Interaction of age and temperature on heat shock protein expression, sperm count, and sperm viability of the adult black soldier fly (Diptera: Stratiomyidae)

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RESEARCH ARTICLE

Abstract

Information regarding black soldier fly (Diptera: Stratiomyidae) adult biology is vital as this is the life stage that produces eggs and thus drives population size. The goal of this study was to determine key biological characteristics of adult black soldier flies as they age in relation to: (1) the thermal preferences (T_{sel}) of males and females; (2) the impact of temperature on heat shock protein expression in males and females; as well as (3) the sperm count; and (4) the sperm viability in males. Aging significantly impacted male and female temperature preferences. Young males (<24-h-old) preferred warmer temperatures (median=24.3 °C, range=19.3-28.2 °C) compared to females of the same age (median=20.2 °C, range=15.4-26.2 °C). However, in older adults (i.e. 72-h-old males and 48-h-old females), temperature preferences converged between 21 and 24 °C. Temperatures tested did not impact *hsp* expression in males or females. However, aging males, but not females, had increased expression of the heat shock proteins (hsp) *hsp70* and *hsp90*. Furthermore, age impacted sperm count but not sperm viability in males. In particular, 48-h-old males had the greatest sperm count (322.5/sample) and sperm viability (60-78%) compared to all other aged males. Thermal data in conjunction with sperm data potentially explain why early thermal segregation behaviour between males and females occurs. Once adult males and females reached 72-h-old and 48-h-old, respectively, they exhibited a common thermal preference, which coincided with the greatest number of viable sperm in males. Forcing adults into environments (i.e. cages) outside these selected preferences could result in premature or delayed mating or low fertilisation rates. Future research exploring cage design and conditions are needed to optimise black soldier fly colony maintenance and fertile egg production, and can leverage information such as the results described here.

Keywords: *Hermetia illucens*, thermal ecology, colony maintenance

1. Introduction

To minimise the challenges imposed by natural environments, mass-rearing facilities often raise insects under controlled conditions (e.g. constant temperatures and high densities) aiming to increase fitness (Berggren *et al.*, 2018; Oonincx and De Boer, 2012; Sheppard *et al.*, 2002). However, the difference between optimal and stressful environmental conditions for insects are often age and sex specific (Bowler and Terblanche, 2008; Nyamukondiwa and Terblanche, 2009). Thus, understanding how both abiotic and biotic factors affects insects over time and across sex will improve their production in mass-rearing facilities.

Males of many insect species are more vulnerable to temperature-driven fertility loss compared to females (Sales *et al.,* 2018; Zizzari and Ellers 2011). Exposure to sub-optimal temperatures (i.e. low and high) not only reduces temperature-sensitive performance, but in some instances also causes irreversible damage in regards to reproduction, such as deformed sperm, temporary or permanent sterility (e.g. sperm or egg death), with reproductive success of

females exposed to the same treatments being unaffected (Sales *et al.,* 2018).

Aging also has adverse effects on physiological processes, as older organisms tend to be less resilient to environmental stress (McHugh and Gil, 2018) with both thermal tolerance and reproductive output dropping off after a certain age (Conti, 2008; Papanastasiou *et al.*, 2011; Rikke and Johnson, 2004). An increasingly common method used to measure environmental stress in insects, including thermal stress, is through monitoring changes in their heat shock protein (*hsp*) gene expression (King and MacRae, 2015; Zhao and Jones, 2012) as these proteins protect cells from thermal damage and thus enhances survival in unfavourable conditions (Lindquist, 1986).

Moreover, not only the expression levels, but type being expressed differ between males and females of the same species. In whiteflies, *Bemisia tabaci* (G.) (Hemiptera: Aleyrodidae), females tend to express greater levels of *hsp90* and *hsp70* when exposed to temperatures ranging from 37.5 to 42.0 °C, while males expressed greater levels of *hsp70* at a more extreme temperatures such as 44 °C (Lu and Wan, 2008).Given the opportunity to actively thermoregulate, both sexes will likely do so in a manner that will maximise their fitness (Angilleta, 2009).

The black soldier fly, *Hermetia illucens* (L.) (Diptera: Stratiomyidae) is currently the most well-known insect mass produced for waste management (Miranda *et al.,* 2019; Shumo *et al.,* 2019) and protein production (Barragan-Fonseca *et al.*, 2017). This is mostly due to its larval stage capacity of digest and recycle a variety of organic products (e.g. brewery waste, food waste from restaurants, and animal waste from farms) (Mertenat *et al.*, 2019; Pleissner and Rumpold, 2018; ur Rehman *et al.*, 2017). Most of the research conducted on black soldier fly biology has focused on the larvae, as this life stage recycles organic material to produce larval biomass that can be used as animal feed. Consequently, relatively little is known about the adult biology, which obviously is the life stage that produces eggs and thus drives population size (see Tomberlin and Sheppard, 2001 for a study on the behaviour of adults in wild populations).

Temperature and age are recognised as important abiotic and biotic factors affecting adult black soldier fly fitness with developmental rates maximised within a specific temperature range (Harnden and Tomberlin, 2016; Holmes *et al.*, 2016; Tomberlin *et al.*, 2009), while sperm production varies by age (Malawey *et al.*, 2019). The black soldier fly industry lacks important information about adult physiology that could impact reproduction and resulting insect production. Therefore, the objectives of this study were to determine key biological characteristics of adult black soldier flies as they age in relation to: (1) the thermal preferences (T_{sel}) of males and females; (2) the impact of temperature on heat shock protein expression in males and females; as well as (3) sperm count; and (4) sperm viability in males.

