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Effects of temperature and diet on black soldier fly, *Hermetia illucens* (L.) (Diptera: Stratiomyidae), development

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ABSTRACT

The black soldier fly, *Hermetia illucens*, is recognised for its use in a forensic context as a means for estimating the time of colonisation and potentially postmortem interval of decomposing remains. However, little data exist on this species outside of its use in waste management. This study offers a preliminary assessment of the development, and subsequent validation, of *H. illucens*. Larvae of *H. illucens* were reared at three temperatures (24.9 °C, 27.6 °C and 32.2 °C) at 55% RH on beef loin muscle, pork loin muscle and a grain-based diet (control). Each of the temperatures and diets were found to significantly ($P < 0.05$) affect all stages of immature growth except for pupation time. Overall, those reared on the pork diet required on average ≈23.1% and ≈139.7% more degree hours to complete larval development than those reared on the beef and grain-based diets, respectively. Larvae reared at 27.6 °C and 32.2 °C required on average ≈8.7% more degree hours to complete development and had a final larval weight ≈30% greater than larvae reared at 24.9 °C. The validity of the laboratory larval length and weight data sets was assessed via estimating the age of field-reared larvae. Grain-diet data lacked accuracy when used to estimate larval age in comparison to estimates made with beef and pork-diet data, which were able to predict larval age for ≈55.6% and ≈88.9% of sampling points, respectively, when length and weight data were used in conjunction. Field-reared larval sizes exceeded the maximum observed under laboratory conditions in almost half of the samples, which reduced estimate accuracy. Future research should develop additional criteria for identifying development of each specific instar, which may aid in improving the accuracy and precision of larval age estimates for this species.

1. Introduction

Forensic entomology is the application of insect or other arthropod evidence to legal investigations [1]. While the first recorded use of forensic entomology dates back to the 13th century in China, it was only in the late 1800s that interest in this field gained momentum [2]. Currently, forensic entomology is organised into three principal areas: urban, stored products and medicolegal entomology [3,4]. Medicolegal entomology concentrates on insects associated with human or other animal remains, and may aid in several aspects of an investigation such as movement of a body postmortem, toxicological analysis, and indication of trauma [5–7]. Despite its range of potential applications, however, entomological evidence is most commonly used in the estimation of the time of colonisation (TOC) of human remains [4]. The TOC can be inferred as the minimum postmortem interval (minPMI), assuming colonisation occurs following death [8,9].

The minPMI is assumed to be most accurately predicted via calculating the age of immature insects found developing in association with a body [4]. This is done via thermal summation models developed by rearing insect species under laboratory conditions to determine the accumulated degree hours or days (ADH or ADD) required for a species to complete each developmental stage or reach a specific larval length or weight [7,10]. Using meteorological data from the crime scene, the time since ovi- or larviposition can be back-calculated from the immature insect age estimate and therefore provide a minPMI [11].

A number of factors are known to influence the growth rates of insects. It is therefore important that laboratory sourced data match the conditions of the crime scene as closely as possible in order to provide the most reliable minPMI in a case [11]. Due to their poikilothermic nature, developmental rates of insects are often published over a range of temperatures, taking into

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consideration the temperatures naturally experienced by each species [10,12,13]. However, few other factors (e.g., food substrate [14]) that could influence the accuracy of subsequent minPMI estimates are taken into consideration.

Colonisation of a body begins externally, at natural orifices and sites of trauma, while later colonising species can be left with a mixture of indistinguishable liquefied tissue and putrid dry remains [6]. The bodily tissue that plays a role in larval diet can therefore differ due to oviposition site selection as well as the time of colonisation. A number of studies have confirmed that tissue type significantly impacts growth rates of several dipteran species [14–17]. Additionally, studies have shown that insects develop at significantly different rates when reared on the same tissue type sourced from different vertebrate species [16–18]. Despite this research, diets used for rearing immature insects are often selected based on their low cost and ease of acquisition rather than their representativeness of crime scene conditions, implementing diets that range considerably, both in terms of the vertebrate species and tissue type [14,17]. Differences in growth rates as a result of differing diets are not observed for all forensically important species, however. In a study by Boatright and Tomberlin [3], larvae of *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae) showed no significant differences in growth when reared on either equine or pork gluteal muscle. Furthermore, research conducted by Stokes et al. [19] suggests that out of pork, sheep and beef tissue, no single vertebrate species is the most representative overall of human tissue. Thus, growth rates of all forensically relevant species need to be determined over a range of species and tissue types in order to confirm or deny the reliability of data from previously published research.

Another aspect of developmental studies that requires attention is the larval growth data recorded, specifically length, weight and instar. Many developmental studies report only one or two of these factors [10,12,14,16,20]. However, a recent study by Núñez-Vázquez et al. [21] demonstrated that no single measurement provides the most accurate larval age estimate throughout the different stages of larval growth. While the instar was never the most accurate predictor of age in their study, it may be the only predictor available if specimens are preserved incorrectly at collection [11].

