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Published in:
Journal of Applied Entomology

DOI:
10.1111/jen.12653

Publication date:
2019

Document Version
Peer reviewed version

Citation for published version (APA):
Gligorescu, A., Toft, S., Hauggaard-Nielsen, H., Axelsen, J. A., & Nielsen, S. A. (2019). Development, growth and metabolic rate of *Hermetia illucens* larvae. *Journal of Applied Entomology*, *143*(8), 875-881. [143]. https://doi.org/10.1111/jen.12653

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ORIGINAL CONTRIBUTION

Development, growth and metabolic rate of *Hermetia illucens* larvae

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1. INTRODUCTION

The human population is expected to reach 9.1 billion by the year 2050, and at the same time, animal production is expected to rise, leading to an increasing requirement for protein-rich fodder sources in the animal production sector (FAO, 2009; Speedy, 2004). Denmark is an important factor in the animal production sector in the EU with a total share of 7.2% of the estimated 22 Mt of pork meat production in the EU in 2013 (Eurostat, 2016). The trend of an increasing human population with an associated increase in animal and meat production will subsequently lead to an increasing need for protein sources in order to secure animal feed (Speedy, 2004). Insect bioconversion of organic waste streams could provide a solution to these challenges by successfully

recirculating viable nutrients and energy that would otherwise be wasted, while providing a more sustainable protein source and thus contributing to food security (van Huis et al., 2013). Thus, insect bioconversion can improve the environmental footprint of vertebrate meats indirectly, through their use as feed. Rearing insects on human inedible wastes and feeding them to larger food animals can boost the protein content of many of these animals and is more environmentally friendly and efficient than growing fields of grains or other feeds, which use land and resources that could otherwise be used to grow food for humans. Among the most promising insect, species to be used as protein source for animal feed is *Hermetia illucens* (Linnaeus, 1758) (Diptera: Stratiomyidae), also known as the black soldier fly (Barragan-Fonseca, Dicke, & vanLoon, 2017).

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Hermetia illucens is an American native insect but now has a worldwide distribution, occupying habitats between 45°N and 40°S. In Europe, the species was first recorded in Malta in 1926 and it currently inhabits mainly the Mediterranean area (Marshall, Woodley, & Hauser, 2015). However, it has been found as far north as the Czech Republic (Roháček & Hora, 2013). The species has three generations per year in subtropical regions and one generation per year in temperate regions (Benelli, Canale, Raspi, & Fornaciari, 2014; Sheppard, Newton, Thompson, & Savage, 1994). The adult flies only serve reproductive purposes and live about 8–20 days depending on temperature, sex, larval food quality and larval development time (Gobbi, Martinez-Sanchez, & Rojo, 2013; Tomberlin, Adler, & Myers, 2009; Tomberlin, Sheppard, & Joyce, 2002). Mating occurs two days after metamorphosis, and the females oviposit about 500 eggs throughout their lifetime at the edge of decomposing substrates (Diclaro & Kaufman, 2009). *H. illucens* larvae live in organic debris and have a short development time of about 20 days at 27°C before migrating into dry areas such as ground vegetation, where they pupate for about 18 days (Diclaro & Kaufman, 2009; Tomberlin et al., 2009).

The larvae of *H. illucens* are scavengers able to feed on a wide range of organic substrates, including municipal organic waste, manure, food waste and human faeces, making them highly suitable for waste management (Diener, Zurbrugg, & Tockner, 2009; Lalander, Fidjeland, Diener, Eriksson, & Vinnerås, 2015; Newton, Sheppard, Watson, Burtle, & Dove, 2005; Nguyen, Tomberlin, & Van Laerhoven, 2013). Furthermore, the larvae of *H. illucens* contain highly valuable proteins and lipids, suitable to be used as feedstuff in aquaculture, poultry and in the swine industry (Magalhães et al., 2017; Cummins et al., 2017; St-Hilaire et al., 2007; Hale, 1973 cited by Newton et al., 2005; Newton, Booram, Barker, & Hale, 1977).

