. The larvae were checked every 8 hours for migration, pupariation or emergence of

adult flies. The time at which the first individual and 50% of the specimens per container entered each of these stages, was recorded.

Significant differences in the time of development between the two populations were observed at both temperatures. At 20 °C, the first specimens in the Mexican population reached all developmental stages a little (< 1 day to < 2 days) earlier than the German *L. sericata* at 20 °C. At 30 °C, the Mexican flies also reached the post-feeding stage slightly earlier (0.2 days). However, at 30 °C, the German flies started pupariation significantly earlier (after 5 days) than the Mexican flies (6.9 days) and the adults from Germany also emerged earlier (10.5 days compared to 13.1 days). The same pattern was observed when looking at 50% of the total number of specimens per container. A comparison with previously published developmental studies was difficult as the experimental design varied widely between studies. However, the results were within the range of most studies. Our study has shown that age estimation can vary widely depending on the population on which the reference data used for the calculations are based. This highlights the importance of using local and population-specific developmental data for estimating the age of blow flies in case work.

Keywords: Forensic entomology, *Lucilia sericata*, Calliphoridae, Development, Geographic variation

INTRODUCTION

Lucilia sericata (Meigen, 1826) is a cosmopolitan, synanthropic blow fly and, due to its preference for dead tissue, often associated with cadavers [1–4]. A recent study at the Institute of Legal Medicine in Frankfurt/Main, Germany showed, that *L. sericata* was present on more than 50 % of all insect infested cadavers [5], making it the most important species in forensic entomology in Germany. Since blow flies are the first colonizers of cadavers, the knowledge of the age of the oldest immature specimen can be used to estimate the minimum time since death, also called minimum post mortem interval (PMI_{min}) [6–8]. Age estimation is possible by e.g. measuring the length of the larvae or by calculating the time that is taken to reach certain developmental stages, like the post-feeding stage, pupariation or emergence at a given temperature [9–11]. Since *L. sericata* is also known to cause myiasis, i.e. the colonization of dead or even living tissues of living humans [12,13] or other animals, such as sheep [14–17], the age of its larvae in e.g. the sores or necrotic tissue can be used to estimate the period of a possible neglect [18]. For all these applications, age estimation often relies on published reference developmental data. These data are often derived from populations in other geographical regions with different climates than the specimens found at a local crime scene. Such different environments with their own climatic conditions might result in diverse phenotypes, visible for example via divergent developmental times that could lead to a loss of information or even a miscalculation of the PMI_{min} due to missing matches between the reference data and the data valid for the case-relevant population. This phenomenon is a feature of so-called phenotypic plasticity [19] and it was shown that the more variation of an environmental factor (e.g. temperature) is experienced by an organisms, the more it will be equipped to cope with greater deviations from that norm as stated in the climatic variability hypothesis [20,21]. This will result in more plastic traits and different distinct phenotypic responses dependent on the heritage environmental conditions [22,23]. Saunders (2000) showed that larval diapause, influenced by the photoperiod experienced by the parent female, is induced by female *Calliphora vicina* in Finland in response to much longer days than those *C. vicina* females

from Scotland [24]. Such variation in thermal plasticity with increasing geographical latitudes was already shown for flies of the family of Drosophilidae [25–29], for beetles [30–32] and even vertebrates like rodents [33].

The degree of comparability of studies by different authors on the same species should always be treated with caution, not only because of possible population-specific differences. It is difficult to say to what extent differences in the rate of development of a species studied in different regions of the world are actually due to possible phenotypic plasticity or to fundamental differences in study design. The latter factor is due to the fact that there is no standardized experimental design for developmental studies in blow flies, which makes it difficult or even impossible to identify population-specific effects between studies. Population-specific differences of development can be masked or even amplified by many other factors affecting insect growth. Such factors could be the type of substrate larvae feed on like e.g. liver or minced meat from pork or beef [34,35], the ~~size of the larval aggregation that is feeding on it and the associated accumulation and increase of~~ thermal heat by larvae aggregations of different sizes [10,36,37], or the type of pupariation substrate (e.g. sand or vermiculite) and the associated differences in the time spent by the larvae in the post-feeding stage [38]. It is therefore important to study possible population differences of the same species in the same experimental setting, ideally in the same laboratory and even with the same personnel. For example, this has been done for the blow fly *C. vicina* Robineau-Desvoidy, 1830, where population-specific differences in developmental time were shown when comparing an English and a German population of *C. vicina* in the same laboratory and applying the same experimental design. Not only the larval length, but also the time required to reach certain developmental stages differed significantly [39].