Núñez-Vázquez et al. [21] also demonstrated the importance of validating laboratory based results. Their study involved rearing immatures in an outdoor environment where they experienced natural fluctuations in temperature and humidity in order to determine the accuracy of their laboratory produced data. To date, very few studies include a validation component within their research [21–24].

Another limiting factor in providing accurate minPMI estimates is the relative lack of developmental data available on later colonising species compared to early colonisers. This is due to the fact that minPMI estimates based on early colonising species are believed to be the most accurate and therefore the most desirable for use in court [6]. However, for bodies discovered during the later stages of decomposition the data from these early colonising species are no longer useful [11]. Instead, information on succession patterns is implemented for minPMI estimates, though this is not thought to be as accurate [4]. As insects are known to colonise bodies throughout all stages of decomposition, the developmental rates of later colonising species could provide more accurate minPMI estimates than insect succession data.

Hermetia illucens (L.) (Diptera: Stratiomyidae), commonly known as the black soldier fly, is a coloniser of decaying material in temperate and tropical regions throughout the world [25–28]. This species has been recognised for its use in reducing animal waste as well as suppressing house fly, *Musca domestica* L. (Diptera: Muscidae), populations through competition [29]. Additionally, the prepupal stage of the black soldier fly has been shown to be an effective alternative food source for farmed animals such as fish and swine [30]. Therefore, the majority of research currently available for this species focusses on determining the optimal conditions for maintaining a year round colony [26,31–34] and achieving efficient waste reduction [29,30].

However, the black soldier fly has been recognised as a potential indicator of the postmortem interval in forensic investigations [27]. The black soldier fly is a late coloniser of human corpses with oviposition typically occurring 20–30 days postmortem [27], though the species has also been observed to colonise within the first week postmortem [35]. Black soldier fly larvae have been used as evidence in a number of cases within the United States [27], Brazil [28] and Italy [36], providing estimates that coincided with other evidence collected from the various crime scenes. In order to provide reliable minPMI estimates, detailed information is required on the growth rates of the black soldier fly when reared in conditions that attempt to mimic those of crime scenes. Currently, only a single study reports the changes in length and weight of black soldier fly larvae throughout their development [37]. This study is also the only one to rear black soldier fly larvae on animal tissue based diets, using pork liver and fish renderings. All other studies examined the growth of this species on artificial [26,31–34,38,39] or manure-based diets [29,30]. Consequently, black solder fly development data based on these diets have historically been applied in forensic entomology case work. The lack of detailed information for this species restricts the reliability of minPMI estimates extrapolated from such data.

The aims of the current study were to determine whether growth rates of *H. illucens* at varying temperatures differ between a commonly used grain-based diet for this species and a vertebrate tissue diet, as well as to observe if developmental differences arise when reared on tissue of different vertebrate species. Additionally, this study aimed to determine whether laboratory produced data for this species can accurately be used to predict the age of field-reared specimens.

2. Materials and methods

2.1. Adult fly colony

Black soldier fly eggs were sourced from a colony established and maintained by the Forensic Laboratory for Investigative Entomological Sciences (FLIES) facility of Texas A&M University, College Station, TX. Larvae were reared indoors on a 70% moisture grain-based diet (dry matter: 30% alfalfa meal, 20% corn meal and 50% wheat bran) [40] ad libitum and transferred as adults to an outdoor greenhouse, maintained at a minimum temperature of 26.7 °C, to allow for controlled egg collection. The colony originated from materials supplied by Phoenix Worms™, Inc., Tifton, GA.

2.2. Diet source

Pork, *Sus scrofa* L., and beef, *Bos Taurus* L., adductor, biceps femoris, semitendinosus, and semimembranosus (commonly known as round) were sourced from Readfield Meats and Deli in Bryan, TX. For each species, meat from a single animal was used. Meat was diced into cubes (approximately 2 cm × 2 cm × 2 cm), divided into 200 g samples and placed into appropriately labelled, sealable 1 L plastic bags. Pork required for the field validation was purchased from Wal-Mart in College Station, TX, and processed similarly. All samples were stored in a freezer at −20 °C. Grain-based diet (dry matter: 30% alfalfa meal, 20% corn meal and 50% wheat bran) was sourced from the supply used for colony maintenance [40].

2.3. Experiment design

A randomised design similar to what was used by Boatright and Tomberlin [3] and Flores et al. [18] was employed. Rearing containers consisted of a 0.7 L plastic Reditainer™ deli container (12.7 cm tall, 11.6 cm top diameter, 8.5 cm base diameter) covered with square cloth (17 cm × 17 cm) and fastened with a rubber band to prevent larval escape or possible contamination.

Specimens were reared in Percival (Model: 136LLVL; Percival Scientific, Inc, Perry, IA) up-right incubators at 24.9 ± 0.4 °C, 27.6 ± 0.4 °C and 32.2 ± 0.5 °C, 55% relative humidity (RH) and 12:12 light-dark cycle (L:D). Rearing containers were placed on the bottom shelf of each incubator with three technical replicates per diet.

