



RESEARCH ARTICLE

Age and calorific restriction impact immature black soldier fly (Diptera: Stratiomyidae) thermal tolerance and preference

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Abstract

Thermal tolerance and preference are traits commonly considered when mass-producing farmed animals as temperature impacts production. In this study, the impact of age and calorific restriction of immature black soldier fly, *Hermetia illucens* (L.) (Diptera: Stratiomyidae) on associated thermal tolerance and preference was examined. Both age (7-d-old for young larvae and 14-d-old for old larvae) and calorific restriction (led to size differentiation, small for calorific restriction and large for non-restriction) within a given stage influenced thermal tolerance (i.e. KR₅₀) and thermal preference. Results indicate the interaction between age and calorific restriction was significant on both larval and prepupal thermal tolerance but not thermal preference. Median heat tolerance KR₅₀ ranged from 46.4 °C (large, old prepupae) to 48.4 °C (large, young larvae). Median cold tolerance KR₅₀ ranged from 21.6 °C (small, young larvae) to 32.1 °C (small, old larvae). Young larvae preferred median temperatures ~3.0 °C greater than old larvae. Large larvae preferred median temperatures ~2.0 °C lower than small larvae. Results from this study indicate ontogeny (i.e. stage of development) and calorific restriction have significant impacts on black soldier fly thermal tolerance and preference. Precise regulation of temperature in an industrial setting is necessary for optimal batch production of the black soldier fly (e.g. survival) and for colony maintenance (e.g. prepupae producing adults and potentially eggs). The same can be said with regards to maintaining consistent age and calorific restriction of immatures produced within each batch as variation in such traits impacts thermal tolerance and preference (e.g. survival to harvest for producing protein or adults for colony) as well. The methods and temperatures used in this study could serve as a foundation for developing standard operating procedures for regulating temperatures experienced by black soldier fly larvae industrially produced.

Keywords

Hermetia illucens – thermal ecology – thermal niche variation

1 Introduction

Organisms, especially holometabolic insects, experience thermal heterogeneity throughout development (Ma *et al.*, 2021) given their distinct morphological and associated ecological differences across life stages. Therefore, thermal tolerance (i.e. the extreme temperatures at which organisms lose certain functions) and thermal preference (i.e. temperatures organisms choose when given choices) may vary tremendously by stage and even by age (Bowler and Terblanche, 2008; Kingsolver *et al.*, 2011). For example, *Drosophila melanogaster* Meigen (Diptera: Drosophilidae), had a cold median lethal temperature (LT₅₀) for old larvae (i.e. 96 h after oviposition) that was ~2 °C greater than for young larvae (i.e. 48 h after oviposition) (Jensen *et al.*, 2007). Similarly, thermal preferences within stage (e.g. late-third-instar vs early-third-instar) *D. melanogaster* differed by ~5 °C (Sokabe *et al.*, 2016). Thermal tolerance and preference are also impacted by body size. For example, the maximum thermal tolerance of the ant, *Atta sexdens* L. (Hymenoptera: Formicidae), increased ~1 °C for every 4 mg of body mass increased (Ribeiro *et al.*, 2012).

Here, we measured the thermal tolerance and thermal preference of immature black soldier fly, *Hermetia illucens* (L.) (Diptera: Stratiomyidae) due to its ecological and agricultural importance. Black soldier fly larvae are able to convert food waste and other organic side-streams to materials of value (e.g. fertilizer) (Diener *et al.*, 2009). Furthermore, the larvae themselves can be used as a feed ingredient for poultry (El-Hack *et al.*, 2020), swine (Biasato *et al.*, 2019), select fish species (Nairuti *et al.*, 2022), and pet food (AAFCO, 2021). Due to its increased economic value over the past two decades, research and industrial production of the black soldier fly has expanded globally. For example, in the 2020 Insects to Feed the World Conference held in Canada, topics specific to black soldier flies represented 43% of all presentations (IFW, 2020).

The black soldier fly is native to the Americas (Kaya *et al.*, 2021; Singh and Kumari, 2019); however, it has spread worldwide from 45°N latitude to 40°S latitude (Sheppard *et al.*, 1994). Minimum critical temperatures have already been determined for select development stages with egg hatching being between 12–16 °C (Holmes *et al.*, 2016) and larval and prepupal stop growth being ceasing at 12–13 °C (Chia *et al.*, 2018). While data from the static method provide useful information on long term exposure, acute exposure allows us to remove competing or interacting variables such as nutrient or water depletion that could alter organisms

thermal tolerance or preference over time (Angilletta, 2009). Considering immature black soldier flies seek out and avoid specific temperatures, and that they partially thermoregulate (McEachern, 2018) within maggot masses (i.e. Allee effect where individual fitness may be altered by population size) (Olea *et al.*, 2019), acute measurements for thermal tolerance and preference are likely of vital importance to mass production efforts. Unfortunately, such data are not available.

Previous studies determined the optimum performance (e.g. growth rate, development time, and survival) of black soldier fly larvae was achieved between 30–35 °C (Chia *et al.*, 2018). The shortest larval development time was also determined by Shumo *et al.* (2019) to occur at ~30 °C. While not the objective of the previous study, the optimum performance temperature (i.e. thermal optimum) determined may indicate actual thermal preference. However, thermal preference is usually lower than the thermal optimum, which may reveal energy allocation strategies, and ultimately suggests that suboptimal (temperature) may actually be optimal (Martin and Huey, 2008). Additionally, these data lack precision as the impact of age and calorific restriction as related to stage has not been determined for the black soldier fly. This limitation is constraining for the black soldier fly industry as such data could be used to optimize its mass production. Both within and across life stages to ensure continuous optimal temperature, and thus production.

Determining thermal tolerance and preference for the black soldier fly is critical for optimizing its industrialization. While novel for the insects as food and feed sector, this concept has a history of application throughout animal (e.g. chicken (Morita *et al.*, 2016) and cattle (Elayadeth-Meethal *et al.*, 2018)) and plant (e.g. tomato (Tarusikirwa *et al.*, 2020) and wheat (Rane and Nagarajan, 2004)) agriculture. In fact, purely from a livestock perspective, identifying thermal tolerances and preferences and implementation of such criteria as guiding parameters has been found to enhance production by optimizing feed conversion (e.g. laying hens (Mashaly *et al.*, 2004)), growth patterns (e.g. piglet (Ross *et al.*, 2015)), and survival (e.g. sheep (Phillips, 2016)).