Several papers have been published on the growth and development of *L. sericata*. The geographical range of these studies covers much of the Holarctic from China to North America [9,38,40–49] and there are

deviations for the total development at e.g. 22°C of up to 5 days, depending on the respective population.

To test whether the developmental data obtained from two geographically distant populations in different climatic zones are comparable, we conducted a developmental study with *L. sericata* from the Frankfurt/Main region, Germany (temperate climate), and an area near Oaxaca de Juarez, Mexico (tropical climate). As this comparison is carried out within one and the same laboratory with identical equipment and staff, significant biasing factors can be excluded. With this study, we add to the sparse data in the Neotropics for this species [44,48] and discuss the occurrence and extent of phenotypic plasticity as a possible factor when using this species in forensic entomology.

MATERIAL AND METHODS

Fly colonies

The Mexican population of *L. sericata* was obtained close to the city of Oaxaca de Juárez (17°03' N, -96°44' W). It is located approximately 1550 m above sea level and characterized by a tropical climate with an annual mean temperature of 23 °C. Approximately 80 specimens were brought to Germany as larvae. After eclosion, their identification as *L. sericata* was verified by means of morphological keys [50,51] and DNA barcoding according to Zehner et al. [52], and a breeding colony was established. The German population was established from individuals sampled in the Frankfurt/Main region (50°06' N, 8°41' E) and kept as a laboratory population for several generations. The annual mean temperature in Frankfurt/Main is 11 °C and it is located 113 m above sea level with a temperate climate. Adult flies of both populations were kept in cages of 35 x 26 x 26 cm at room temperature (approx. 22 °C) and 12:12 L:D with sugar and water provided *ad libitum*. Once a week, beef liver was offered as a protein source and oviposition medium. In order to minimize the possible effects of adaptation of the Mexican flies to the new laboratory environment, only F1 generation flies were used for the present study.

Developmental study

Beef liver was offered for oviposition. After 3 hours, the liver and eggs were removed and placed in two climatic chambers (Binder KB 53, E3.1), each set to a working temperature of 20 °C and 30 °C respectively. After emergence of the larvae, the larvae were placed in five groups of 30 larvae each on an excessive amount of approximately 20 g of minced meat (beef/pork) in a plastic cup. Each plastic cup was placed separately in a container measuring 12 x 12 x 8 cm. A total of five replicates (chronologically separated ovipositions) with five containers per replicate were carried out.

Three times a day, every eight hours, migrating (post-feeding) larvae, puparia or adult flies were counted. After each observation time, the containers were placed in different positions in the chambers. When 50 % of the larvae had left the minced meat, a thin layer of small animal litter was added as a pupariation medium for the larvae. Once all larvae were present in the litter, the remaining meat was removed from the containers. As soon as white prepupae were visible, they were counted as pupariated larvae [53]. After all specimens in a container had pupariated, puparia were transferred to 50 ml tubes with perforated lids and animal litter until emergence. The time when the first individual per container and when 50% of the specimens per container reached a new developmental stage was reported.

Statistical analysis

Accumulated degree hours (ADH) were calculated by adding up the hourly temperature values without subtracting a species-specific threshold. Differences in the developmental time between German and Mexican flies were tested for significance via Wilcoxon rank sum test using the package rstatix, version 0.7.0. [54] for RStudio version 1.4.1103 [55].

RESULTS

The time required to reach the different developmental stages (post feeding, puparia and adult flies) was mostly (highly) significantly different for both populations of *L. sericata* at both 20 °C and 30 °C (Fig. 1).