2.4. Egg development

Eggs intended for rearing were collected from the colony using methods outlined by Sheppard et al. [31]. Eggs <6-h-old were used in this study. Using a paintbrush dampened with deionised water, eggs originating from multiple females were placed in a 30 mL plastic cup (4 cm height, 4 cm top diameter, 2.7 cm base diameter) and homogenised by mixing with the dampened paintbrush. The homogenised eggs were weighed using a laboratory balance (Model: Adventurer Pro AV64; Ohaus Corporation, Pine Brook, NJ) and divided evenly in weight (representing approximately 15,000 eggs) between three 30 mL plastic cups. Each cup was placed within an empty rearing container and placed into each incubator in the centre of the bottom shelf.

Minimum time to egg hatch was determined by observing the eggs within the incubators every 2 h after an initial 48 h interval. Time until egg hatch was recorded for eggs collected on three different occasions. Resulting larvae within an incubator were transferred as batches of 250 larvae over a period of 6 h into separate 30 mL plastic cups. In order to avoid hatch bias, larvae were transferred in groups of 10 to each technical replicate cup until a total of 250 larvae per cup were reached. Cups with larvae were placed within a prepared rearing container in an inverted manner to ensure larvae would come in contact with the assigned diet. Three technical replicates were used per treatment, each containing 40 g of the respective diet at the time of larval transfer.

2.5. Larval and pupal development

Observations were made daily to determine if larvae needed to be fed. Assigned diets were provided in 40 g aliquots as needed. Pork and beef were thawed overnight in a refrigerator at 4 °C and allowed to reach room temperature (≈24 °C) prior to being used. Larvae were sampled 36–42 h after egg hatch. Sampling after the initial observation was carried out daily for the first five observations. Following this, sampling was carried out every 48 h to ensure sufficient larvae survived through to the adult stage.

During each observation, three larvae were sampled haphazardly from each technical replicate in order to avoid sampling a progressively diminishing population growth-rate [10]. Larvae were placed in hot water (≈80 °C) for approximately 10 s [41] and transferred to a labelled Petri dish. Larval length was measured using a stereo microscope (Model: Meiji Techno EMZ-8TR; Meiji Techno America, San Jose, CA) with a USB microscope camera attachment (Model: INFINITY1-3C; Lumenera Corporation, Ottawa, Ontario, Canada) and the accompanying software INFINITY ANALYZE and CAPTURE v6.5.2 (Lumenera Corporation, Ottawa, Ontario, Canada). Larval weights were measured individually with a laboratory balance (Model: Adventurer Pro AV64; Ohaus Corporation, Pine Brook, NJ). During initial observations, larvae were not large enough to register individually on the laboratory balance. Consequently, they were weighed as a group for each replicate. Lengths and weights were measured within an hour of killing larvae. Larval lengths and weights were recorded as outlined previously until prepupae were observed in a given replicate. It should be noted that, as described in previous studies [26,29–32,38,42,43], the term "prepupae" refers to the mobile, non-feeding final larval instar of *H. illucens*.

Prepupae were identified by having a dark brown-black dorsal and ventral integument and reduced, darkened mouth parts [38]. Prepupae were collected and transferred to a single 30 mL plastic cup, labelled appropriately and covered with a breathable paper towel to allow for pupation. Cups containing prepupae were returned to the assigned incubators for each corresponding replicate and observed daily for pupal formation and adult emergence. For development time from prepupa to pupa and pupa to adult, only the appearance of the first individual for the respective stage for a given replicate was recorded as this indicated the minimum time for development to reach this stage as well as the minimum time spent in this stage. This approach was carried out for each replicate until approximately 25% of the initial 250 larvae had reached the prepupal stage or a week had passed after the last observation of a prepupa.

2.6. Field validation

Methods were adapted from Núñez-Vázquez et al. [21]. Eggs were collected from the onsite colony using methods previously discussed. Approximately 0.0100 g aliquots of harvested eggs (approximately 500 eggs) were placed on a damp 4.5 cm diameter disc of filter paper and placed atop 20 g of diced pork round within a rearing container (0.7 L plastic Reditainer™ deli container; 12.7 cm tall, 11.6 cm top diameter, 8.5 cm base diameter). Twelve technical replicates were prepared and placed in a 76 cm × 91 cm × 60 cm metal framed cage with a 280 μm mesh amber lumite screen (BioQuip Products, Inc., Rancho Dominguez, CA) on a 1 m high table. This cage prevented possible contamination by other insects. Data loggers (Model: HOBO U12-012; Onset® location) were placed within the mesh cage in order to monitor temperature and humidity on an hourly basis. The described setup was located outdoors in a shaded location within the FLIES facility grounds.

