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Establishment of developmental charts for the larvae of the blow fly *Calliphora vicina* using quantile regression

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ABSTRACT

Developmental data of necrophagous blow fly species can be used to estimate a minimum postmortem interval (PMI_{min}) in death investigations by estimating the age of larvae sampled from the cadaver. The most important parameter used in this age estimation is the increase in larval length during growth. Larval length can be compared to species-specific reference data to get an age estimate for the larval specimen. The exploration of this type of data and the use of an appropriate statistical method are the major challenges in evaluating forensic entomological data sets. In Europe, *Calliphora vicina* is one of the most dominant species with forensic entomological relevance. Despite its frequency and importance, there are currently no published developmental studies for German populations of this blow fly that can be referenced for larval age estimations. This is regrettable because the geographical origin of different populations may lead to phenotypic plasticity in the same species and population-specific growth patterns that differ from published data sets.

To address this shortcoming, the objective of the present research was to generate growth data for *C. vicina* which can be used for age estimation in German casework.

We present, for the first time, local developmental data for the larval stages of German *C. vicina*, reared at three constant temperatures (15, 20 and 25 °C) and compare the results with published studies on *C. vicina* development from elsewhere. To analyse the development we chose a quantile mixed effects model because of its robustness and insensitivity towards outliers. Quantile regression was developed as an extension of the linear model to estimate rates of change in all parts of the distribution of a response variable and to discover more useful predictive relationships between variables. By applying a linear quantile mixed effect model, we estimated the 0.1 and 0.9 quantile functions of the larval age for each temperature. Graphically, these quantile functions act like reference bands and therefore, plots for larval stages 1–3 can be used to estimate the age of a larva. Nevertheless, the post-feeding stage still remains difficult to describe with linear models and need to be further investigated. A comparison of the results from this present study with the currently applied methods for age estimation of *C. vicina* still demonstrates the importance of collecting growth rates for local blow fly populations and of standardising experimental designs and statistical analyses.

1. Introduction

Some necrophagous fly species are able to colonise a dead body hours, or even minutes, after death and their offspring feed and develop on the decomposing remains. Since the life cycle of insects is considered a precise species-specific biological clock [1–4], a fairly accurate calculation of the minimum time since death, or the minimum postmortem interval (PMI_{min}), is possible through age estimation of these juvenile stages [1,5]. For these purposes, reference growth data, generated under laboratory conditions, are applied for forensically important taxa [2,6].

As the fly family Calliphoridae belongs to the most common and abundant taxa used in a forensic context, the majority of the studies focus on blow flies (Diptera: Calliphoridae). The most important parameter in estimating the age of their larvae, and

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thus the time since colonisation, is the increase in body size (length or, less commonly, weight) over time [7], with size positively correlating with age for the biggest part of the larval life time [2]. By taking the ambient temperature at the crime scene into consideration and by estimating the stage of development of the larvae found on the corpse, subsequent age determination after species identification is usually done through curvilinear regression [8], isomegalen diagrams [9], isomorphen diagrams [10,11] or accumulated degree hours or days (ADH/ADD) [8].

Unfortunately, there are some methodological and biological sources of confusion in PMI_{min} estimation which are obviously undesirable in this context. Due to differences in experimental set up and performance [13,16–18] e.g., variable rearing techniques or different food substrates [19,20], some forensic scientists may find different developmental rates for the same forensically important species. In practice, this may lead to variable PMI_{min} predictions, depending on the data set used as a reference [7,12–15]. Furthermore, an experimental sampling design with little, or even no, replication and independence between samples and treatments, and experimental conditions that do not cover a representative spectrum of natural variability, weakens the inference value [21]. Another source of discrepancy may be attributed to the geographical origin of different populations which can lead to phenotypic plasticity within the same species [2,13,16,22]. The concept of phenotypic plasticity leads to disputes about whether so-far published data might be valid only for the geographic area from which the examined breed was obtained [2,6,12,13,16]. As a consequence, more development data for local blow fly populations need to be generated. Such data should be supported by appropriate statistics that provide the required level of statistical accuracy, as this is an essential part of the current recommendations for the evaluation of forensic evidence [23]. Simple linear models do not reflect the data adequately because larvae grow in a non-linear fashion and data often show dependent and heteroscedastic structures [15,24,25].