2. Materials and methods

Black soldier fly colony

Hermetia illucens adults used in experiments came from a colony maintained at the Forensic Laboratory for Investigative Entomological Sciences (F.L.I.E.S.) Facility at Texas A&M University in College Station, Texas, USA and were reared following the methods of Sheppard *et al.* (2002) and Cammack and Tomberlin (2017). Neonates (~10,000) <24-h-old were placed in a 500-ml plastic container with 200 g of Gainesville diet (Hogsette, 1992) at 70% moisture and stored in a walk-in environmental chamber (30.0±1.7 °C, 80% RH, and 16:8 L:D). After seven days, 500 larvae allotments were removed from the container and placed in 1-l plastic containers (n=10 containers). Approximately 60 g of Gainesville diet at 70% moisture were provided daily until ~40% of the larvae reached the prepupal stage, at which point feeding was terminated (Tomberlin *et al.,* 2002). Resulting prepupae were sifted and consolidated into 35 cm (l) × 21 cm (w) × 12 cm (h) plastic pans. Each plastic pan was placed inside a 30×30×30 cm plastic cage (Bioquip Products Inc., Rancho Dominguez, CA, USA). Plastic cages containing pans with prepupae were then placed in a Percival® incubator set at 27 °C, 70% RH, and 12:12 light:dark (L:D) cycle (Holmes *et al.*, 2017; Tomberlin *et al.*, 2009). Prepupae were monitored every 12 h for adult emergence. As a standard practice, flies emerging within the first 24 h were excluded from the experiment due to insufficient numbers (Lin, 2016). Adults emerging during the subsequent 12 h were collected, sexed (Oliveira *et al.*, 2016), and used in the thermal preference study. For the *hsp* expression, as well as sperm viability and count experiments, <12-h-old adults were transferred from the plastic pan into 1 l-glass Mason jars (n=10 flies of one sex per jar) covered with a breathable organza fabric. A wet cotton ball was placed in each jar to provide water *ad libitum*. A total of 10 jars (i.e. 10 technical replicates) were used per sex/treatment for the sperm count and viability experiments. Jars containing flies were maintained in the Percival incubator previously described were rotated daily within the incubator to reduce biases.

Thermal gradient set up

Two thermal gradients were constructed based on the design described by Anderson *et al.* (2013). Each gradient consisted of an aluminium sheet (85×20 cm) bent at the ends (10 cm on each end bent at 90°) so as to place one end into an ice bucket and the other in a hot water bath (WB02A11B Digital General-Purpose Water Bath, 2

l Capacity, 120 V/60 Hz, PolyScience®, Niles, IL, USA). This setup provided a range of surface temperatures from 10 °C to 50 °C (Figure 1). Temperatures along the gradient were recorded using four fine-wire fast-response k-type digital thermocouples (Model A0188598; Gain Express Inc., Hong Kong, China) affixed to the surface of the aluminium plate, at 2, 17, 37, and 51.5 cm along its length. To keep flies in contact with the aluminium plate, a closed arena (53×18×4 cm) constructed of Plexiglass was affixed to the surface of the aluminium plate. Vents were cut into the sides of the arena to allow airflow but were small enough to prevent flies from escaping (Figure 1A). Preliminary studies were conducted to ensure stability of temperature regime employed (Figure 1B) (Schilman and Lazzari, 2004).

Thermal preferences

The thermal preferences of males and females from seven age groups (<24, 48, 72, 96, 120, 144, and 168 h post pupal emergence) were measured. These ages were selected to represent sexually active adults up to senescence (Malawey *et al.*, 2019). For each sex and age group, ten individuals were placed in the middle of a thermal gradient and allowed to explore the thermal arena for 20 min before data recording began. Note, placement of flies in the middle