The goal of this study was to quantify the impact of age and calorific restriction on thermal tolerance and preference of immature stage of black soldier flies (i.e. larvae and prepupae). While such data could provide insights into the potential range expansion of wild populations due to climate change, such data are also vital for the industrial production of this species. By identifying the thermal tolerance and preference as the

insect develops through its life cycle, criteria can be developed for optimizing growth, survival, and reproductive potential depending on the life stage as has been accomplished for other livestock. Such precision not only increases production but also decreases costs per unit volume of insect produced.

2 Materials and methods

Colony maintenance

The black soldier fly colony used for the following experiments was established in January 2014 from eggs purchased from Phoenix Worm[®], Inc., Tifton, GA, USA. The colony was maintained at the Forensic Laboratory for Investigative Entomological Sciences (F.L.I.E.S. Facility) at Texas A&M University in College Station, TX, USA. This strain originated from a laboratory colony maintained at the Coastal Plains Experiment Station, University of Georgia, Tifton, GA, USA in 1998. Adopted from Cammack and Tomberlin (2017), detailed colony management procedures were described in Li *et al.* (2021). The Gainesville diet (Hogsette, 1992) with ~15% protein and ~36% soluble carbohydrate (Bellezza Oddon *et al.*, 2022), which contains 50% wheat bran, 30% alfalfa meal, 20% corn meal, and moisturized to 70% water content, was used as a larval food.

Black soldier flies used in this study were raised from eggs, which were collected from the colony maintained in a greenhouse in the F.L.I.E.S Facility. Eggs were hatched in a laboratory walk-in Rheem[®] environmental chamber (Asheville, NC, USA, set to $\sim 27.0 \pm 0.5$ °C, $\sim 60.0 \pm 5.1\%$ relative humidity (RH), 14 h light/10 h dark). To raise different size black soldier flies, either 500 (for larger size due to no calorific restriction) or 2000 (for small size due to calorific restriction) newly hatched larvae were hand-counted and transferred to separate cups (Great Value[®] 532 ml, from Walmart, AR, USA) containing 40 g Gainesville diet (days 1-8) and then transferred to larger ($\sim 1,893$ ml) cylinder plastic containers (Airlite[®] 6451, Omaha, NE, USA) on day nine. Detailed feeding procedures are described in Addeo *et al.* (2021). Both young (7-d-old) and old (14-d-old) larvae, and young (1-d-old) and old (7-d-old) prepupae were collected during the larval-rearing process for thermal tolerance and preference experiments. To maintain the same population size among treatments, cohorts (i.e. containers) with individuals collected for experiments were discarded, and only intact cohorts were allowed to continue feeding until use. To better represent the biological explanation for size differences,

'large size' will be noted as control, ad libitum, or fed, while 'small size' will be noted as calorie restriction or starvation through the discussion.

Thermal tolerance

Methods were modified from Huey *et al.* (1992). A water bath (PolyScience[®] WB10, Niles, IL, USA) (i.e. for heating) or a steel pan (Update International[™] SPH1006, China) (i.e. for cooling) was used to regulate the water temperature. Plastic tubes (VWR[®] 15 ml, Petaluma, CA, USA) (15 ml) with biodegradable packing peanut lids (MT Products[®] PACKPNT-0.60FT-Parent, NJ, USA) were placed in the water to ensure a homogenous temperature inside the tubes, allow airflow to prevent asphyxiation, and to prevent flies from escaping the tubes. Black soldier flies were individually placed into a 15 ml centrifuge tube (described above). Tubes were placed in groups of ten within pre-cut (to fit in the water bath) PVC R-round bullnose structures (CUSTOM[®] H7802SP98, Vietnam) to hold them vertically in place within the water bath (or pan). Two threaded rods (EVERBILT[®] 6.35 mm \times 30.5 cm, from Home Depot, GA, USA) were tied underneath the bullnose structures with one on each side of the ten tubes to help the bullnose with tubes to partially sink in the water and keep the tubes submerged. The water level was maintained below the opening of tubes and above the lower end of the inserted packing peanut lids. The tubes were subjected to either cooling or heating at constant rates of 1 °C/min. The water inside the bath was periodically stirred with a plastic cooking spoon to ensure thermal homogeneity. Before formal tests, four digital k-type thermocouples (Model A0188598; Gain Express Inc., Kowloon, Hongkong, China) were randomly inserted into the tubes, where thermal homogeneity was checked during heating/cooling protocol (explained below). For formal tests, one digital k-type thermocouple was randomly inserted in one of the tubes and served a temperature reference during experiments rather than the temperatures shown on the digital screen of the water bath as the external water often differed from the temperatures within the tubes due to thermal lag.

For heat (i.e. warm) tolerance, the starting temperature was 27 °C, and was increased to 53 °C at a rate of 1 °C/min. Each replicate began with a total of X tubes, after which 10 tubes were removed from the bath every 1 °C shift and assessed for instant mobility. The tubes pulled out were immediately placed horizontally on a white towel for observation. Individuals that were visibly mobile were noted as knockdown resistant, while those immobilized were noted as knocked down. For

cold tolerance, since only tiny mobility was observed under 27 °C, the starting temperature was set to 34 °C for an activation. When the temperature was reduced from 34 °C to 14 °C ten tubes were pulled out every 2 °C decreased. Knock down temperature was defined as the temperature at which larvae or prepupae lose the ability to effectively locomote (i.e. stop crawling or muscle spasms) (Huey *et al.*, 1992). For heat tolerance, a total 3,840 individuals were tested (2 stages × 2 ages × 2 sizes × 8 temperatures × 3 replicates × 10 individuals per replicates × 2 trials). For cold tolerance, a total 5280 individuals were tested (2 stages × 2 ages × 2 sizes × 11 temperatures × 3 replicates × 10 individuals per replicates × 2 trials). Eggs from different rearing batch was used in each trial.