Past studies mainly focused on the ability of using *H. illucens* larvae for organic waste management or as feedstuff, whereas only few scientific studies describe development and growth of the larvae (Kim et al., 2010; May, 1961; Oliveira, Doelle, & Smith, 2016; Pujol-Luz, Pitaluga De Godoi, & Barros-Cordeiro, 2016;

Schremmer, 1986). Disturbingly, different numbers of larval stadia are reported in these papers. Thus, May (1961) and Kim et al. (2010) claim there are 6 larval stadia while Schremmer (1986) reports seven larval stadia. Furthermore, no literature was found to deal with the metabolic rate of different *H. illucens* larval instars.

Investigation of development, growth and metabolism of different larval instars could lead to a better understanding of larval performance and could further be used to optimize *H. illucens* bioconversion systems that aim to convert organic waste streams into larval biomass. In this work, some important parameters for understanding the life cycle, growth and metabolism were investigated. An important goal was to definitively determine the number of larval stadia through measurements of head capsules and microscopic observation of a moult between the 6th and the 7th larval stadia.

2. MATERIAL AND METHODS

2.1. Insect rearing and abiotic factors recorded

The stock of black soldier flies was provided by Dansk Insekt Protein ApS, Skjern, Denmark, and a colony was established at Roskilde University. The adult black soldier flies were bred in a wooden frame cage (100 × 63 × 110 cm) covered with mosquito net and maintained in a climate room at Roskilde University on a 14-:10-hr light/dark regime at 26 ± 1°C and 27% relative humidity. The adult flies were provided with water and sugar during their entire life, and 2 cm diameter rolls of corrugated cardboard were placed in the cage as oviposition substrate. The cardboard rolls were checked and replaced daily in order to ensure that all eggs were oviposited within 24-hr intervals. This secured a relatively synchronized development of the larvae during the experiment. Eggs from the same corrugated cardboard were transferred to a transparent plastic box (11 × 11 × 6 cm) containing moist wadding. The plastic box was placed in front of a LED panel used as light source for the colony until egg eclosion was observed about four days later. About 500 neonate larvae were transferred to a plastic box (17 × 14 × 11 cm) covered with a plastic lid; holes of one mm were made in the lid in order to ensure air circulation. The larvae were reared on 100 g pulverized Gainesville medium (50% wheat bran, 30% alfalfa hay and 20% maize mixed with water at a ratio of 10 g medium to 17 ml water (70% moisture) according to Tomberlin et al. (2002)). New freshly made medium was added to the plastic box every other day, until more than 50% of the larvae had reached the non-feeding (pre-pupal) stadium, distinguished by its dark brown colour, about 17 days later. This feeding strategy was adapted in order to minimize the handling effect on larval development (Nguyen et al., 2013). We consider that the daily addition of fresh medium and the daily removal of larvae as they grew larger would minimize the effects of scramble competition on larval growth.

The temperatures at the surface of the medium in the larval rearing box (mean 26.4 ± 0.7°C) were recorded every 5 min using a Hobo® U23 Pro v2 data logger (Onset® Computer Corporation).