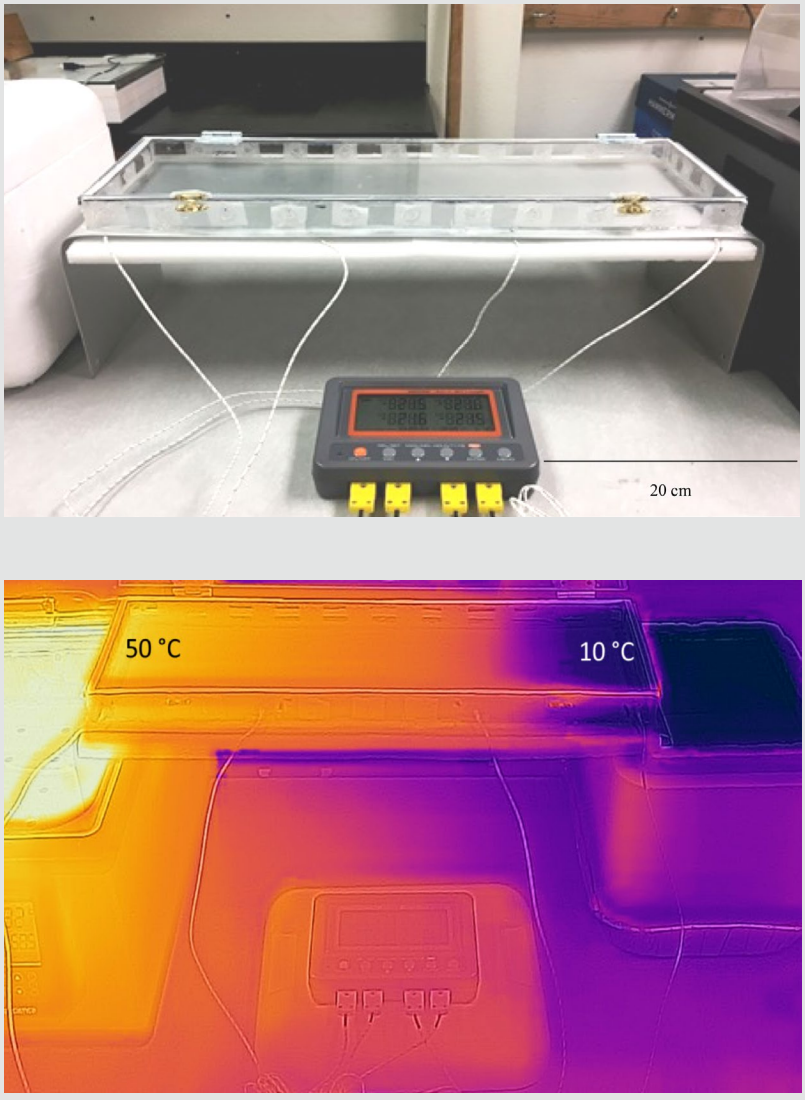

Figure 1. Image of (A) thermal gradient setup and (B) thermal variation of gradient, used to assess thermal preference of *Hermetia illucens* adults. Note the thermal gradient represented by the arrow pentagon created by a warm water bath and a cold water bath placed in each extremity of the metal plate, providing a temperature range of ~10-50 °C (±2 °C). The temperatures of the gradient were continuously recorded during experiments using four equidistant fine-wire fast-response digital thermocouples affixed to the aluminium plate (photography: Travis W. Rusch).

of the gradient was to prevent cold-trapping at the lower end of the gradient (Giraldo *et al.*, 2019) and to avoid heat shocking at the warm end of the gradient. After the 20-min exploratory period, a digital video camera (Canon® VIXIA HF R700, Tokyo, Japan) placed 1.5 m directly above the thermal gradient recorded the spatial positioning of the flies (i.e. the value on the x-axis within the range of 0 to 51.5 cm of the thermal gradient) for 1 h. To estimate the temperature at any position of the thermal gradient, the slope of the temperature gradient was estimated using a third-degree polynomial equation for each replicate/trial/sex within the age categories using the temperatures recorded from the four fine-wire fast-response digital thermocouples. Videos were analysed using Ethovision software (Noldus, Wageningen, the Netherlands), whereby the data point position of each fly on the surface of the thermal gradient was recorded every 5 s. A Cartesian coordinate system was established in Ethovision, with 0 cm starting from the cold side and 51.5 cm reaching the end of the hot side, to track the position of flies on the surface of the thermal gradient. Two trials with four replicates per trial per age group and sex were performed, with males and females tested independently.

Heat shock protein expression assay

The physiological response to environmental stress was tested through analysis of expression of the heat shock proteins *hsp70* and *hsp90*. Three temperatures were selected based on the temperature threshold for the population of this species: 18 °C (low temperature) (Holmes *et al.*, 2016), 27 °C (control temperature) (Harnden and Tomberlin, 2016), and 33 °C (high temperature) (Tomberlin *et al.*, 2009). Adult flies at early ages (72-h-old females and 96-h-old males) and late ages (144-h-old females and 168-h-old males) were exposed to the respective temperatures for 1 h in a I-36LLVLC8 Percival® incubator (Percival Scientific, Inc., Perry, IA, USA) before the adults were flash frozen in 100% ethanol mixed with dry ice and then stored in a -80 °C freezer until RNA extraction. Each fly was counted as a replicate and a total of two trials were performed for males and females, respectively (n=3 flies/age/sex/temp/trial). The humidity and photoperiod were set to previously determined optimal conditions described for this species (12:12 L:D; 70% RH) (Holmes *et al.*, 2017; Tomberlin *et al.*, 2009).

To extract RNA, individual adults were homogenised in 1 ml of TRI-Reagent® (Molecular Research Center, Inc., Cincinnati, OH, USA) and total RNA was extracted according to the manufacturer's protocols. Phase separation was performed with 4-bromoanisole (Molecular Research Center Inc., Cincinnati, OH, USA), and RNA was precipitated in ice-cold isopropanol and washed with 70% ethanol. RNA was resuspended in 99 µl of DEPC water with 1 µl of SUPERase (Invitrogen, Carlsbad, CA, USA). Quality (260/280 ~ 2.0, 260/230 ratio ~2.0-2.2) and quantity of RNA extractions were assessed with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc, Wilmington, DE, USA) to ensure concentrations of at least 500-700 ng/µl. Two µg of RNA from each sample were treated with amplification grade DNase I (Invitrogen) and converted to cDNA using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA, USA), as per the manufacturer's protocol. After cDNA synthesis, all samples were stored at -20 °C. Quantitative PCR (qPCR) was performed on a 10 µl reaction using 2 µl of the cDNA solution mixed with Sso Fast EvaGreen Supermix in a BioRad CFX96 Real-time system (Bio-Rad Laboratories, Hercules, CA, USA) for each of three biological replicates per age/sex/temp/trial. This was done to measure CT values and relative gene expression. Each sample was replicated once to obtain an average CT value between the two technical replicates. Quantification of *hsp70* and *hsp90* expression was performed using the Bio-Rad CFX96 Real-Time System with a C1000 Thermal Cycler (Bio-Rad Laboratories), using the primer and protocol information shown in Supplementary Table S1. *16s* and *ef1-a* were used as reference genes (Supplementary Table S1) after they were determined to be stable according to M-values estimated by the function geneStabM of the package SLqPCR in R (Kohl, 2007). All primers were selected based on their previous use with black soldier fly (Giannetto *et al.*, 2017). All runs were accompanied by a non-template control and a reverse-transcriptase control sample to ensure no genomic contamination. To estimate relative gene expression for each gene of interest, $2^{-\Delta\Delta CT}$ was calculated for each gene by using average CT values across the two technical replicates within each time point. The CT value for each target gene was standardised against the geometric mean of the reference genes.