Thermal preference

Following similar methods from Dillon *et al.* (2009) and Malawey *et al.* (2021), the thermal preferences of black soldier fly larvae and prepupae were measured. An aluminum plate (85 × 20 cm) was used for the surface of the thermal gradient. The plate was bent at 90° approximately 10 cm from each distal end length-wise to create an “U” shape with arms (Supplementary Figure S1), one of which was placed in a water bath (mentioned above) maintained at 85 °C, while the other arm was placed in a polystyrene box (27 × 22 × 22 cm) maintained at ~0 °C using ice and water. Consequently, a linear thermal gradient from 60 °C to 15 °C on the surface of the aluminum plate was achieved (details described below). During the experiments, temperature of the gradient remained consistent; room fluorescent lights (GE®, Ecolux w/Starcoat, F32T8, SPP41, ECO, Hg E, 32 W, 4100K, MA, USA) remained on during the experiment.

The gradient was divided into ten lanes with aluminum foil walls to allow testing individual larva or prepupa at the same time without direct interaction with each other. The surface temperatures of the thermal gradient were measured by placing four digital k-type thermocouples (mentioned above) spaced at 2, 18, 34, and 52 cm along the surface of the aluminum gradient at ~5 cm away from the edge. A Cartesian coordinate system was used to estimate the temperature where each larva or prepupa was located.

Thermal preference experiments were conducted without feed or substrate. Individual larva and prepupa were released into the center of each lane, which was approximately 30 °C, on the thermal gradient and allowed to explore and adjust to the condition of the thermal gradient for 5 min. After the initial exploration

period, the spatial positioning of the individual was captured at 5 min intervals for 30 min by a phone camera (iPhone® 8, 12-megapixel wide-angle f/1.8 camera, Cupertino, CA, USA) placed ~60 cm directly above the thermal gradient by hand. In total 480 individuals were tested (2 stages × 2 ages × 2 sizes × 3 replicates × 10 individuals per replicates × 2 trials).

Statistical analysis

Generalized linear models (GLM with logistic regression, with family set as binomial) were applied to test how black soldier fly larval and prepupal thermal tolerance were impacted. Data had binomial distributions with “1” coded for knockdown resistant and “0” coded for knocked down. Initially, logistic regression was used to test the main effects of stage, age, size, trial, and temperature, as well as the interactive effects (i.e. three-way interaction) among stage, age, and size on maximum critical temperature (CT_{max}) and minimum critical temperature (CT_{min}). Logistic regression was used to examine interaction effects between trial and other factors on CT_{max} since the full model indicated that trial was significant ($P < 0.05$). Since all the interactive effects between stage, age, and size were determined to be significant in the full model, for simplification, further analyses were done by assessing each combination of stage and age with logistic regression. Doing so, allowed for the impact of size and temperature on both CT_{max} and CT_{min} to be determined. Odd ratio (OR) with 95% confidence interval (CI₉₅) and median knockdown temperature (KR₅₀) also were calculated in each data subset (i.e. data with specific stage, age, and size).

Linear mixed effect models (LME) were applied to test the thermal preference of different ages and sizes of larval and prepupal black soldier flies. Heat-killed individuals were removed from the full data set. To clarify, the heat-killed individuals were determined by data points that no longer changed by time on the high (i.e. >45 °C) temperature side, which is in accordance with the observation that non-heat-killed individuals keep wandering along the thermal gradient. Based on our *a priori* hypotheses, the main effects of time and trial as well as a three-way interaction effects among stage, age, and size were tested. Group ID (individuals tested together were signed with the same group ID, $n = 10$) was nested within time as a random effect. The weight component in the LME model was explored to have the best model fit by comparing the Akaike information criterion (AIC) with maximum likelihood. Restricted residual maximum likelihood (REML) was specified in the best model. Further subset data were pooled by stages

(i.e. larvae and prepupae). The LME model was applied to each of the subset data as well since significant ($P < 0.05$) interactions between stage and other factors were determined. The package emmeans was used for pairwise comparison (Lenth *et al.*, 2018). Analyses were conducted in R version 4.0.3 (R Core Team, 2015) with the significance set at $P < 0.05$.

3 Results

Immature black soldier flies CT_{max}

Knockdown resistance differed by trial ($z = 4.209$, $df = 1$, $P < 0.001$), but trial did not interact with other main effects of stage ($z = 0.256$, $df = 1$, $P = 0.798$), age ($z = 1.625$, $df = 1$, $P = 0.104$), and size ($z = -1.630$, $df = 1$, $P = 0.103$). The trial effect ($OR = 1.8$) was smaller than the effect of temperature ($z = -26.812$, $P < 0.001$, $OR = 8.4$) and interaction effects between stage and age ($z = 4.210$, $df = 3$, $P < 0.001$, $OR \geq 5.0$), stage and size ($z = 6.157$, $df = 3$, $P < 0.001$, $OR \geq 10.8$), as well as age and size ($z = -5.524$, $df = 3$, $P < 0.001$, $OR \geq 4.8$). Therefore, trial was not separated in subsequent analyses. Further data analyses were done with a subset of data pooled by a combination of stage and age for a more direct application for potential industrial uses.

Subset data analyses determined size had strong impacts ($z = -7.171$, $df = 1$, $P < 0.001$, Figure 1A) on CT_{max} among young larvae. The knockdown resistance of large, young larvae ($KR_{50} = 48.4 \pm 0.1$ °C) were eight times greater ($OR = 8.0$, $IC_{95} = 4.55-14.29$) than small, young larvae ($KR_{50} = 47.3 \pm 0.1$ °C) (Figure 1A). Size did not impact ($z = -0.842$, $df = 1$, $P = 0.400$) (Figure 1B) old larvae CT_{max} . The knockdown resistance of large, old larvae ($KR_{50} = 47.9 \pm 0.1$ °C) were similar to small, old larvae ($KR_{50} = 47.8 \pm 0.1$ °C) (Figure 1B).