A container was randomly selected every 72 h for analysis. All live larvae within the selected container were removed from the substrate and hot water killed before preservation within 70% ethanol. Sampling continued until either the prepupal stage was reached or no replicates remained. Only 20 larvae per container (approximately 10% of surviving larvae within a given container) were selected for measurements. Larvae were haphazardly selected, while attempting to avoid those with apparent growth retardation. Length and weight of the preserved samples were measured in the same manner as previously described. Linear regressions of laboratory produced growth data were used to estimate the ADH required to reach the minimum and maximum larval lengths and weights of those selected from each field-reared container. The average minimum ADH required for egg hatch was added to each calculated value to account for time since oviposition. Average temperature and RH recorded for the duration of the field validation was 29.5 ± 4.2 °C and 71.0 ± 16.3%, respectively. Data of larvae reared at 27.6 °C were therefore used to produce TOC estimates.

2.7. Statistical analysis

All statistical analyses were conducted using SAS JMP 11.0.0 statistical software (SAS Institute Inc., Cary, NC) and Microsoft Office Excel version 2010 (Microsoft Corp, Redmond, WA). A two-way analysis of variance (ANOVA) was used to compare the minimum ADH (dependent variable) required for

H. illucens to complete each developmental stage across diets and temperatures (independent variables). Post hoc Tukey's honestly significant difference (HSD) test was used to separate least square means in the event of a significant *F* test ($P < 0.05$). Differences in final larval length and weight observations (dependent variables) across diets and temperatures (independent variables) were compared in the same manner as above. ADH was calculated using a lower developmental threshold (LDT) of 12 °C [44] and the following formula:

$$\text{ADH} = (\text{Ambient Temperature } (^{\circ}\text{C}) - \text{LDT } (^{\circ}\text{C})) \times \text{time (h)}$$

3. Results

3.1. Life-history traits

Minimum ADH required for *H. illucens* to complete each developmental phase when reared on different treatments are presented in Table 1. Temperature was not found to significantly ($F_2 = 3.1006$; $P < 0.1189$) affect the minimum ADH required for egg hatch. A significant ($F_3 = 16.7901$; $P < 0.0001$) interaction between temperature and diet for predicting the minimum ADH required to complete larval development prior to appearance of prepupae was determined. Overall, larvae reared on a grain-based diet were observed to require the fewest ADH to reach the prepupal stage following eclosion while those reared on pork loin muscle required the most ADH to complete larval development. Similarly, a significant ($F_3 = 4.0835$; $P = 0.0281$) interaction between temperature and diet for predicting minimum ADH required to complete the prepupal stage was determined; however, no clear trend was observed. Temperature and diet had no significant ($F_3 = 1.7519$; $P = 0.2024$) effect on minimum ADH required to complete pupal development.

Final mean larval length and weight over different treatments are presented in Table 2. A significant (length; $F_4 = 5.2156$; $P = 0.0011$; weight; $F_4 = 6.0749$; $P = 0.0004$) interaction between temperature and diet for predicting both final mean larval length and weight was determined. Overall, larvae reared at 32.2 °C and 27.6 °C were ≈30% heavier than those reared at 24.9 °C, but only ≈5% greater in length.

Changes in larval length and weight across treatments from egg hatch until the first observation of prepupae are presented in Figs. 1 and 2, respectively. Larvae reared on a grain-based diet and at the highest temperature (32.2 °C) developed into prepupae in the fewest degree days (152 DD), while those reared on pork and at the same temperature required the most degree days to develop into prepupae (556 DD). Development of larvae reared on beef was consistently faster than those reared on pork. Two of three technical replicates of 32.2 °C pork were lost at time points of 152 DD and 394 DD. A technical replicate of 27.6 °C pork was lost at 370 DD and a technical replicate of 24.9 °C pork was lost at 435 DD. At each of these time points, sample size was reduced by three for the respective temperatures.

Table 2
Mean final larval length (mm) and weight (mg) of *Hermetia illucens* ($N = 9$) prior to prepupal observations when reared on diets of grain, beef and pork at three temperatures at 55% RH.

Temperature (°C)	Diet	Length (mm) ± SD	Weight (mg) ± SD
32.2	Grain	16.53 ± 0.49E[a]	126.5 ± 13.8B
	Beef	19.36 ± 1.80AB	187.7 ± 37.9A
	Pork[b]	20.28 ± 1.49A	183.9 ± 43.2A
27.6	Grain	18.78 ± 1.24ABC	178.4 ± 25.9A
	Beef	19.35 ± 1.75AB	182.2 ± 34.6A
	Pork[c]	17.30 ± 1.19CDE	134.5 ± 22.7B
24.9	Grain	16.79 ± 0.60DE	109.1 ± 7.8B
	Beef	18.21 ± 1.59BC	134.7 ± 29.6B
	Pork[c]	18.15 ± 0.97BCD	138.8 ± 18.8B

[a] Different capital letters within a column indicate significant difference ($P < 0.05$).
[b] $N = 3$.
[c] $N = 6$.