Sperm count and viability analysis

The same methods and conditions described for rearing, selecting, and housing adult black soldier flies were used to assess sperm count and viability (see the '*Black soldier fly colony*' section above) with one modification: jars containing adults (n=10/jar) were placed in Percival incubators set at either 20.0, 24.0, or 27.0 °C (±0.5 °C, SD). Temperatures were selected based on the data available regarding optimal development of the black soldier fly (Booth and Sheppard, 1984; Shumo *et al.*, 2019). A total of ten jars, each containing ten flies, were placed in each incubator. The humidity and photoperiod were kept at optimal conditions described for this species (27 °C; 12:12 L:D; 70% RH) (Holmes *et al.*, 2017; Tomberlin *et al.*, 2009). Flies were exposed to treatments for at least 12 h prior to initiating sperm viability measurements to allow for a given temperature to have potential effects on the flies. To diminish the impacts of air temperature on the results, all flies from the same temperature treatment were dissected within 15 min of their removal from the

incubator. Over the course of the experiment, jars were randomly rotated daily within incubators to reduce biases.

Semen collection and sample preparation

Males from the same seven age groups (<24, 48, 72, 96, 120, 144, and 168 h post eclosion) were examined for sperm count and viability when exposed to 20, 24, or 27 °C for 12 h in a Percival incubator with RH of 70% RH and a 12:12 (L:D). A single jar containing ten adult males from each temperature was removed daily from each temperature. All flies from each jar were dissected following methodology of Eckel *et al.* (2017). The testes and seminal vesicles were removed from each individual using dissecting tools under a binocular stereoscope (SW-2B13-V331, AmScope™, Stephens city, VA, USA). Samples from each age/temperature cohort were placed into labelled 1.5 ml microcentrifuge tubes containing 30 µl of sperm diluent (0.24 g HEPES, 0.88 g NaCl and 1 g BSA diluted in 100 ml of diH_2O) to maintain physiological conditions in order to improve sperm longevity for dual florescent staining and counting (Rzymski *et al.*, 2012). Dissecting needles (insect pins morpho black enameled No. 2, catalog number: 1208B2, Bioquip Products Inc.) were used to cut the submerged tissues (testes and seminal vesicles) to release sperm. Sperm diluent was also used during dissection, whereby abdomens were immersed during dissection to ensure that sperm viability was preserved. After allowing the sperm to flow into the sperm diluent used for dissecting for 10 s, 6 µl of mixture of sperm and sperm diluent were collected and combined with an additional 30 µl of sperm diluent, 3 µl of sybr-14 (1 mM in DMSO) (Life Technologies™, Carlsbad, CA, USA) and 3 µl of propidium iodide (PI, 2.4 mM in water) (Life Technologies), which differentially stained viable (green) and non-viable sperm cells (red), respectively (Collins and Donoghue, 1999). The prepared tubes were then gently inverted to homogenise the samples and kept in darkness for approximately 10 min to allow interaction between sperm, sybr-14 and PI and facility dye uptake (Eckel *et al.*, 2017). The semen from three males per each age/temperature cohort were pooled for the sperm count and viability analysis following procedures also adapted from Eckel *et al.* (2017). The viability and count of the sperm for each treatment were analysed using the Nexcelom Cellometer® Vision CBA Image Cytometer (Nexcelom Biosciences LLC, Lawrence, MA, USA).

Statistical analysis

Nonparametric tests were used for all assays because the groups tested violated the assumption of normality and homogeneity of variance. For the thermal preference, sperm count, and viability assays, the Kruskal-Wallis test (H) was used to determine significant differences among ages; when significant differences were determined, a Wilcoxon method for pairwise comparisons was used to determine differences between two independent treatments. Bonferroni corrections were applied to all *P*-values to compensate for multiple comparisons (Newson, 2002). Statistical significance prior to Bonferroni corrections was set at a α=0.05. The thermal preference (T_{sel}) was established based on the central 50% (median) of the spatial position, which were transformed to surface temperatures (described above), observed on the thermal gradient for all individuals per age/sex. The 25% and 75% quartiles were set as the lower and upper limits of the T_{sel} range (Hertz *et al.*, 1993; Kirchhof *et al.*, 2017) and were presented as the interquartile range (IQR). A Nominal logistic regression was performed to assess interaction effects between temperature and age on sperm count and viability. All analyses were conducted in JMP pro 14.3 (SAS©). For *hsp* expression, ANOVA was performed with the aov() function in the package 'stats' in R. 3.6.1. Tukey's HSD tests were performed with the function TukeyHSD() in the package 'stats' in order to test for significant differences in gene expression among sex, age and temperature groups. In order to verify no differences with parametric and non-parametric analyses, the *hsp* data were also analysed with Kruskal-Wallis test using the kruskal.test() function from the 'stats' package and a post-hoc Dunn's test with correction for multiple tests using the dunnTest() function from the package FSA. Results for these analyses were similar to the ANOVA and Tukey's HSD, so ANOVA and Tukey's results are presented here. Statistical analyses were conducted in R 3.6.1, with the level of significance set at $P<0.05$.

3. Results

Thermal preference (T_{sel})

Without considering age as a factor, typical of mass-production facilities, males and females appear to select similar median T_{sel} (male median=22.3 °C; Q1-Q3 range: 16.6-28.6 °C; female median=21.5 °C; Q1-Q3=16.4-25.4 °C) (Supplementary Table S2). However, upon closer examination, aging significantly impacted the T_{sel} displayed by males (H=15,841.5, df=6, $P<0.0001$) and females (H=5,089.8, df=6, $P<0.0001$). Males <24-h-old T_{sel} temperature preference was warmer (median=24.3 °C; Q1-Q3=19.3-28.2 °C) (Supplementary Table S3, Figure 2), than females at the same age (median=20.2 °C; Q1-Q3: 15.4-26.2 °C) (Supplementary Table S4, Figure 3). Male T_{sel} preference gradually decreased by 3.4 °C until age 72-h-old ($P<0.0001$) and then increased to 23.6 °C at age 168-h-old ($P<0.0001$). Female T_{sel} increased by 3.8 °C from age <24-h-old to 48-h-old ($P<0.0001$) and then decreased by 2.3 to 21.7 °C at 168-h-old (Figure 3).