Size had moderate impacts ($z = 2.349$, $df = 1$, $P = 0.019$) (Figure 1C) on young prepupae CT_{max} . The knockdown resistance of large, young prepupae ($KR_{50} = 47.7 \pm 0.1$ °C) were approximately two times lower ($OR = 1.96$, $CI_{95} = 1.12-3.46$) than small, young prepupae ($KR_{50} = 48.0 \pm 0.1$ °C) (Figure 1C). Size had strong impacts ($z = 6.760$, $df = 1$, $P < 0.001$) (Figure 1D) on old prepupae CT_{max} . The knockdown resistance of large, old prepupae ($KR_{50} = 46.4 \pm 0.1$ °C) were approximately seven times lower ($OR = 6.9$, $CI_{95} = 4.02-12.4$) than small, old prepupae ($KR_{50} = 47.4 \pm 0.1$ °C) (Figure 1D).

Immature black soldier flies CT_{min}

No significant difference ($z = -1.362$, $df = 1$, $P = 0.173$) between trials for black soldier fly CT_{min} was found.

Temperatures ($z = 30.721$, $P < 0.001$) as well as an interaction between age and size had significant impacts ($z = 10.187$, $df = 3$, $P < 0.001$) on CT_{min} .

Subset data analyses showed size had strong ($z = 12.82$, $df = 1$, $P < 0.001$) (Figure 1E) impacts on young black soldier fly larvae CT_{min} . The knockdown resistance of large, young larvae ($KR_{50} = 27.9 \pm 0.3$ °C) were 24 times greater ($OR = 24.3$, $IC_{95} = 15.17-40.30$) than small, young larvae ($KR_{50} = 21.6 \pm 0.3$ °C) (Figure 1E). Size had no significant ($z = -1.412$, $df = 1$, $P = 0.158$) (Figure 1F) impacts on CT_{min} among old larvae. The knockdown resistance of large, old larvae ($KR_{50} = 31.3 \pm 0.4$ °C) were similar to small, old larvae ($KR_{50} = 32.1 \pm 0.5$ °C) (Figure 1F).

Size had slight ($z = 2.261$, $df = 1$, $P = 0.024$) (Figure 1G) impacts on young black soldier fly prepupae CT_{min} . The knockdown resistance of large, young prepupae ($KR_{50} = 27.4 \pm 0.6$ °C) were 1.4 times lower ($OR = 1.4$, $IC_{95} = 1.04-1.79$) than small, young black soldier fly prepupae ($KR_{50} = 25.4 \pm 0.3$ °C) (Figure 1G). Old prepupae were not mobile at room temperature (~ 27 °C) without stimulation (i.e. touching or heating). Therefore, knockdown resistance was not recorded for any of the old prepupae tested (i.e. all data were noted as knocked down; Figure 1H).

Black soldier flies thermal preference

Thermal preference differed by trial ($F = 21.156$, $df = 1$, $P < 0.001$), but trial did not interact with other main effects of stage ($F = 0.665$, $df = 1$, $P = 0.420$), age ($F = 0.033$, $df = 1$, $P = 0.857$), and size ($F = 0.057$, $df = 1$, $P = 0.8122$). Population distributions along the thermal gradient were drawn with trial separated (Figure 2), but an overall distribution was also given (Supplementary Figure S2) for better illustration of ontogenetically differences. Moreover, the interaction between stage and age ($F = 123.455$, $df = 1$, $P < 0.001$), stage and size ($F = 31.230$, $df = 1$, $P < 0.001$), as well as age and size ($F = 1.087$, $df = 1$, $P < 0.001$) were significant. Further data analyses were done with subset data pooled by the stage.

For larvae specifically, no significant interaction between age and size ($F = 0.180$, $df = 1$, $P = 0.676$) was found. Young larvae (27.1 ± 0.5 °C on average) preferred greater ($F = 16.439$, $df = 1$, $P < 0.001$) temperatures than old larvae (23.9 ± 0.6 °C on average). Large larvae (24.3 ± 0.5 °C on average) preferred lower ($F = 10.054$, $df = 1$, $P = 0.005$) temperatures than small larvae (26.7 ± 0.6 °C on average). More specifically, small, young larvae (27.2 ± 0.9 °C in trial 1 and 29.0 ± 1.0 °C in trial 2) preferred the highest temperatures, followed by large,