The first specimens of the Mexican population at 20 °C reached the post feeding (PF) stage significantly earlier (after 2106 ADH or 4.4 days, $p < 0.001$) than the first specimens of the German population, which required 2813 ADH (5.9 days) to reach this stage (Table 1). Although the time differences for pupariation and eclosion of the adult flies between the two populations were not significant ($p = 0.072$ and $p = 0.48$, respectively), the first specimens from the Mexican population reached the corresponding stages again earlier. Looking at the time at which 50 % of the specimens per container had reached the respective stages of development, it is again noticeable that the Mexican *L. sericata* reached all stages earlier and the overall development was faster (23.5 days compared to 24.7 days, Table 1).

At 30 °C, the first specimens of the Mexican population also reached the PF stage slightly earlier than the German population (0.2 days, $p < 0.001$). However, the German flies developed significantly faster at 30°C than the Mexican flies (Fig.1, Table 1). The first German specimens started pupariating after 5 days (3610 ADH), whereas the Mexican *L. sericata* did not start until 6.9 days (5002 ADH). For eclosion, the difference was 2.6 days ($p < 0.001$). This temporal difference increased even more when the time of pupariation and eclosion of the adult fly was considered for 50 % of the specimens per container (4.7 days and 5.3 days, respectively).

However, the time taken to reach the different stages differed significantly within the German population subjected to constant 20 °C and 30 °C. The first individuals of the German population required significantly less ADH to reach the PF stage at 30 °C (> 1000 ADH difference, $p < 0.001$) than at 20 °C (Fig. 2 A). These time differences became even greater when considering the occurrence of the first imago (> 1500 ADH difference, $p < 0.001$ and > 3000 ADH difference, $p < 0.001$, respectively). This trend was almost similar for 50 % of all individuals reaching the respective stages. Again, all differences between the development time

at 20 °C and 30 °C were highly significant ($p < 0.001$).

In the Mexican population, the ADH required for the first individuals to reach the PF stage and emerge as adults was significantly lower at the constant temperature of 30 °C (> 450 ADH difference, $p < 0.001$ and > 1000 ADH difference, $p < 0.001$, respectively). However, this trend was reversed when comparing 50% of all individuals per container. Again, PF stages were reached significantly earlier by larvae developing at 30 °C than at 20 °C ($p < 0.001$). However, pupariation started earlier at 20 °C than at 30 °C ($p < 0.001$). The ADH required until adult emergence were also higher for immatures at 20 °C, but not significant ($p = 0.18$).