Hsp expression assay

M-values for *ef1-α* and *16s* were below the accepted value of 1.0 and were considered reliable for use as housekeeper genes (Supplementary Table S1). No effect of trial was

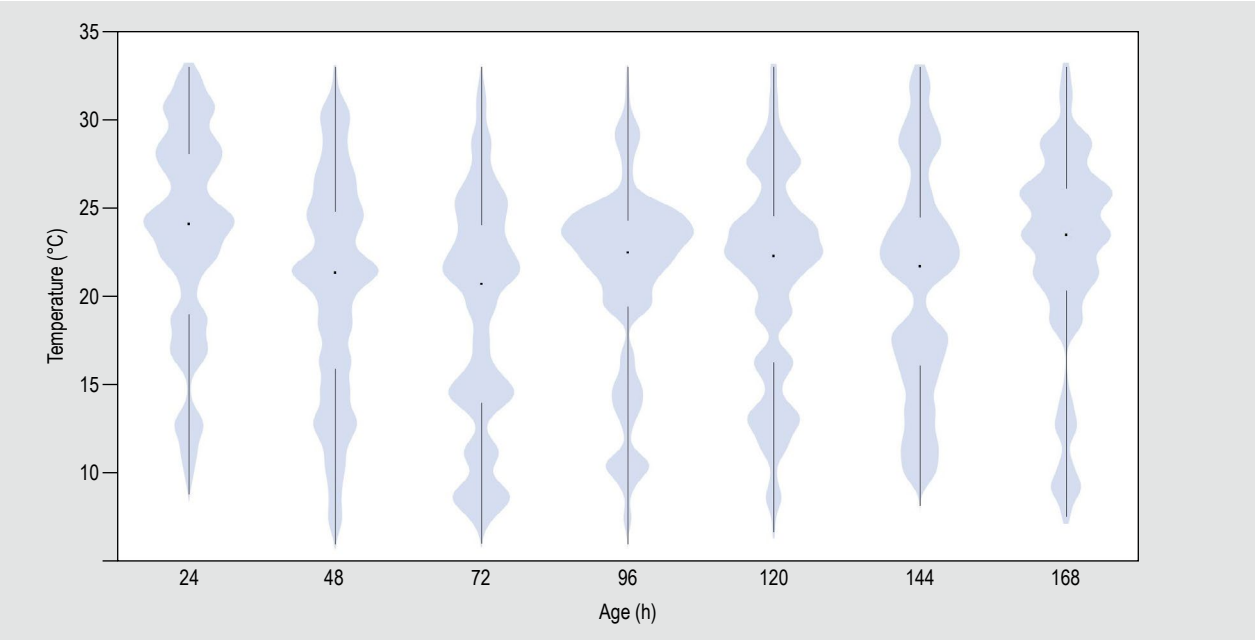

Figure 2. Violin plots of the thermal preference (T_{sel}) represented by the median for aging (h) adult male *Hermetia illucens* at 27.0±1.7 °C, 70% RH, and 12:12 L:D. Variance is given in interquartile range (IQR); IQR: median-Q1 (lower values); IQR: Q3-median (upper values); (*P*<0.05).

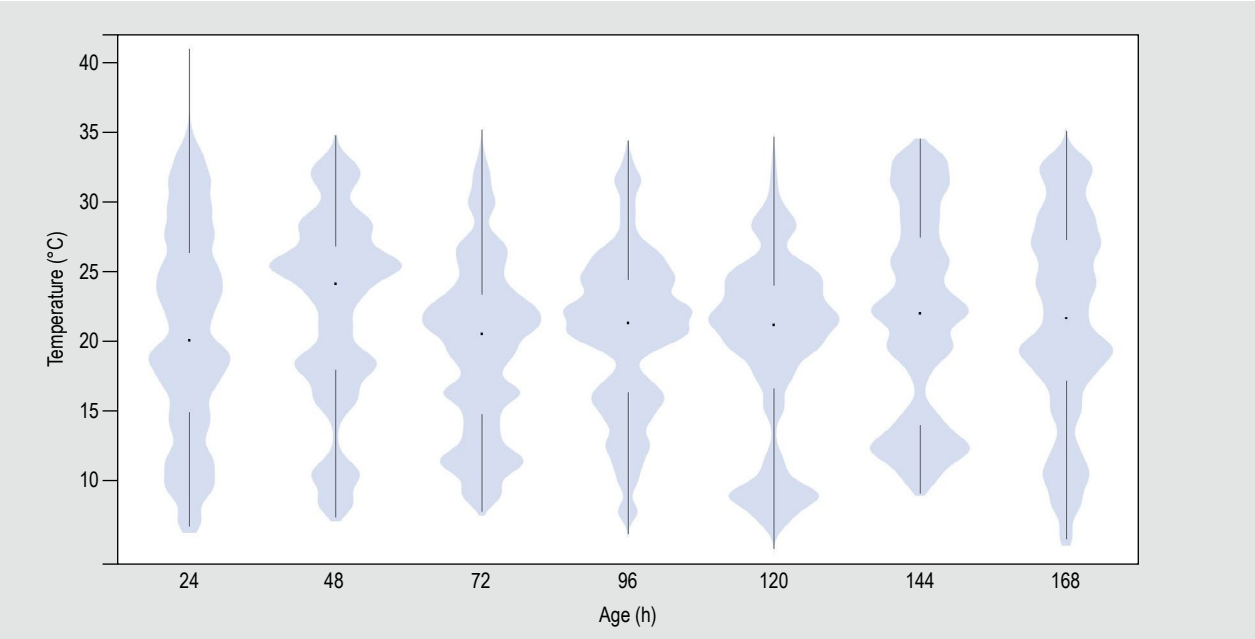

Figure 3. Violin plots of thermal preference (T_{sel}) represented by the median for aging (h) adult female *Hermetia illucens* at 27.0±1.7 °C, 70% RH, and 12:12 L:D. Variance is given in interquartile range (IQR); IQR: median-Q1 (lower values); IQR: Q3-median (upper values); (*P*<0.05).