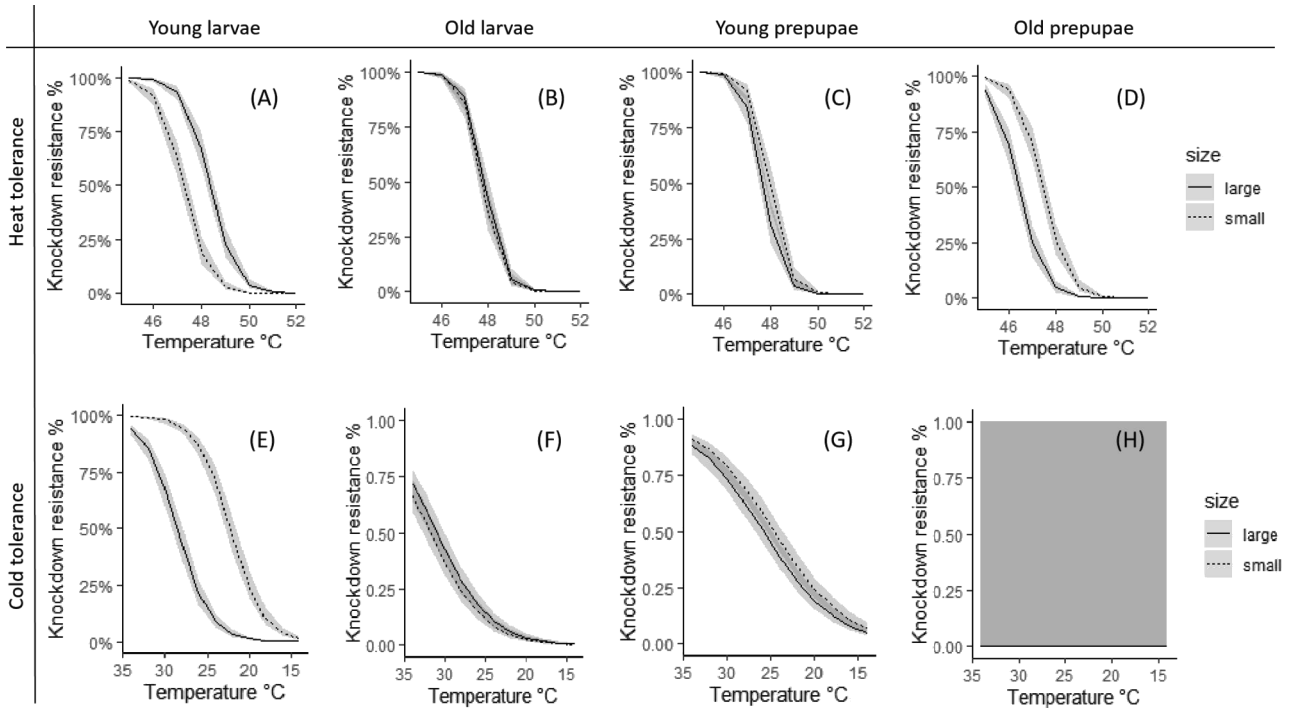


FIGURE 1 Impacts of size and age on heat tolerances (A-D) and cold tolerances (E-H) of black soldier fly larvae and prepupae. Regression curves showing the effect of size on black soldier fly heat tolerance was age and stage dependent, A) large young larvae more tolerant to heat than small young larvae, not much differences were determined between sizes for (B) old larvae and (C) young prepupae, while D) large old prepupae were less tolerant to heat than small old prepupae. The effect of size on black soldier fly cold tolerance was age and stage dependent as well, which (E) large young larvae were less tolerant to cold than small young larvae, not much differences were determined between sizes for (F) old larvae and (G) young prepupae, and (H) no records could be made for old prepupae since none of them moved during cold tolerance experiments.

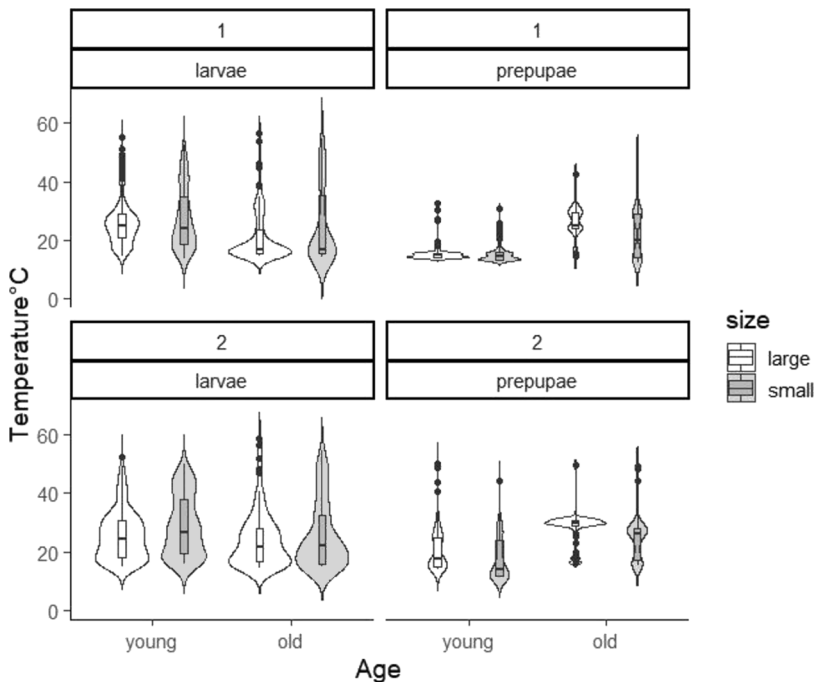


FIGURE 2 Impacts of size and age on thermal preferences of black soldier fly larvae and prepupae. Data are facet wrapped by trial number (e.g. upper panel referring to trial 1 and lower panel referring to trial 2). The thermal preference decreased ontogenetically except old prepupae. Large larvae preferred lowered temperature than small larvae (e.g. left panel), but large prepupae preferred greater temperatures (e.g. right panel).

young larvae (26.6 ± 0.8 °C in trial 1 and 25.6 ± 0.9 °C in trial 2), small, old larvae (24.8 ± 1.4 °C in trial 1 and 25.6 ± 1.1 °C in trial 2), and large, old larvae (21.0 ± 0.8 °C in trial 1 and 24.0 ± 1.1 °C in trial 2) (Figure 2).

For prepupae specifically, no significant interaction between age and size ($F = 1.705$, $df = 1$, $P = 0.207$) was determined. Young prepupae (17.3 ± 0.6 °C on average) preferred lower ($F = 78.117$, $df = 1$, $P < 0.001$) temperatures than old prepupae (25.1 ± 0.6 °C on average). Large prepupae (22.6 ± 0.6 °C on average) preferred greater ($F = 12.167$, $df = 1$, $P = 0.002$) temperatures than small prepupae (19.8 ± 0.6 °C on average). More specifically, large, old prepupae (25.9 ± 0.8 °C in trial 1 and 28.2 ± 0.7 °C in trial 2) preferred the highest temperatures, followed by small, old prepupae (21.8 ± 1.0 °C in trial 1 and 24.5 ± 0.8 °C in trial 2), large, young prepupae (15.6 ± 0.8 °C in trial 1 and 20.7 ± 0.9 °C in trial 2), and small, young prepupae (15.6 ± 0.8 °C in trial 1 and 17.5 ± 0.8 °C in trial 2) (Figure 2).

4 Discussion

This study determined the impact of stage, age, and size on immature (i.e. larvae and prepupa) black soldier fly thermal tolerance and preference. Data indicate heat tolerance of immature black soldier flies decreased (i.e. KR_{50} differed by ~ 2.0 °C) through development but such variation in response decreased (i.e. KR_{50} differed by ~ 0.6 °C) in the small size (i.e. calorie restricted) group. The ability to move with respect to cold tolerance decreased with age within stage, with young larvae that were calorie restricted being able to locomote at much lower temperatures (i.e. KR_{50} differed with ~ 6.3 °C). Thermal preference of immature black soldier flies decreased (i.e. median ranged from 29.0 °C to 15.6 °C) ontogenically except for old prepupae indicating this stage might be fixed (less mobility observed around the point released). Meanwhile, as mentioned earlier, calorie restriction (i.e. resulted in small size) triggered larvae to spend more time at higher temperatures (i.e. ~ 2 °C and ~ 7 °C increased in thermal preference on median and the third quartile, respectively, with higher proportion and longer distribution tails toward the warm side) than control but resulted in lower prepupal thermal preference (i.e. 2.8 °C difference in median). Such data are critical for developing strategies for optimizing black soldier fly production at an industrial scale (applications and recommendations discussed below).