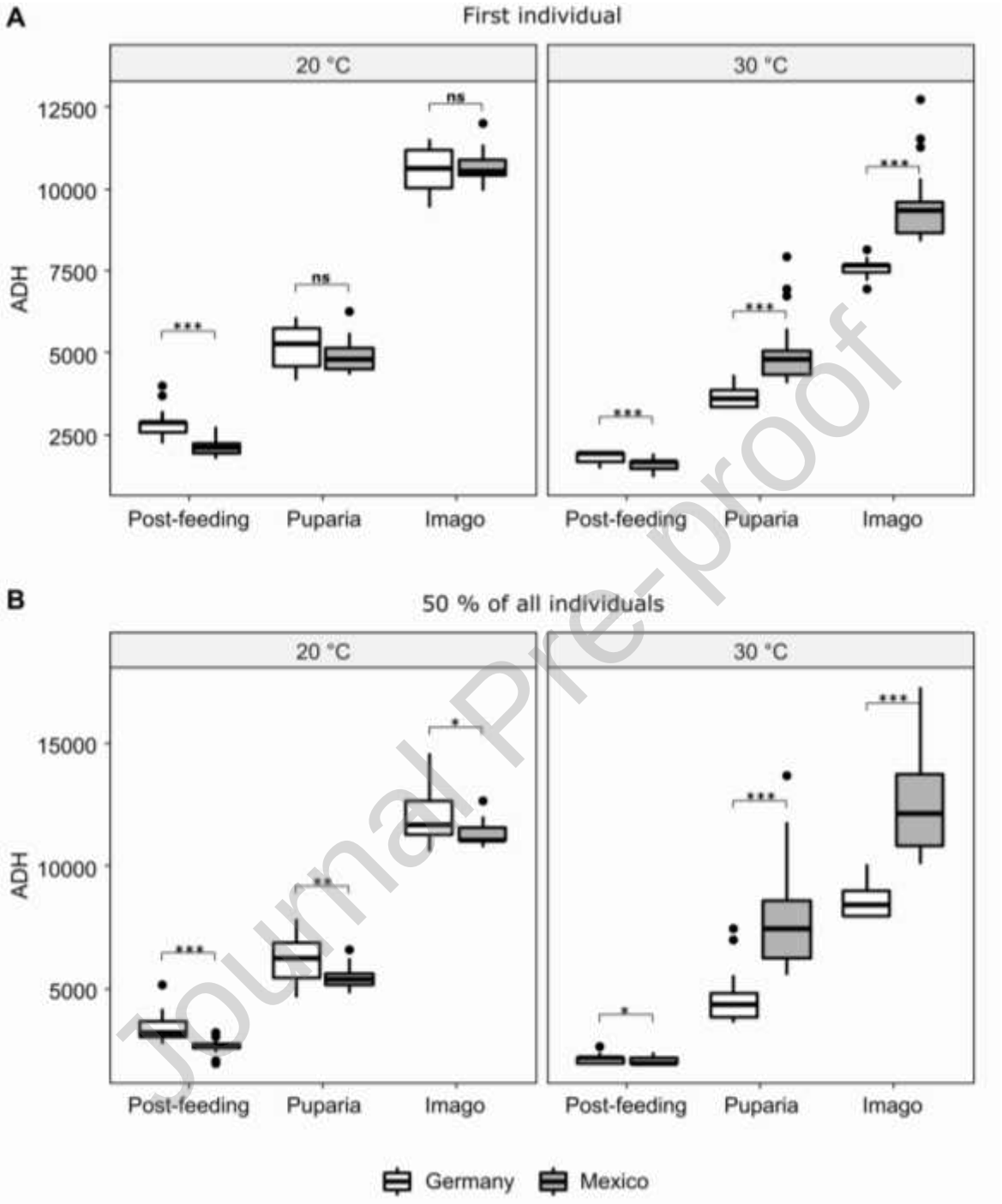

Figure 1: Comparison of developmental time needed to reach the post-feeding, puparial and adult stage in ADH for a Mexican and German population of *L. sericata* at constant 20 °C and 30 °C, respectively. (a) ADH required for the first individual per container to reach each developmental stage. (b) ADH needed until 50 % of all specimens per container reached the respective developmental stages. ADH were

calculated by accumulating the hourly temperatures without subtracting a lower developmental threshold.