3.2. Field validation

Age estimates of field-reared larvae based on laboratory length and weight data are presented in Table 3. Individual larval weights were unable to be measured at the first sampling point (ADH = 2916) due to low equipment sensitivity and were therefore deemed as inaccurate estimates. Grain-diet data were unable to be used to accurately predict the true age of larvae at any of the sampling points. Estimates based on pork-diet data encompassed the true ADH more frequently than estimates based on beef-diet data (≈55.6% and ≈38.9%, respectively). Excluding estimates based on grain-diet data, larval length data provided fewer estimates that encompassed the true ADH than larval weight data (≈38.9% and ≈55.6%, respectively). For both length and weight data, estimates were generally more accurate later in development, although estimate ranges were also larger later in development. When laboratory based larval length and weight data were utilised in conjunction, the actual ADH were able to be accurately predicted for ≈55.6% of sampling points when using beef-diet data and ≈88.9% of sampling points using pork-diet data. A single container of field reared specimens was observed to have prepupae at 16,946 ADH.

4. Discussion

In this preliminary study, significant differences were observed between the minimum ADH required to complete

Table 1
Minimum accumulated degree hours (ADH) (LDT = 12 °C) required by *Hermetia illucens* ($N = 3$) to complete each stage of development when reared on diets of grain, beef and pork at three temperatures at 55% RH.

Temperature (°C)	Diet	Oviposition to hatch ADH ± SD[b]	Hatch to prepupa ADH ± SD	Prepupa to pupa ADH ± SD	Pupa to adult ADH ± SD
32.2	Grain	1238.9 ± 19.0A[a]	4969.2 ± 228.5C	2747.2 ± 228.5A	2747.2 ± 228.5A
	Beef		12,423.0 ± 0.0A	2424.0 ± 395.8A	3393.6 ± 685.6A
	Pork		–	–	–
27.6	Grain	1248.0 ± 25.5A	5928.0 ± 0.0C	1872.0 ± 0.0AB	2870.4 ± 176.5A
	Beef		10,046.4 ± 0.0B	1747.2 ± 353.0AB	2745.6 ± 353.0A
	Pork		12,480.0 ± 1310.4A[c]	1123.2 ± 374.4B[c]	3369.6 ± 0.0A[c]
24.9	Grain	1290.0 ± 21.1A	5237.4 ± 0.0C	1032.0 ± 145.9B	3715.2 ± 0.0A
	Beef		8952.6 ± 252.8B	1135.2 ± 386.1B	2373.6 ±1437.4A
	Pork		12,822.5 ± 154.8A[c]	1857.6 ± 309.6AB[c]	3250.8 ± 154.8A[c]

Dash (–) indicates omitted data as $N = 1$.
[a] Different capital letters within a column indicate significant difference ($P < 0.05$).
[b] ADH until hatch was not measured across different diets.
[c] $N = 2$.