Heat tolerance decreased ontogenically

Large, young larvae had the greatest heat tolerance ($KR_{50} = 48.4$ °C), while large, old prepupae were the least tolerant ($KR_{50} = 46.4$ °C). Remaining treatments were in between ($KR_{50} = 47.0$ – 48.0 °C) (Figure 1). Therefore, heat tolerance of the large size group (i.e. more feed provided per larva daily) declined significantly through development (i.e. from young larvae, old larvae, young prepupae, to old prepupae in order).

Such differences in thermal tolerance can be a result of mobility across life stages. Those being less mobile, such as larva versus adult, expected to have greater thermal tolerance (Bowler and Terblanche, 2008). For example, adult kelp flies, *Paractora dreuxi* Seguy (Diptera: Helcomyzidae), had ~ 5 °C lower heat tolerance than larvae (Klok and Chown, 2001). To some extent, the same appears to be the case with the black soldier fly (i.e. young larvae having lower crawling speed than old larvae). However, the old prepupae appear to be the exception to the rule if considering old prepupae are the least mobile at room temperature (e.g. ~ 27 °C). But the old prepupae tested were indeed still active when experiencing high temperatures (e.g. >40 °C). Of course, being mobile at high temperatures provides individuals, such as old prepupae, with the ability to thermoregulate simply by moving from one microclimate to another.

Conversely, the greatest heat tolerance of large, young larvae could be an adaptation to high substrate temperature experienced due to larval aggregative feeding (Li *et al.*, 2023), which is how larvae are mass produced by industry. However, it should be noted that large larval densities in nature are also common (Tomberlin, personal communication), for example, massive (e.g. millions) larval masses have been found in layer hens and swine house (Burtle *et al.*, 2012) Therefore, thermal resistance may be enhanced during the young larval stage particularly.

In fact, the strain of black soldier fly used in the current experiments has a long adaptive history to industrial scale production (e.g. 10,000 larvae in a single container (Yang and Tomberlin, 2020)). At such a high density, the substrate can heat up to 40 °C when young larvae are actively feeding and then decreases to room temperature ~ 27 °C after feed consumption (i.e. at the time that the larvae were 14-d-old in most scenarios) (Beesigamukama *et al.*, 2021). The young larvae (7-d-old) tested in this experiment are actively feeding and aggregating while the old larvae (14-d-old) had reduced their feeding and were no longer tightly aggregating. The prepupae were also not feeding or aggregating, so their

behavior for a lower upper thermal tolerance makes sense in this case too.

Cold tolerance decreased as individuals aged within stage

When temperature decreased, mobility in old larvae ($KR_{50} = 31\text{--}32\text{ }^{\circ}\text{C}$) and old prepupae (no movement) was less active than for young larvae ($KR_{50} = 27.9\text{ }^{\circ}\text{C}$) or young prepupae ($KR_{50} = 27.4\text{ }^{\circ}\text{C}$) (Figure 1). Such responses have been documented for other species where older individuals within a given stage are less mobile than younger individuals. Žd'árek *et al.* (2002) determined the distance crawled by flesh fly larvae, *Sarcophaga bullata* (Parker) (Diptera: Sarcophagidae), decreased from ~ 9 cm to 0 cm per every two minutes within 120 min before pupariation at a constant room temperature. While the physiological mechanisms regulating such a response are not known for the black soldier fly, the decreasing mobility as related to cold tolerance as individuals aged within stage could be due to increased production of leucopyrokinin (LPK), a neuropeptide regulates larval movements and the onset of pupation, which was determined for the flesh fly previously discussed (Žd'árek *et al.*, 2002).

While the cooling approach (adding ice to decrease the water temperature by $\sim 1\text{ }^{\circ}\text{C}/\text{min}$) is commonly used (Wharton and Raymond, 2015; Wu *et al.*, 2018) to measure cold knockdown temperatures, there are challenges. Considering the short time window allowed for observation (i.e. notes need to be taken within 10 s before the body temperature increased again after being pulled out from the water bath) and the decreasing mobility of the larvae and prepupae during temperature decline, knock-down temperatures recorded in this study should be much greater than the minimum critical temperatures causing an absolute long-term cessation in mobility (e.g. the mobility KR_{50} was determined to be $\sim 30\text{ }^{\circ}\text{C}$ in this study, which should not cause mobility out of function). Noted that in our thermal preference study, larval movements were recorded below $15\text{ }^{\circ}\text{C}$. Larvae/prepupae examined in the current experiments did stop crawling for ~ 10 s at the temperature identified as the corresponding knocked down temperature. Therefore, cold tolerances recorded in this study demonstrates a relationship between temperature, stage, age, and size as related to mobility, which partly reflects the locomotory patterns. However, it should be noted that the black soldier fly locomotory pattern do differ between the mature larvae and prepupa, in which a relatively quick and continuous movement of prepupae was recorded in comparison to the

6th instar (Giannetti *et al.*, 2022). Given the limitations of this measurement approach, additional consideration of this trait is warranted. For example, a static assay instead of ramping assay may be required.