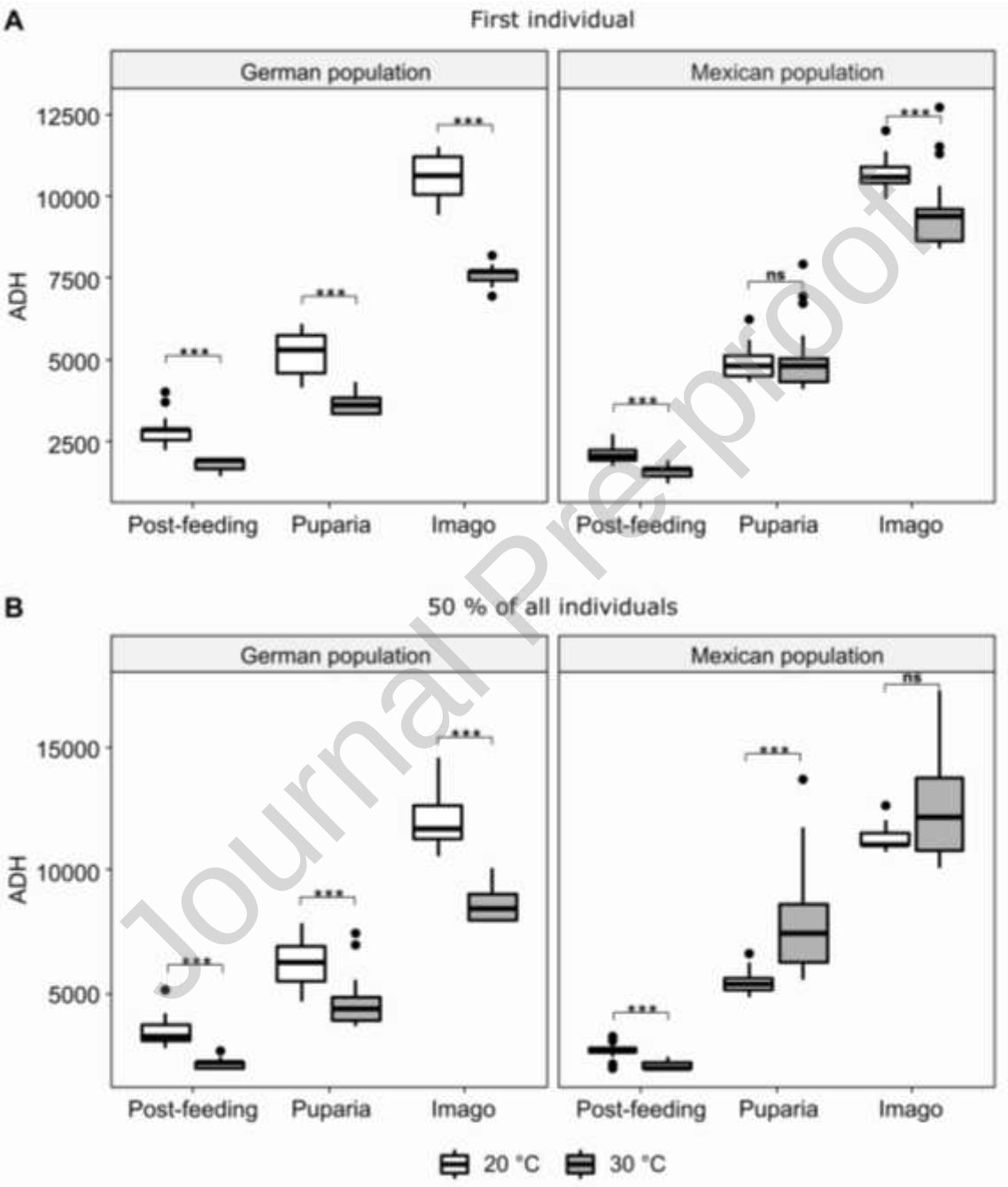

Figure 2: Differences in the developmental time required to reach the post-feeding, puparial and adult stages of ADH with particular emphasis on a comparison between developmental times at 20°C and 30°C

for both populations of *L. sericata* (German and Mexican). (a) ADH required for the first individual per container to reach each developmental stage. (b) ADH required for 50% of all individuals per container to reach each developmental stage. ADH were calculated by accumulating hourly temperatures without subtracting a lower developmental threshold.

Table 1: Mean time in ADH including standard deviation taken by the first individual and 50% of *L. sericata* to reach the post-feeding (PF), puparial (P), and adult stage (A)

	20 °C		30 °C	
	Germany	Mexico	Germany	Mexico
1st PF	2813 ± 395	2106 ± 239	1805 ± 141	1622 ± 169
1st P	5160 ± 607	4877 ± 434	3610 ± 306	5002 ± 942
1st A	10613 ± 682	10592 ± 464	7594 ± 301	9446 ± 1066
50 % PF	3476 ± 699	2611 ± 336	2150 ± 206	2030 ± 158
50 % P	6202 ± 1000	5411 ± 449	4571 ± 1022	7940 ± 2201
50 % A	11858 ± 1018	11284 ± 468	8628 ± 737	12440 ± 2099

DISCUSSION

The current study provides information on the applicability of developmental data from non-local populations of *L. sericata* in forensic casework. We have proved significant differences in the developmental time between the German and Mexican populations of up to 2.6 days for the first specimen per container to develop from egg to imago and 5.3 days when 50 % of the specimens per container were considered.