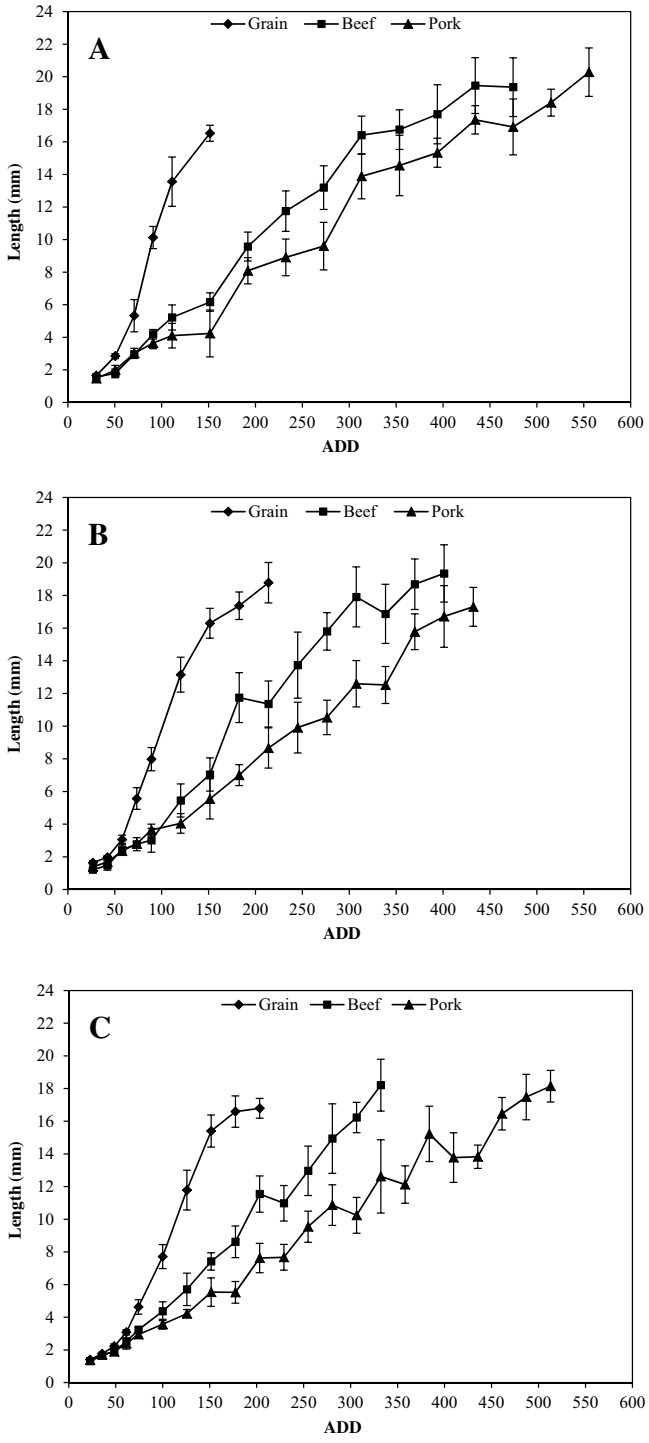

Fig. 1. Larval length (mm) ± SD of *Hermetia illucens* ($N = 9$) developing at three temperatures (A: 32.2 ± 0.5 °C; B: 27.6 ± 0.4 °C; C: 24.9 ± 0.4 °C) at 55% RH over accumulated degree days (ADD) from egg hatch when reared on diets of grain, beef and pork.

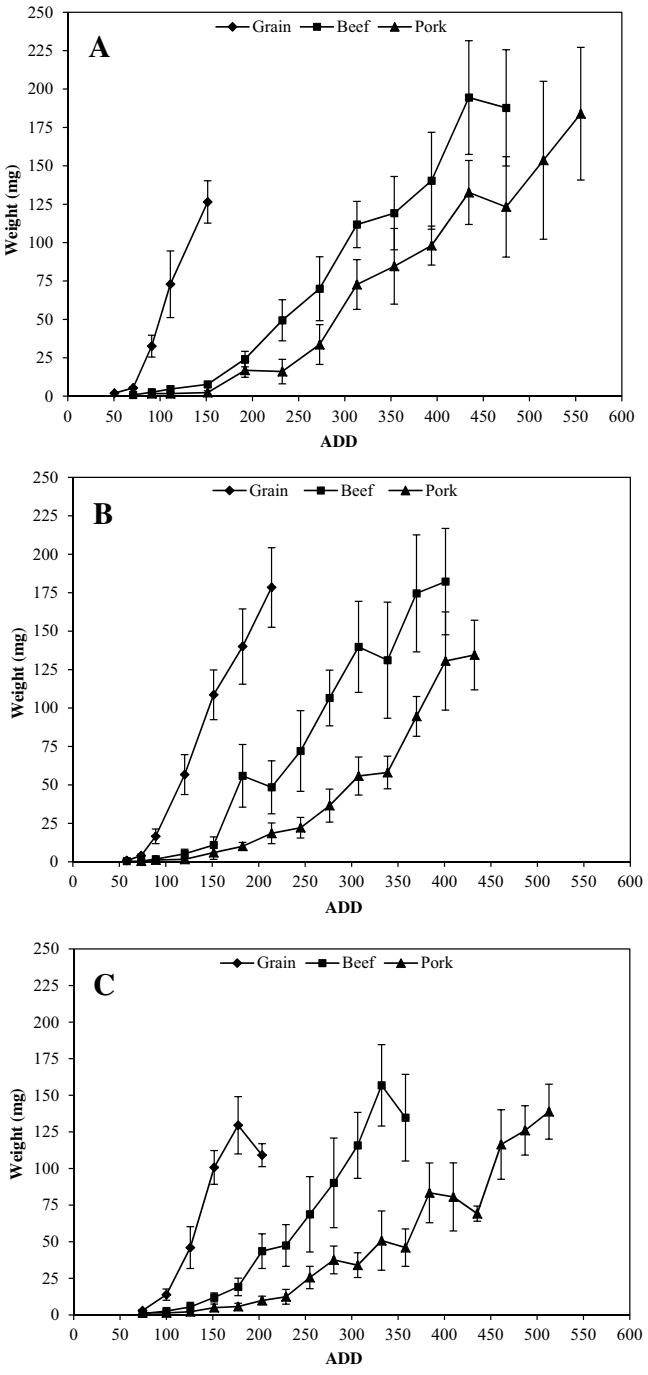

Fig. 2. Larval weight (mg) ± SD of *Hermetia illucens* ($N = 9$) developing at three temperatures (A: 32.2 ± 0.5 °C; B: 27.6 ± 0.4 °C; C: 24.9 ± 0.4 °C) at 55% RH over accumulated degree days (ADD) from egg hatch when reared on diets of grain, beef and pork.

larval development on each diet within each temperature, though larvae reared on pork at 32.2 °C were not able to be included in the analysis. Overall, grain-reared larvae required the fewest ADH at each temperature to complete larval development, while those reared on pork required the greatest ADH.