Thermal preferences decreased ontogenically

Outside of old prepupae (discussed below), the thermal preferences, much like with heat tolerance, decreased ontogenically (e.g. from $\sim 26.1\text{ }^{\circ}\text{C}$ for young larvae and $\sim 22.0\text{ }^{\circ}\text{C}$ for old larvae to $\sim 18.2\text{ }^{\circ}\text{C}$ for young prepupae) (Figure 2 and Supplementary Figure S2). Such a decrease in thermal preferences appear to be in accordance with those typically experienced by black soldier flies naturally where young, feeding, larvae experience high temperatures (Shishkov, 2020) and prepupae reside in substrates that have cooled following larval feeding (Chen *et al.*, 2019). For example, when mass produced, the substrate (i.e. different combinations of sawdust and brewer's spent grain) temperature increased to $\sim 40\text{ }^{\circ}\text{C}$ in the initial 2-3 days, decreased to $\sim 30\text{ }^{\circ}\text{C}$ around day 5, and subsequently to $\sim 26\text{ }^{\circ}\text{C}$ around day 10 when feeding finished (Beesigamukama *et al.*, 2021; Li *et al.*, 2023). Similar patterns have been determined for the house fly, *Musca domestica* L. (Diptera: Muscidae), whose mature larvae select temperatures $\sim 20\text{ }^{\circ}\text{C}$ lower than preferred by larvae actively feeding (Deal, 1941; Thomsen and Thomsen, 1937). These data are critical for industrialization of the black soldier fly as these thermal pattern responses can be used to develop programmed temperature shifts in facilities as through the production cycle as a means to maximize growth, survival, and feed conversion.

Calorie restriction shifted thermal tolerances

Not like the large immatures, smaller individuals had similar heat tolerance throughout ontogeny (Figure 1). Reduced size in some instances has been linked to heat tolerance reduction. This has been demonstrated for some ant species (Baudier *et al.*, 2015) where size, which was linked with lipid content and thermal inertia, impacted heat tolerance which is in line with our young larval data but opposite to the old prepupal data (see discussion below).

Limiting nutrition resources (e.g. calorie restriction, fasting, and starvation) to an individual could enhance heat tolerance (DeVries *et al.*, 2016; Gotcha *et al.*, 2017; Mutamiswa *et al.*, 2018). While we did not find an example for immature dipterans, similar results have been recorded for starved adults. Natal fruit flies, *Ceratitis rosa*, Karsch (Diptera: Tephritidae) had $\sim 0.5\text{ }^{\circ}\text{C}$ greater critical thermal maximum than the control, which was

fed (Gotcha *et al.*, 2018). Similarly, starved African fig flies, *Zaprionus indianus* Gupta (Diptera: Drosophilidae), had a 1.5 h longer heat knockdown duration than the control (Kalra *et al.*, 2017). This phenomenon is not restricted to Hexapoda as it was also recorded for the amphipod, *Gammarus fossarum* Koch (Amphipoda: Gammaridae), where its survival time was ~1.7 min longer at 33.5 °C when starved (Semsar-Kazerouni *et al.*, 2020).

Each of these examples demonstrates cross-tolerance where one stress factor impacts tolerance of another stress factor. The existence of cross-tolerance indicates that shared physiological protective mechanisms are involved in the evolutionary adaptation to stressful environments, which is indeed the case considering heat waves in nature often coincide with droughts and subsequent food shortages (Bubliy *et al.*, 2012). This explanation is in line with the ephemeral nature of black soldier fly larval habitats. Black soldier flies are omnivorous saprophages that can develop on a wide range of organic wastes; such a high substrate flexibility usually translates into high developmental plasticity (Seyedalmoosavi *et al.*, 2022). Metabolism modification could be a potential explanation allowing for diet dependent thermal tolerance by the black soldier fly. Starvation was determined to be able to induce hypometabolism (i.e. lower metabolic rates), for example, starved *D. melanogaster* third instars and adults had lower (i.e. 2-5 $\mu\text{l}/\text{mg}/\text{h}$ CO₂ depended on population) metabolic rates than their fed counterparts (Brown *et al.*, 2019). Lower metabolism means lower oxygen requirements at certain temperatures. In support of this explanation, hypoxia tolerance and heat tolerance correlated up to 30% for *D. melanogaster* (Teague *et al.*, 2017).

In the case of the current study, increased heat tolerance was recorded for calorie restricted old prepupae but not larvae or young prepupae. It is possible, while not recorded, small, old prepupae had lower metabolic rates than the large, old prepupae considering the whole larval stage of the small size group were under calorie restriction and the prepupal stage is no longer eating. This account is supported by the study mentioned in the previous paragraph where diet-induced hypometabolism can be stage dependent with starved younger instars not differing in metabolic rate from their fed conspecifics but third instars and adults did (Brown *et al.*, 2019).

Small, young larvae experienced intermittent locomotive abilities at 21.6 °C, which was much lower than for large, young larvae (KR₅₀ = 27.9 °C). Therefore, calorie restriction potentially enabled young lar-

vae to be more active at low temperatures. Calorie restriction may induce mobility to facilitate food exploration and acquisition, which has been determined in vinegar flies (Diptera: Drosophilidae) (Yu *et al.*, 2016), blow flies (Diptera: Calliphoridae) (Mahishi *et al.*, 2021), and honey bees (Hymenoptera: Apidae) (Vázquez and Farina, 2021). Interestingly, and a potential future area of research, increased mobility in starved *Drosophila* was determined to be regulated by an adipokinetic hormone (i.e. AKH, a neuropeptide widely found in insects) (Yu *et al.*, 2016), indicating the importance of considering ontogeny and nutrient status on mobility researches. Understanding the plasticity of mobility helps to explain the natural geographical distribution of insects. (Stange and Ayres, 2010)

Of course, as noted earlier, old prepupae seem to be an outlier in terms of heat response, which begs the question- why? One potential explanation is the Bogert effect (i.e. greater thermal tolerance phenotypic plasticity in less mobile stage) (Klockmann *et al.*, 2017). For example, a study on the heat hardening capacity in *D. melanogaster* determined the immobile life stages (puparia and pupae) had higher plasticity in heat tolerance than larvae and adults (Moghadam *et al.*, 2019). Our results may reveal that when facing stresses (i.e. calorie restriction), the less mobile stage (i.e. old prepupae) of black soldier flies showed greater plasticity on heat tolerance (i.e. increment was greater than young prepupae and old larvae). Such greater plasticity may be important for old prepupae to survive during the less mobile stage (i.e. right before the metamorphosis).