The few data available so far suggest that age determination can be flawed when relying on data from geographically separated populations [39,56,57], which can have a great impact on case work. Based on our data, a discrepancy of up to 3 days in the PMI_{min} estimate would be possible if the reference values of the corresponding *L. sericata* population in Mexico or Germany were used. Such mismatches could have drastic consequences for forensic investigations. These differences in the rate of development at different temperatures could be a result of selection processes due to the native climate of the region of origin, as stated in the climate variability hypothesis [20,21]. While the German *L. sericata* are predominantly active during summer (i.e. from June to August) and absent during cooler periods [5,58], it has been shown that Mexican *L. sericata* can colonize pig cadavers in the Coahuilan semidesert in Mexico even during winter and spring (February to April), with monthly mean temperatures of 19 °C to 26 °C [59,60]. Therefore we expect that the German population subjected to a wider temperature range (annual mean temperature of 10.5 °C and seasonal variations of up to 20 °C [61]) may be more resistant to temperatures outside its developmental optimum (such as 30 °C for longer periods), while an organism from a tropical climate (i.e. Mexico with a mean annual temperature of 23 °C and little fluctuation [62]) is more adapted to and dependent on a smaller temperature range. This is also evident from the obtained data, where the Mexican population needed significantly more time to pupariate and to complete its total development at 30 °C than the German population (Fig. 1). The idea of accumulating a certain amount of energy (heat) to complete each developmental stage [63] leads to the assumption of a linear relationship between

increasing temperature and developmental rate. Therefore, we would suspect to see a significant reduction in the time required to complete their development when the temperature increases from 20 °C to 30 °C, which was observed for the German population of *L. sericata* (Fig 2). However, this trend is not visible in the Mexican population, where some individuals took the same or even slightly more time to develop (Fig. 2) compared to 20 °C. However, the total accumulated heat that is required to for each species to complete its development might not be the same for all populations of the same species, as it was shown to increase with increasing latitude ([64,65]. So when comparing the differences in developmental time between the German and Mexican population, for example at 20 °C, this could be due to the fact that the German population generally needs more thermal energy to complete its development or Mexican *L. sericata* is better adapted to this temperature and therefore less stressed.

The linear relationship between temperature and developmental rate should make it possible to compare growth studies at different temperatures, which has led to the creation of thermal summation models. In theory, each species has its own thermal requirements and a species-specific upper and lower developmental thresholds (UDT/LDT). However, previous studies have shown that there is less variation in the upper thermal limits between species or populations from different latitudes, than in the lower thermal limits [25,30,66–68]. The LDT in particular plays a crucial role in estimating the age of developing insects based on thermal summation models and was shown to increase with decreasing latitude [65,69]. This trend was also confirmed by Honek, who recalculated developmental thresholds for 605 species in 14 insect orders [70]. However, changes in the LDT between populations can lead to major implications in forensic casework that is relying on developmental data from geographically distinct populations. For example, the commonly used reference value of 9°C for *L. sericata* is derived from a Lithuanian population [10]. It has also been confirmed for populations from Germany and China [49,71]. However, Reibe et al. observed a lower LDT of 8 °C for a population from Austria [72] and *L. sericata* from Ecuador may even

have a higher LDT than 10 °C, as larvae were not able to complete their development at this temperature [48].

Our data show similarities or matches to the results of other studies, but also striking discrepancies (Fig. 3). At 20 °C, the development of the fastest specimen took 22.1 days for both of our examined populations, which is close to 20.3 days at 20.7 °C for a Canadian population [43] and 24.3 days at 19 °C for Chinese *L. sericata* [49]. In contrast, an US population needed 54.4 days to complete its development at 19 °C [41]. At 30 °C, the first individuals of the German population completed their development from egg to adult within 10.5 days. This is within the range of previous observations (Fig. 3). French and Austrian *L. sericata* both had a total development time of 11.2 days at 30 °C [9,47]. And the duration was similar for a Chinese population with a total developmental time of 11.4 days at 31 °C [49]. The time required from egg to adult for populations from the United States at 29 °C [42] and Ecuador at 30 °C [48] were more similar to the Mexican population in the current study, at approximately 12 days and 13.1 days, respectively. Only a few studies were conducted with *L. sericata* at temperatures below 16 °C and show extremely different developmental times, e.g. 120 days at 12.7 °C [46] and 62 days at 12 °C [47]. This highlights the need for more developmental studies, in particular at lower temperatures close to the LDT.