Several factors are likely responsible for the differential development observed between diets. The most influential factor was likely the nutritional content difference of animal and plant based diets, which would support why larvae reared on pork and beef tissue had ADH values more similar to each other than to those reared on a grain-based diet. While it was also evident from visual observations that diet texture varied between the grain and meat diets, there is no consensus on the effect of diet consistency on larval growth. A study by Clark et al. [16] reported no difference in growth rates of *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) as a consequence of different food structures, while Niederegger et al. [17] observed faster growth rates of *Calliphora vicina* Robineau-Desvoidy and *Calliphora vomitoria* (L.) (Diptera: Calliphoridae) when reared on minced meat compared to unprocessed tissue.

Table 3
Minimum to maximum larval age percent estimates (ADH) of field-reared *Hermetia illucens* ($N = 20$) based on laboratory larval length and weight data of *H. illucens* reared at 27.6 °C and 55% RH on each diet.

Actual ADH in field	Grain		Beef		Pork	
	Length	Weight	Length	Weight	Length	Weight
2915.7	66.0–74.7	–	71.3–87.9	–	75.0–97.4	–
4140.6	57.4–70.4	69.4–71.2	71.0–95.9	84.7–88.5	80.9–114.6[b]	105.9–111.6
5208.5	64.9–84.2	58.3–69.4	93.4–130.3[b]	73.8–97.2	114.4–164.2	94.0–129.8[b]
6391.0	69.1–88.5	53.4–82.4	107.2–144.3	72.5–133.6[b]	135.2–185.4	95.5–188.9[b]
7716.7	61.0–79.6	49.5–78.7	95.9–131.5[b]	71.3–132.7[b]	121.6–169.8	96.3–190.2[b]
9090.6	61.2–75.0	44.1–67.2	99.5–125.9[b]	64.8–113.5[b]	127.6–163.4	88.2–162.6[b]
10,381.2[a]	46.4–54.1	38.3–54.7	73.3–88.1	56.1–90.5	93.1–113.1[b]	76.3–128.9[b]
11,536.0	47.6–58.3	47.4–74.4	77.2–97.6	77.7–134.6[b]	99.0–126.6[b]	110.3–197.3
12,848.9	42.0–48.1	38.3–52.3	67.9–79.5	60.9–90.2	86.9–102.6[b]	85.5–130.3[b]

Dash (–) indicates equipment not sensitive enough to provide larval weight measurements.
[a] $N = 8$.
[b] Estimate range encompasses the actual ADH.

Statistical analyses also revealed significant differences between treatments during the prepupal stage, though there was no clear overall trend in the minimum ADH required to complete this stage. A possible reason for this is due to varying densities of prepupae following their transfer from the main feeding containers to substrate-free containers. A study by Holmes et al. [34] showed that black soldier fly prepupae developed into pupae faster when provided with a pupation substrate. In high density containers, other prepupae may have provided a pseudo-substrate in which they could bury themselves, reducing time to pupate. Conversely, frequent disturbance by the movement of other prepupae may have inhibited their ability to develop in pupae.

As suggested in previous studies, the vertebrate species selected as a diet source can have significant effects on insect immature development. Clark et al. [16] compared the immature development of *L. sericata* when reared on a range of tissues from beef and pork. They observed that larvae of *L. sericata* reared on pork tissue had more rapid developmental times and were of greater final lengths than their beef-reared counterparts. However, in the present study, developmental times were significantly faster for beef-reared larvae of *H. illucens* compared to pork-reared larvae, while larvae only differed significantly in size when reared at 27.6 °C where beef-reared larvae were longer and heavier than those reared on pork. This shows that caution should be taken when applying assumptions across species.

A number of previous studies have also suggested that the tissue type selected as a diet substrate is also a significant factor in larval growth, with liver resulting in the slowest development compared to other body tissues [14–17]. This cannot be confirmed for *H. illucens* as larvae in the present study were reared on only a single type of tissue. Additionally, research by Nguyen et al. [37] is the only previous study to observe developmental times of *H. illucens* when reared on animal tissue. Nguyen et al. [37] reared larvae on six different waste diets, which included two animal based diets of pork liver and fish rendering, at a constant temperature of 28 °C. The minimum time taken for *H. illucens* to reach the prepupal stage when reared on either pork liver or fish renderings was nearly identical to the developmental time in the present study of larvae reared on a grain-based diet at a similar temperature. This suggests that the development of *H. illucens* is also affected by different tissue types sourced from the same animal species, with development on liver surpassing that of loin muscle. It also suggests that prior data published on black soldier fly development on grain-based diets [26,32] may be acceptable for use in a crime scene, should liver be determined as the most representative tissue consumed by black soldier fly larvae on a human body. However, caution should be taken when comparing the results of each study due to a number of differences in the experimental methodology, most notably, the age at which larvae were subjected to different treatments. In the present study, newly-eclosed larvae were transferred directly to their respective treatments while Nguyen et al. [37] reared larvae for 4 days (≈20% of larval developmental time) on control poultry feed before transferral to other diets. Therefore, further tissue related studies would be needed before firm conclusions could be made.