2.2. **Larval measurements**

Once egg eclosion had occurred, 10 larvae were randomly sampled daily for 18 days. The larvae were weighed and preserved in 70% ethanol for 24 hr before the width of their head capsule (mm) was measured. As in May (1960) and Kim et al. (2010), the first six larval instars were distinguished based on the head capsule measurements. From previous studies of larval development in the black soldier fly (May, 1960; Kim et al., 2010; Schremmer, 1986), it is assumed that a change in head capsule width indicates a moult and that all early moults (i.e., prior to the moult between the 6th and 7th stadia; see below) lead to an increase in head capsule width. The larvae collected during the first 4 days of the experiment were weighed using a four decimals after milligram electronic scale (Mettler Toledo XP6; Mettler-Toledo A/S), and hereafter, the larvae were weighed using the four decimals after grams electronic scale (Mettler AE 163). Furthermore, the head capsule measurements of the larvae collected during the first three experimental days were measured using the Cell D 3.4 software connected to a compound microscope (Olympus CX41), and hereafter, the head capsules of elder larvae were measured by Cell D 3.4 software connected to a stereo microscope (Olympus SZ61). In addition to the daily head capsule measurements used to identify the actively feeding instars, ten 6th instars that showed signs of beginning ecdysis were placed in 70% ethanol for 24 hr. Based on these, the possible presence of a 7th instar larva inside the exuvia of the 6th instar larva, as claimed by Schremmer (1986), was observed under a stereomicroscope. Furthermore, the differences in morphological characteristics of the head capsules between the 6th and 7th instar larvae were assessed. The head capsules of 6th and 7th instar larvae and the ecdysis situation between the two stadia were inspected in a drop of Euparal (Carl RothGmbH+ Co) and photographed using Cell D 3.4 software connected to a stereo microscope (Olympus SZ61) equipped with a SC30 camera.

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2.3. **Specific metabolic rate measurements**

The investigations of the metabolic rate consisted of measurements of the heat release from *H. illucens* larvae under isothermal conditions at 27°C by using the calorimeter DSC-7707 (Hart Scientific, Pleasant Grove, UT; Nielsen, Jensen, Kristensen, & Westh, 2006). Due to the small size of first and second instars, the metabolic rate of these was not measured. Thus, the specific metabolic rates of 3rd, 4th and 5th instars (n = 9 of each), 6th instars (n = 4) and the 7th instars (n = 9) were measured.

The calorimeter had four removable steel ampoules (1 cm^3) which were sealed with a threaded lid and closed with a gasket prior to the heat release measurements. One of the four ampoules was kept empty and used as a thermal reference, while the other three were employed in individual measurements of larval heat release. At the start of each experiment day, a blind measurement was performed on each of the three ampoules (H1, H2 and H3). After that the heat release data from these ampoules were plotted in a graph and visually checked.

This was done in order to identify the data points at which thermodynamic equilibrium was achieved. Based on these data points, an average value of the blind measurements (AVG blind in µW) was calculated for each ampoule.

The larvae belonging to different stadia were sampled from the medium and transferred gently and quickly into individual ampoules. These were hermetically closed and transferred to the calorimeter. The heat release was recorded every 30 s on all three ampoule samples for 1 hr. After this step, the data were plotted into a graph and visually inspected for reaching a plateau (thermal equilibrium); an average value of the heat release at the plateau for the individual larva (AVG heat in µW) was subsequently calculated. Furthermore, the tested larvae were weighed and their wet weight (WW in mg) was noted. After this, the AVG blind was subtracted from the calculated AVG heat, multiplied with −1 and divided by the WW of each individual larva, in order to estimate the specific metabolic rate (SMR in µW/mg), according to the Equation 1.

1

$$\mathrm{SMR} = \frac{(\mathrm{AVG\,heat} - \mathrm{AVG\,blind}) * -1}{\mathrm{WW}}$$

2.4. **Statistics**

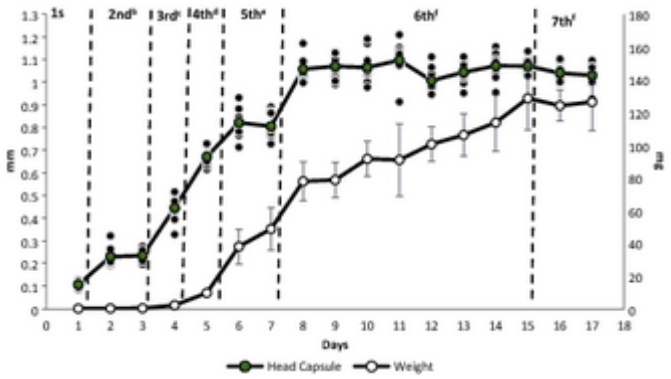
The statistics were performed using the IBM SPSS software ver. 24 (IBM Corp). The daily head capsule data as well as the SMR and the body mass of different instars were tested using a general linear model (GLM) for statistical significance (α = 0.05), followed by Tukey's post hoc test for multiple comparisons. The data on SMR and body mass of different instars were square root transformed in order to ensure homogeneity of variance (Levene's test).