seen for qPCR results for each sex, and therefore all results were combined among trials ($P>0.05$). When investigating expression of *hsp70* and *hsp90* for females, no significant differences were found between early and late age individuals, or by temperature treatments (Figure 4, $P>0.05$). However, when investigating expression of *hsp70* and *hsp90* for males, significant differences in expression were found between early and late aged male adults (*hsp70* $P<0.05$; *hsp90* $P<0.05$; Figure 5), but not significantly by temperature ($P>0.05$; Figure 5). Overall, males appeared to express higher levels of *hsp*, especially as they aged (Figure 4 and 5). When comparing fold-change values in

expression between sexes, a significant interaction was seen between sex and age (*hsp70* $P<0.05$; *hsp90* $P<0.05$) supporting significant differences in *hsp* expression between males and females of different ages, with notably higher expression in males in some ages than females, and vice versa. The Tukey's HSD of the complete model, corrected for all tests, did not find any significant differences between any female age or temperature treatments, however, although expression was significantly higher for males than females (Tukey HSD, *hsp70* $P<0.001$, *hsp90* $P<0.001$). When looking within males, *hsp70* expression was significantly higher in all late age treatments than in early treatments, aside from the 33 °C treatment ($P<0.05$; Figure 5). Expression of *hsp90* was significantly higher in late aged males across all treatments, except the 33 °C treatment in early aged adults, which was not significantly different from the late age treatments ($P<0.05$; Figure 5).

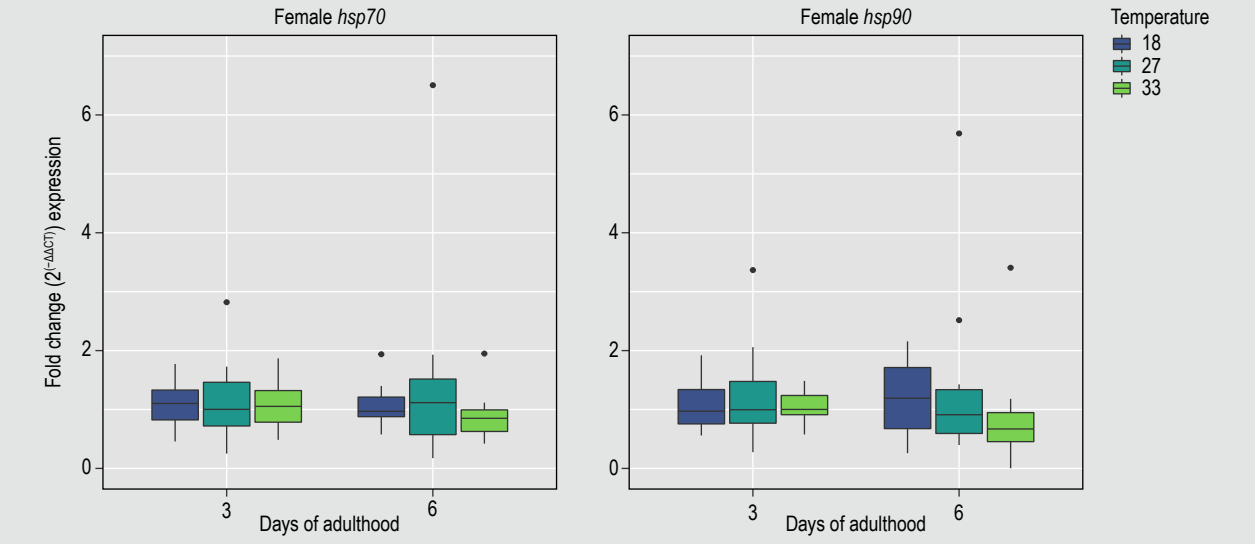

Figure 4. The fold change ($2^{(-\Delta\Delta CT)}$) expression of *hsp70* and *hsp90* in females at two ages across three temperatures. The middle lines of boxes represent the median expression with the edges of the boxes representing the first and third quartiles. Dots represent points determined to be outliers from ggplot2 software package. No significant differences were found between temperatures, days of adulthood, or genes (Tukey HSD, $P>0.30$) Adult ages of males and females are different because males emerge a day ahead of females, thus adult flies from the same age cohort will have post-emergence ages that differ.