We are therefore still a long way from understanding in detail the causes and extent of geographical variability in the growth of different populations of a species. . What can and should be done as soon as possible, however, is to harmonize the design of growth studies so that their data can be reliably compared. Almost every study uses a different diet for the larvae. While some studies used beef liver [38,40,42,43,46], others used beef muscle [47,48], pork meat [49], an artificial diet [44] or minced meat (current study). However, the choice of the diet can influence the developmental rate of blow fly larvae. Clark et al. tested the development of *L. sericata* on bovine and porcine lung, liver and kidney tissues and observed a significantly slower development on liver than on the other substrates and, in addition, an overall faster development on porcine tissues [34]. Slower growth rates of blow flies on liver tissue have

also been recorded by other studies [57,73–75]. Because moisture is an important factor during development, one explanation could be that liver dries faster than the other substrates [38,75]. Furthermore, as observed by Niederegger et al., processed substrates, such as minced meat, resulted in faster and more stable growth than the other substrate types [74]. In addition, Bernhardt et al. suggest porcine minced meat as a suitable surrogate for human tissue in developmental studies of blow flies [76]. However, even minced meat does not have the same fat and nutrient content as a decomposing human body. This is because the availability of certain structures and nutrients depends on the body parts and organs, which the larvae feed on.

Other factors were also shown to affect the development time of the immature specimens, such as the pupariation substrate, which can modify the time spent in the post-feeding stage. Tarone and Foran observed that a transfer of post-feeding larvae to a new and dry substrate shortened the time to pupariation and that the type of substrate also played an important role [38]. The pupariation substrate used varies, such as sand [38,44,47,49], sawdust [9,40,41,43,45], diatomites [48], pine shavings [46], or small animal litter (current study), or is sometimes not mentioned at all in the materials and methods section [42].