3. **RESULTS**

The controlled age of eggs used to start the larval culture secured a synchronized development of the larvae. With very few exceptions, all 10 larvae collected on the same day clearly were the same instar according to the head capsule measurements (Figure 1). Thus, one individual on day 2 and one on day 4 could be either instar 2 or instar 3; also, one individual on each of days 5, 6 and 7 could be either instar 4 or instar 5. Overall, the head capsule widths were found to increase stepwise, showing that *H. illucens* larvae pass through six stadia with significantly different head capsule widths (F_{16} = 480.1, $p < 0.001$; Figure 1). The larvae fed actively during these first six stadia. After day 15, the larvae turned darker brown in colour, stopped eating and started to migrate out of the substrate (“Wanderlarven” of Schremmer, 1986). The head capsule measurements indicated no difference from the actively feeding 6th instar. Microscopic examination, however, revealed that a moult had occurred (Figure 2). Thus, the dark wandering larvae from the last two days of our experiment

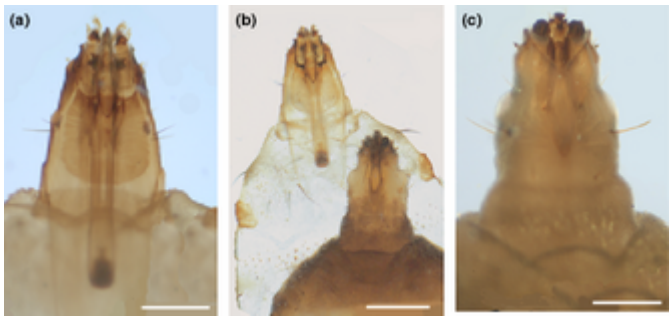
(days 16–17) were separate 7th instar larvae. Although no significant difference was seen between the head capsule widths of the 6th instar and the 7th instar (Figure 1), their head capsules were found to have different morphological characteristics (Figure 2). The 6th instar larvae have functional mouthparts, while the 7th instar larvae have reduced mouthparts. The 7th instar also has a different form of the head, the eyes are more prominent, and the top of the head is bent down. It is used during forward movement as a hook to fix the head into the substrate, when the larva is moving forward by body contraction. The last instar larvae are wandering about for some time to find an acceptable place for pupation; soon they become immovable and the skin hardens to form a puparium. This larval development in *H. illucens* was previously described by Schremmer (1986).

Fig. 1



Growth in head capsule width (mm) and mass (mg) of *Hermetia illucens* larvae measured daily (mean ± *SD*). Larval instars 1–6 are delimited by vertical dotted lines based on statistical analysis of head capsule widths (indicated by different superscript letters in upper horizontal row). The 7th larval instar was separated based on observation of a moult (Figure 2)

Fig. 2



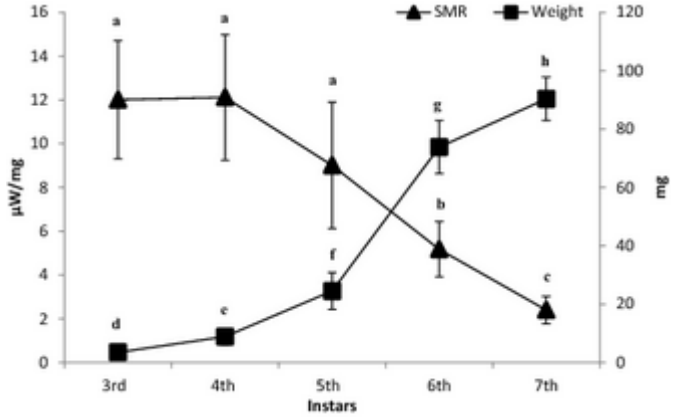
(a) Anterior end of the 6th larval instar of *Hermetia illucens* larva (Scale: 0.5 mm). (b) Ecdysis process observed between the 6th and 7th instars where the 7th instar is seen inside the exuvia of the 6th larval instar (Scale: 1 mm). (c) Anterior end of the 7th larval instar (Scale: 0.5 mm)