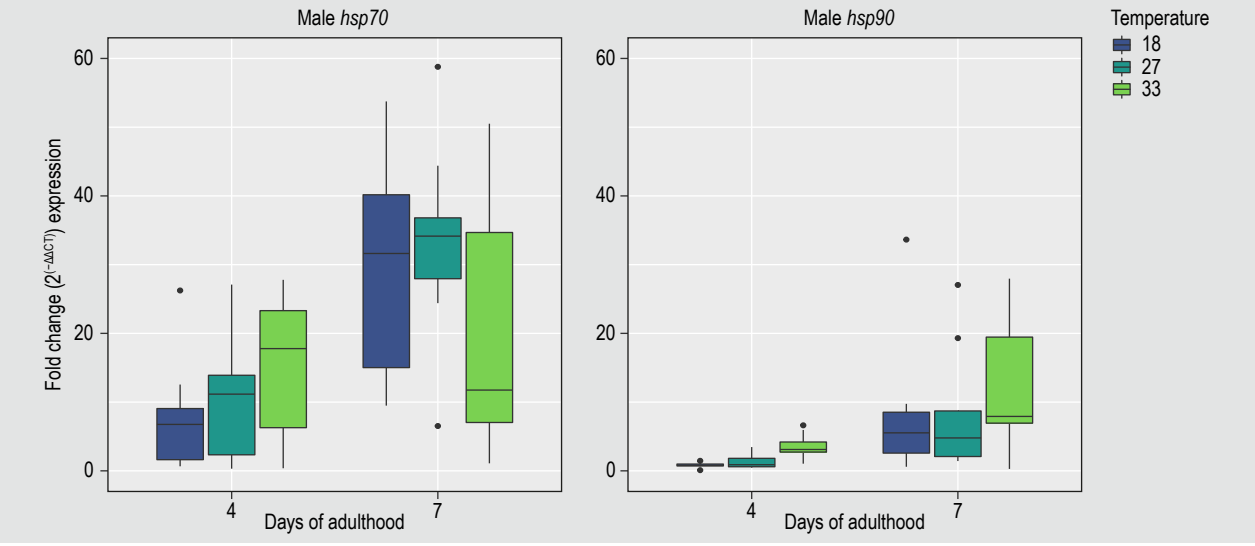

Figure 5. The fold change ($2^{(-\Delta\Delta CT)}$) expression of *hsp70* and *hsp90* in males at two ages across three temperatures. The middle lines of boxes represent the median expression with the edges of the boxes representing the first and third quartiles. Dots represent points determined to be outliers from ggplot2 software package. Adult ages of males and females are different because males emerge a day ahead of females, thus adult flies from the same age cohort will have post-emergence ages that differ. Significant differences in expression for both *hsp70* and *hsp90* were found between days of adulthood (Tukey HSD, $P<0.0001$). No significant differences were seen by temperature treatment (Tukey HSD, $P>0.30$).

Sperm count and viability

Age significantly impacted sperm count (Supplementary Table S5) and viability (Supplementary Table S6) of males (H=39.88, df=6, P<0.05 and H=17.35, df=6, P<0.05, respectively). Adults 48-h-old had significantly more sperm cells (Supplementary Table S5) (median=322.5 cells; Q1-Q3 cells=214.5-429.3), while 168-h-old males had 50% fewer sperm cells (median=167.0 cells; Q1-Q3=96.0-275.3) (P=0.0023; Figure 6). Regarding sperm viability (Supplementary Table S6), 48-h-old males had significantly greater sperm viability (median=78.3%; Q1-Q3=70.5-81.2%), while 144-h-old males had 25% lower viability (median=59.5%, Q1-Q3=55.9-63.1%) (P<0.05; Figure 7). There was no statistically significant effect of temperature on sperm count or viability (H=1.11, df=2, P>0.05 and H=0.45, df=2, P>0.05, respectively). Moreover, there was not a statistically significant interaction between age and temperature on sperm count or viability ($F_{12,126}$=0.37, P>0.05 and $F_{12,126}$=1.30, P>0.05, respectively).

4. Discussion

Thermal preferences of adult black soldier flies varied based on sex initially, but mostly converged as they aged. These finding are similar to those determined for other insect species. The kissing bug, *Rhodnius prolixus* Stål (Hemiptera: Reduviidae), which has a similar Western hemisphere distribution as the black soldier fly, exhibited an average thermal preference of 25.0 °C for males and 25.4 °C for females (Schilman and Lazzari, 2004). The same was determined for male and female of *Drosophila tripunctata* Loew (Diptera: Drosophilidae) in the laboratory with both sexes displaying a similar thermal preference of 21.5 °C (Dillon *et al.*, 2009).

In our study, young adult males (<24-h-old) preferred a warmer T_{sel} of 24.3 °C, while females of the same age preferred a cooler T_{sel} of 20.2 °C. However, females selected warmer temperatures as they aged until they reached 48-h post eclosion, while males progressively selected cooler temperatures until they reached 72-h-old. A similar relationship has been observed for male and female *Drosophila virilis* Sturtevant (Diptera: Drosophilidae), whereby males select significantly lower temperatures on a thermal gradient than females (Yamamoto, 1994).

The findings that virgin black soldier fly females preferred a higher T_{sel} than males during the initial 48-h post eclosion could be due to differences in physiological mandates. Females, being synovigenic (Jervis *et al.*, 2001), develop eggs during the first 48 h prior to mating (Tomberlin and Sheppard, 2002), while males emerge with mature sperm and thus do not need to undergo spermiogenesis after eclosion (Malawey *et al.*, 2019). Interestingly, the wasp *Ooencyrtus nezarae* Ishii (Hymenoptera: Encyrtidae), which is also synovigenic, responds similarly to black soldier fly, with females exposed to warmer temperatures being more fecund than females exposed to cooler temperatures (Aung *et al.*, 2011). The data reported by Chia *et al.* (2018) with black soldier fly females supports the results found in the present study, whereby adults maintained at 30 °C had