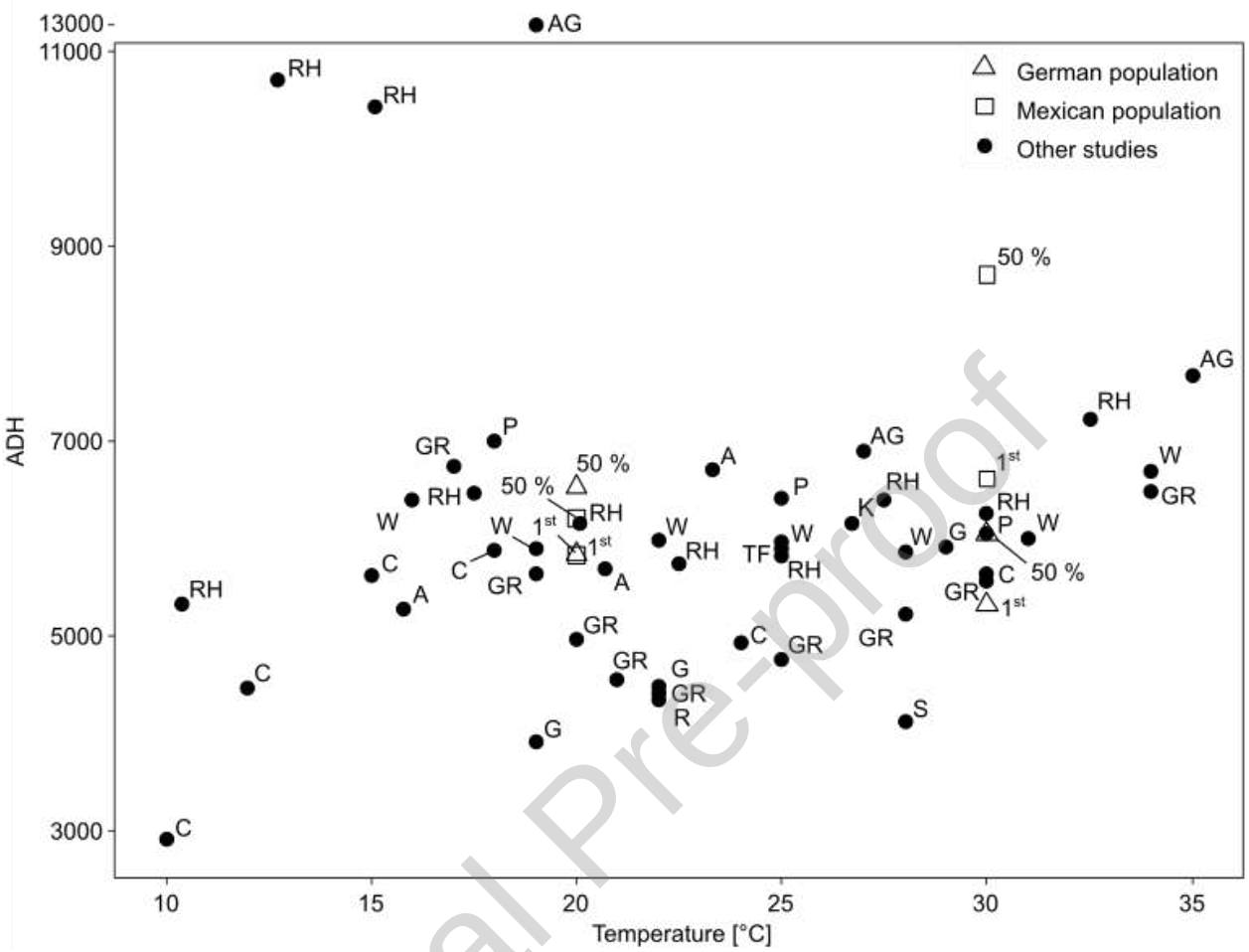

Figure 3: Comparison of previously published growth studies of *L. sericata* with ADH needed for the development from egg to adult emergence at different constant temperatures, based on Amendt et al. 2011 [8]. ADH were calculated with a developmental threshold temperature of 9 °C. Triangles and rectangles represent data collected during the present study for German and Mexican *L. sericata*, respectively. Black circles represent previous studies and are marked with the corresponding abbreviation (K = Kamal 1958, United States [40]; AG = Ash and Greenberg 1975, United States [41]; G = Greenberg 1991, United States [42]; A = Anderson 2000, Canada [43]; GR = Grassberger and Reiter 2001, Austria [9]; TF = Tarone and Foran 2006, United States [38]; R = Rueda et al. 2010, Colombia [44]; S = Saleh et al. 2014, Iran [45]; RH= Roe and Higley 2015, United States [46]; C = Cervantés et al. 2018, France [47]; P = Pruna et al. 2019, Ecuador [48]; W = Wang et al. 2020, China [49]). Please note that the recordings of Ash and

Greenberg at 19 °C are outside the range of the graph and thus are displayed above with a corresponding label.

Conclusion

The present study shows that development data from other regions should not be used uncritically as reference values for local populations when estimating the age of immature blow flies. At the same time, the comparison of different developmental studies highlights the hitherto neglected problem of study design in forensic entomology. In order not to negligently postulate population-specific differences, it should first be clarified to what extent other factors of the study may be responsible for possible differences.

Funding

The research project was supported by the German Academic Exchange Service (DAAD) with funds from the German Cooperation for Sustainable Development GmbH (GIZ) on behalf of the German Federal Foreign Office. Project ID (DAAD): 57594060.

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Conflicts of interest

The authors declare no conflict of interest.

Highlights

- Mexican and German populations of *L. sericata* differ in their development times
- Mexican *L. sericata* had a shorter development time at 20°C than German flies
- At 30 °C, German *L. sericata* pupariated and eclosed earlier than the Mexican flies
- Differences in study design make the comparison of developmental studies difficult