The larvae showed very fast development during most of the actively feeding stadia until they became 6th instars (Figure 1). Thus, the larvae hatching from

the eggs (first instar) became second instar larvae in less than 24 hr. They stayed in the second stadium for two days. From this stage, an even faster development occurred, as the third and fourth stadia lasted only one day each. After that the larvae remained in the fifth stadium for two days. During the 6th stadium, they then spent eight days as actively feeding larvae before moulting into the 7th stadium. The growth in larval body mass followed an S-shaped curve with accelerating growth during stadium 1–5 and decelerating growth during stadium 5–6 (F_4 = 418.8, $p < 0.001$). The lack of feeding by the 7th instar larvae is witnessed by a constant body mass since the end of the 6th stadium (Figure 1).

The SMR was similar across the earlier larval instars (3rd, 4th and 5th) but significantly reduced by the 6th and even more so by the 7th instar larvae (F_4 = 39.2, $p < 0.001$; Figure 3).

Fig. 3



Specific metabolic rate (SMR) and body mass of the *Hermetia illucens* larvae used for metabolic measurements (mean ± *SD*). Points with the same letter are not significantly different (GLM test, $\alpha < 0.05$, followed by Tukey's post hoc test for multiple comparisons). The increase in body mass between the 6th actively feeding instar and the 7th instar in Figure 3 is because the 6th instar larvae were measured just after they entered this instar, cf. Figure 1

4. **DISCUSSION**

The results on head capsule widths (Figure 1) and of the microscopic examinations (Figure 2) show that *H. illucens* passes through six actively feeding stadia before entering the 7th non-feeding stadium. Our results thus confirm those of Schremmer (1986) who found that the last actively feeding larval instar (the 6th instar) and the 7th instar have different morphological characteristics and are separated by a moult, thus making them two different larval instars. Previously, May (1961) claimed that *H. illucens* larvae pass through only five actively feeding stadia before entering the non-feeding "pre-pupal" 6th stadium. However, comparison of May's head capsule measurements (May, 1961 table 1) with ours (Figure 1) indicates that she may have missed the first instar, which is very small and of short duration; the exuviae may be easily overlooked. Like Schremmer (1986), May (1961) also reports the occurrence of

a moult between her 5th instar and the “pre-pupal” instar. Also, the results of Kim et al. (2010) differ from ours, as they claim that *H. illucens* larvae have six stadia before pupation. Their measurements of actively feeding instars 1–6 are similar to ours, but there is no mention of the non-feeding migratory larval instar. However, the “pupa” measured by Kim et al. (2010 table 1) must be this instar, that is identical with our 7th instar and with May's pre-pupa. In fact, it is the exuvia of the pre-pupa that forms the puparium in *H. illucens* (see May, 1961 figs 6–7). Thus, the results of all four studies may converge on the agreement that *H. illucens* has seven larval stadia with six actively feeding and growing instars and one non-feeding migratory instar that pupates within its exuvia.

Contrary to the fast development seen in the present paper (17 days for full larval development), Kim et al. (2010) found that *H. illucens* experienced a considerably slower development (28 days) despite being maintained at similar temperatures (27°C in Kim et al., 2010). Furthermore, the body masses of larval instars (3rd = 3.9, 4th = 22.1, 5th = 66.3 and 6th = 185.3 mg/larva) obtained by Kim et al. (2010) were higher than ours. Such differences may be explained by the different methods and dietary regimes used in the two studies as well as due to different handling. Thus, in the present investigation, the larvae were sampled daily, while May (1961) and Kim et al. (2010) sampled exuviae after each moulting episode. Harnden and Tomberlin (2016) found that different diets (Pork, Beef and Gainesville) affect the development and the mass of different larval instars. However, it is not fully understood to what extent different handling procedures and diet qualities impact the development and body mass of *H. illucens* larvae; this could therefore represent an important topic for further research.