Calliphora vicina (Diptera: Calliphoridae) is a blow fly widely distributed throughout Europe [26] and one of the most important necrophagous species used as entomological evidence in crime investigations. To analyse its development we chose a quantile mixed effects model because of the robustness of the method and its insensitivity towards outliers [27]. Quantile regression allows prediction of the relationship between variables for every kind of probability distribution [28] and was developed as an extension of the linear model to estimate rates of change in all parts of the distribution of a response variable [29].

In most of the regression analyses the straight line is aligned precisely with respect to the observations and they thus focus on the mean. This is a particular problem for heterogeneous data [29], where the assumption of normally distributed residuals with a mean of 0 and a variance of σ^2 is wrong. Hence, focusing only on the mean in heterogeneous models may lead to erroneous conclusions because there is more than a single slope describing the relationship between x and y [30]. In contrast, in quantile regression the straight line is based on a certain quantile, so that for example, when focusing on the median, 50% of the observations are below and 50% above this straight line.

With quantile regression one can describe the whole distribution of a response variable and thus accomplish inference on conditional quantile functions. Where conventional regression highlights only a single measure for the conditional distribution of a response variable and assumes normal error distribution, the conditional quantiles in the quantile regression show a more complete picture and may detect information about relations where mean regression estimates none [29].

Hence, quantile regression is a good way to discover more useful predictive relationships between variables without making an assumption about the error distribution.

Conditional quantile functions can then be used to construct reference bands which contain a certain percentage rate of the results of a study. For example, we use 0.1 and 0.9 quantile functions to construct a 80% reference band around the larval age. At low sample sizes (minimum sample size of 118) [31], the estimation of the 0.1 and 0.9 quantile functions has the advantage that the accuracy of the estimators for these quantile functions is higher than for the 0.05 and 0.95 quantile functions. This is due to the fact that more observations are below the 0.1 quantile and above the 0.9 quantile. To get precise estimations for the quantile functions, a sufficient number of observations "from both sides" is required. Therefore, at low sample sizes, one should use 0.1 and 0.9 quantile functions. For constructing 0.05 and 0.95 quantile functions, a minimum sample size of 377 is suggested [31,32].

The objective of the present research was (1) to generate local developmental data for German *C. vicina* under three constant temperatures, (2) to compare the results with published studies on *C. vicina* development, (3) to statistically analyse its development, and (4) to give estimators of the larval age that can be used in an expert opinion because they are supported by quantile functions as reference bands.

2. Materials and methods

2.1. Fly rearing and specimen collection

About 50 wild *C. vicina* were caught in 2010 at two sites in Frankfurt am Main, Germany which are about 3 km apart. They were trapped using beef liver as bait and a sweep net. Adult flies were held in two rearing cages at room temperature (average temperature approximately 18 °C, 60% RH) and a 12:12 L:D cycle. They were provided with water and sugar *ad libidum*.

Every two days beef blood was offered as a source of protein and once a week, the flies were given beef liver for 3 h to allow for oviposition. After these 3 h the liver was checked for eggs and, if eggs were present, transferred to a LinTek MKKL 600/2 incubator, set at 25 ± 1 °C. Constant temperature was monitored using a DS1922L Temperature Logger iButton (Maxim/Dallas), with measuring performed every hour. Twenty-four hours after transferring the eggs into the incubator, hatched larvae were used for further experiments.

2.2. Experimental set-up and sampling

In 2012, growth experiments with a total of 1800 larvae were carried out at three constant temperatures, 15 ± 0.5 °C, 20 ± 0.5 °C and 25 ± 0.5 °C (these values cover the range of the most common mean temperatures in Germany, see also Discussion). Each temperature run was repeated twice. The runs consisted of ten 200 mL plastic cups that were cut in half, and placed in a 12 cm × 12 cm × 8 cm plastic container that was filled with 2 cm of sawdust, which served as medium for pupariation. Approximately 50 g of ground meat (half beef, half pork) were placed in the shortened plastic cups and 30 hatched larvae were transferred from the oviposition medium to the meat with a dampened paint brush. The plastic containers with the cups were moved daily within the incubators to avoid possible incubator-specific effects. Additionally, each of the runs was arbitrarily assigned to one of three incubators [21]. Three times a day (8 am, 1 pm and 6 pm), five containers were randomly chosen and five larvae sampled, killed with boiling water and then placed in 96% ethanol to avoid post-mortem changes [33,34]. Within 1 h the larval length was measured to 0.1 mm using a geometrical micrometre [35], the weight was recorded to the nearest 0.001 g using an electronic balance, and the larval stage was determined under a