The field validation component of this study gave an indication of the accuracy of the laboratory produced data when applied to larvae reared in naturally occurring temperatures. When using larval length and weight in conjunction, ranges produced by minimum and maximum larval measurements encompassed the true larval age for 55.6% and 88.9% of observations for beef and pork diets respectively, while grain-diet data were unable to be used to accurately predict the true ADH at any sampling point. When converted into days, however, the estimate ranges spanned several days, with an average range of accurate estimates of ≈10.0 d for both the beef and pork diets. Considering the late colonisation of bodies by *H. illucens* and the ≈10.0 d range associated with this [27], narrower accurate larval age estimates are therefore particularly preferred for this species.

Inaccurate and wide estimate ranges observed in the validation component could be due to several reasons. Firstly, more than half of all samples contained larvae that exceeded the maximum length and a third of the samples contained larvae that exceeded the maximum weight observed under laboratory conditions, despite originating from the same colony. It is also possible that the accuracy and precision of estimates were affected by the preservation of field-reared samples in 70% ethanol, which laboratory reared specimens were not subjected to, and the variation in the duration of preservation of these samples. Research by Adams and Hall [45] showed that larvae preserved in 80% ethanol had lengths that varied depending on the duration of preservation, potentially deviating from the pre-preservation length. This could therefore explain why length based estimates were less accurate than weight based estimates. Other factors that may have influenced larval age estimates include the loss of a pork replicate at 27.6 °C, which would have hindered the observed variation, while the use of constant rather than fluctuating temperatures may have altered larval development. Another factor that should be taken into account is the differences in life history traits as a result of genetic variance that can exist between different populations [47,48]. While in the present study laboratory and field-reared larvae were sourced from the same colony, in a practical setting wild populations may develop at different rates to laboratory reference colonies.

One of the most evident limitations in the current study was the inability to easily distinguish larval instar. Unlike other species of

fly, the morphological changes of *H. illucens* as it progresses through the six larval instars are not well documented [38,39]. In this study, only the final prepupal instar was able to be accurately identified. Further research into these morphological changes would be beneficial for two key reasons. Firstly, in the instance of improper specimen preservation at a crime scene, larval length and potentially larval weight are unable to be accurately measured. Therefore estimates based on larval instar are the preferred method. Secondly, the results of this study show that larval lengths and weights can vary considerably despite being of roughly the same age. Measurement of instar would account for overlaps in larval size between instars as well as likely providing more accurate estimates in the circumstance that field data exceeds the size of laboratory data, as was experienced in this study. The measurement of age based on instar is also not likely to be limited by equipment sensitivity, as is the case for larval weight measurements.

Due to time constraints the experiment was unable to be repeated with rotation of incubator temperatures. Consequently, effects due to unknown differences between incubators are unable to be eliminated as a possible cause of differential development in this study. Several other aspects of methodology could be improved on in future, including the individual monitoring of prepupal and pupal stage duration, validation of these stages, and the provision of a pupation substrate. It should also be noted that larvae were periodically provided with fresh muscle tissue; however, due to the typically late colonisation of this species, the tissue available on a corpse at this time would be substantially decomposed. Additionally, it is possible all muscle tissue has been consumed by other species prior to colonisation by *H. illucens*. Therefore, data collected from human cadavers as a means of determining the most representative diet, in terms of tissue type and freshness, for this species would be valuable. Furthermore, infrequent sampling and small sample sizes are notable limitations of the experimental methodology in this study. An increase in larval density within rearing containers would allow for an increase in sampling resolution and therefore would provide more informative growth rates throughout larval development. Increased density would also allow for increased sample size, providing a more accurate representation of the sample population's weight and length at each observation. As shown in a study by Richards and Villet [46], both of these factors can subsequently affect the accuracy of minPMI estimates, and therefore it is recommended that future studies include larger sample sizes as well as more frequent sampling.

This study is only the second to record changes in larval length and weight of *H. illucens* during development [37], as well as the first to rear larvae of this species on beef and pork muscle tissue. Additionally, this study is among very few studies in forensic entomology to assess the accuracy of laboratory produced data through comparison to field-based development. Overall, the results of this study suggest that both temperature and diet significantly influence the development of *H. illucens* during the egg, larval and prepupal life stages. Due to variation between the present and previous studies, firm conclusions cannot be made about the effects of tissue type on black soldier fly development nor whether previously published data are able to be used accurately in a forensic setting. Limitations caused by larger field-reared specimens and a lack of instar distinction resulted in either large estimation ranges or low accuracy of estimates. The results of this study further highlight the necessity of considering larval diet both when determining developmental rates and when implementing the data in a forensic context. Additionally, it demonstrates the importance of recording multiple aspects of larval growth and the validation of these data in order to provide reliable minPMI estimates in the field.

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