Calorie restriction shifted thermal preferences

Like with thermal tolerance, thermal preferences of the calorie restriction group also decreased ontogenically (e.g. from ~28.1 °C for young larvae, ~25.2 °C for old larvae, to ~16.6 °C for young prepupae) similar to the control group. But the impact of calorie restriction on thermal preference was stage dependent. Calorie restricted larvae preferred greater (i.e. ~2.0 °C for the young and ~2.7 °C for the old) temperatures than control, while calorie restricted prepupae preferred lower (i.e. ~1.6 °C for the young and ~3.9 °C for the old) temperatures than control (Figure 2 and Supplementary Figure S2).

Downstream effects of starvation include a lower thermal preference which in turn reduces metabolism (Scharf, 2016) as briefly discussed previously. However, while the prepupal data from the current study support such a phenomenon, larval data do not. Unfortunately, while no other insect-related examples similar to the larval responses observed could be located in the literature,

starved crabs, *Cancer magister* (Dana) (Decapoda: Cancridae) tend to spend more time in high temperature environments searching for food (provided only at high temperatures), indicating fed crabs avoiding the heat and/or potential predators that may be attracted to the food (Curtis and McGaw, 2012).

As a final note with prepupae, the older individuals within this stage were not active and thus not as informative. They tended to remain in the vicinity where they were released on the thermal gradient, even though, calorie restricted, old prepupae distributed more toward the cold than control, indicating the calorie restricted, old prepupae either prefer cooler temperatures or more mobile than the control. The former reason can be mutually confirmed by the thermal preference of young prepupae, which are still active in locomotion in nature. The latter reason may also contribute since starvation-induced hyperactivity has been determined in adult *D. melanogaster* (Yang *et al.*, 2015).

Potential explanation of differences between trials

Though trial effects were detected, no interactive effects were found between trial and other variables. The heat tolerance in trial one was lower than trial two. Meanwhile, the thermal preference in trial one was lower than trial two. Eggs used for thermal preference, heat tolerance, and cold tolerance in trial one were collected on date April 29th, 30th, and May 1st, respectively, while in trial two, eggs were collected on date May 2nd, 3rd, and 4th, respectively. As described in the previous methods, eggs were collected in a greenhouse. Unfortunately, there were no temperature data recorded in the greenhouse. But according to the local climate history (Wunderground.com), maximum temperatures from April 29th to May 4th were 27.8, 27.8, 29.4, 31.1, 31.7, and 32.8 °C, respectively. Therefore, on average, eggs used in trial two experienced a ~3.5 °C increment in maximum temperatures than eggs used in trial one. Trial differences determined in this study may reveal the importance of cross-stage (Gray, 2013) and even cross-generation (Watson and Hoffmann, 1995) thermal acclimation studies in black soldier flies.

Limitations of current study

First, the cold tolerance method used in this study (i.e. decreased temperatures until movements paused) as previously discussed can only determine the relationship between temperature and movement. One should pay attention to the difference in temperatures causing immobility, chill-coma, and mortality (Macdonald *et al.*, 2004; Ransberry *et al.*, 2011). Thermal tolerances can

be determined by many types of measurements. Future studies can use another type of measurement (i.e. chill-coma recovery time or static assay instead of ramping) as a supplementary reference.

Second, the thermal preference determined may be skewed due to limitations of the method used. The insect releasing point was approximately 27-28 °C, which was the midpoint of the thermal gradient. Therefore, the thermal preferences determined mostly shifted towards the cold side from the releasing point, indicating black soldier fly immatures tested may prefer lower temperatures than their thermal optimum (i.e. 27-30 °C) reported (Chia *et al.*, 2018). However, one notable limitation of the thermal gradient is that small ectotherms, such as black soldier fly larvae and prepupae (<2 cm length), are easily cold-trapped, resulting in thermal preferences biased to the low temperature (Giraldo *et al.*, 2019). Further studies can measure the temperature dependent crawling speed so that data can be transformed accordingly to correct the cold-trapped bias. Considering the tested individuals were able to wander back and forth based on observation, the low temperature range may only be able to decrease the crawling speed without ceasing mobility. In addition to the impact of cold on movement, it is worth considering that thermal preference could be considered to reflect a random walk from the release point, which was not accounted for here but might explain peaks around the placement location and at lower temperatures. However, this could be addressed in the future by releasing from different points along the gradient. An additional consideration for this experiment as it relates to application would be the different environments between the thermal gradient and the real rearing condition. Neither the larvae nor the prepupae were provided any substrates during measurements, which was not the case in the reported thermal optimum studies (Chia *et al.*, 2018). If the latter explanation contributed to the observation in this study, data should partially reveal thermal optimum differences among food acquisition, assimilation, growth, and development.

Third, the different groups (i.e. 2,000 larvae versus 500 larvae per container) used to generate different sized individuals interacted with diet fed. Large individuals were produced at low density and high amount of feed and vice versa for the small individuals. Further study should consider size more carefully in experimental design by setting diet or density and only shift one to remove the covariation.

5 Conclusion

To the best of our knowledge, this study provides the first records of thermal tolerance (in terms of knock-down temperatures) and preference for black soldier fly larvae and prepupae. Results from this study highlighted the significant phenotypic variation of immature black soldier flies in their thermal tolerance and preference within and across stage of development. Rearing temperatures could be set and monitored through the growth cycle where larvae are initially reared at a warmer temperature then gradually decreases over time. Doing so should reduce stress and potentially optimize feed conversion, body size, and survival. Also, fine scale management of feed composition and pattern as a means to produce larvae with little variation in size then can also be used as a means to develop temperature programming allowing for optimal production as well.

Supplementary material

Supplementary material is available online at: <https://doi.org/10.6084/m9.figshare.26212835>

Author contributions

Chujun Li: methodology, validation, data curation, formal analysis, writing – original draft preparation, conceptualization, methodology. Nicola Francesco Addeo: data curation, review & editing; Travis W. Rusch: methodology, review & editing; Thomas M. Chappell: formal analysis, review & editing. Aaron M. Tarone: review & editing. Jeffery K. Tomberlin: conceptualization, resources, supervision.

Conflicts of interest

The authors declare no conflict of interest. Any opinions, findings, conclusion, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the view of United States Department of Agriculture.

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