The body mass of the larvae was found to follow a sigmoid curve with accelerating growth in the first five instars and decelerating growth in the sixth instar. Scriber and Slansky (1981) describes that in general, the greatest overall growth and food consumption usually take place in the penultimate and final larval stadia. This pattern is believed to be related to differences in energy allocation and metabolism during the earlier and the later stadia. In the earlier stadia, the larvae are mainly focusing on rapid development and primarily allocate energy to catabolism, which leads to high metabolic rates. In *H. illucens*, however, after reaching a certain size (instar 4, Figure 1), the metabolic rate decreases (Figure 3), as the larvae increasingly focus on building body mass and storing the energy reserves (anabolism) required for metamorphosis and for reproduction in the adult life stage (Schmolz & Lamprecht, 2000). The results obtained from the calorimetric measurements of *H. illucens* larvae support these arguments. Thus, the SMR of *H. illucens* larvae decreased with increasing mass and development stage (Figure 3). Acar, Mill, Smith, Hansen, and Booth (2004) found that the specific metabolic rate of lady beetles (*Harmonia axyridis* (Pallas, 1773)) decreased with increasing larval development stage, and Schmolz, Kösece, and Lamprecht (2005) obtained similar results for European honeybee drones (*Apis mellifera* Linnaeus, 1758).

As a step towards implementing *H. illucens* in bioconversion systems, it would be an advantage to predict the waste conversion efficiency depending on waste composition and conversion efficiencies under various temperature regimes. Such predictions can be made by aid of simulation modelling. Simulation modelling is no magic tool, but requires a detailed knowledge of essential biological processes such as the temperature-dependent larval growth and larval waste conversion efficiency. A full understanding of these processes under a wide range of environmental circumstances will provide the knowledge necessary to develop simulation models of their population development and of the amount of waste they consume. A metabolic pool model (Gutierrez, 1996) seems to be a good choice of model type for this purpose, as this kind of model is ideal for simulating predator–prey interactions, and it can be programmed to take different temperature regimes into account through the utilization of a physiological time scale as defined by the degree-day concept or the Gauss equation (Taylor, 1981). In the case of black soldier flies, the predator will be the soldier fly and the prey will be the waste or different types of waste. The results concerning the growth of *H. illucens* larvae are challenging for a detailed modelling of their growth, and in turn for their conversion efficiency of waste, as they show a rather constant growth measured in grams per day, but a decreasing relative growth rate (measured in g/g/day) in 5th and especially 6th instar. Most often models use a specific growth rate in units of g/g/time for the entire juvenile stage (Gutierrez, 1996) or at least for each instar (Axelsen, 2009). Looking at the growth curve of 5th and 6th instars, it is clear that the mass-dependent growth rate is decreasing as the growth is not exponential. Therefore, the results from this investigation will make it necessary to make the growth rate mass or age specific, which will be new to most population dynamical model types.

5. **CONCLUSION**

The results presented here confirms that *H. illucens* larvae pass through six actively feeding stadia followed by a separate migratory 7th stadium (pre-pupa) as previously described by Schremmer (1986). The first six instars can be easily identified using head capsule width measurements while the 7th instar can be distinguished from the 6th instar by differences in morphological characteristics as well as colour and behaviour. Furthermore, larval metabolism decreased as a function of increasing larval body mass and larval instar number. The results regarding larval growth are important for population dynamical modelling, as the growth rates of the actively feeding 5th and 6th instars are not constant.

6. **ACKNOWLEDGEMENTS**

We thank two anonymous reviewers for valuable comments.

7. **AUTHOR CONTRIBUTION**

Author AG, ST and SAN conceived and designed research. AG, ST and SAN wrote the paper. AG, ST and SAN analysed data and conducted statistical

analysis. JAaA and HHN contributed material. All the authors read and approved the final manuscript.

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