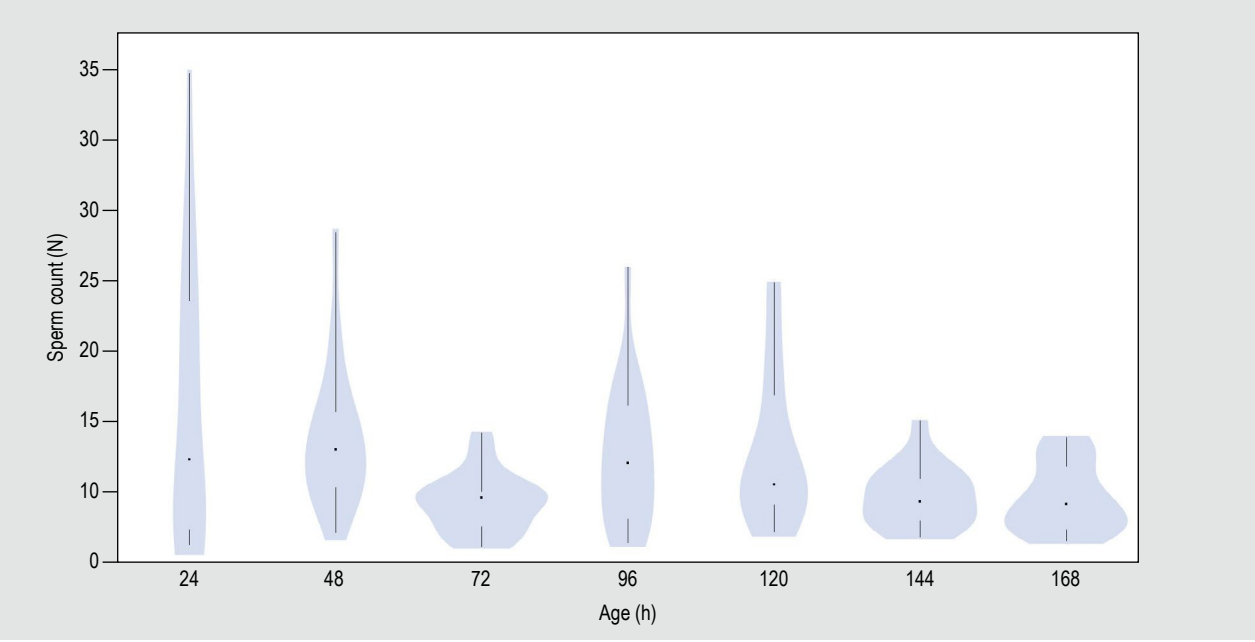

Figure 6. Violin plots of sperm count represented by the median for aging (h) adult male of *Hermetia illucens* at 27.0±1.7 °C, 70% RH, and 12:12 L:D. The variance is given in interquartile range (IQR); IQR: median-Q1 (lower values); IQR: Q3-median (upper values); (P<0.05).

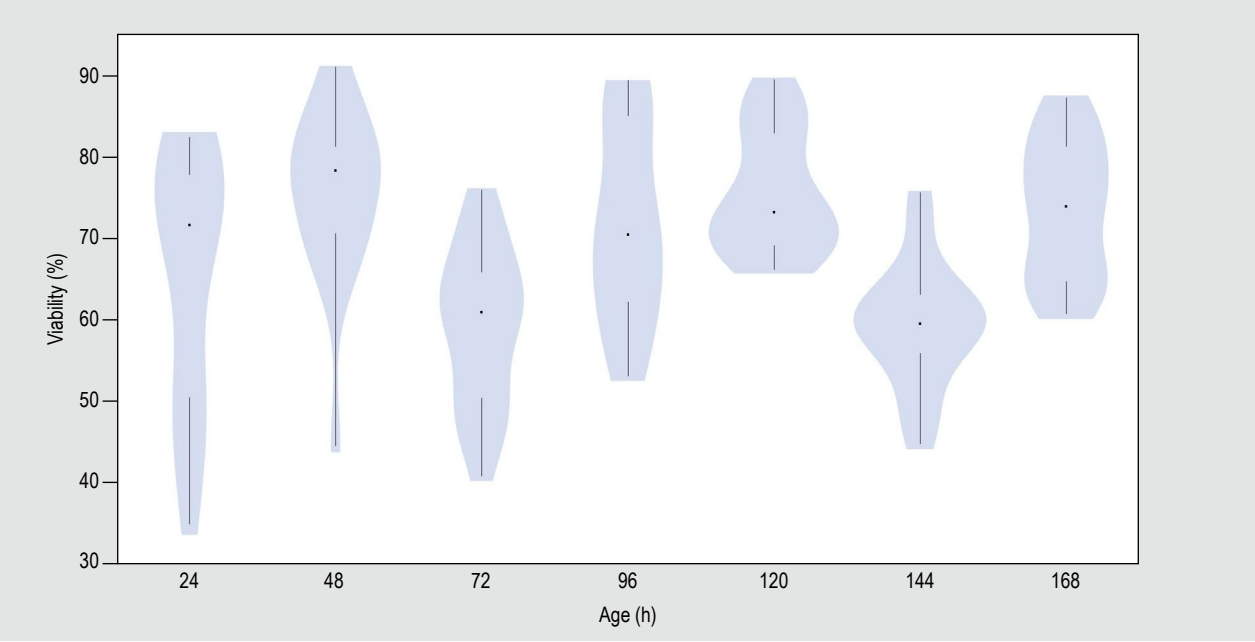

Figure 7. Violin plots of sperm viability represented by the median for aging (h) adult male of *Hermetia illucens* at 27.0±1.7 °C, 70% RH, and 12:12 L:D. The variance is given in interquartile range (IQR); IQR: median-Q1 (lower values); IQR: Q3-median (upper values); (*P*<0.05).

more viable eggs (80% viability) than those kept at 15 °C (viability below 11%) (Chia *et al.*, 2018).

Our finding that males preferred lower temperatures as they aged could potentially be used during management procedures of indoor settings by establishing a temperature that would enhance sperm viability over time. Males used in the present study were virgins and black soldier fly males are known to emerge with a full batch of functional sperm (Malawey *et al.*, 2019); therefore, maintaining a full complement of sperm and mating for the greatest duration of time could result in greater fitness for black soldier fly males as it could allow for maximum sperm transfer to females. As black soldier fly males had significantly increased *hsp70* and *hsp90* expression as they aged, seeking out cooler temperatures in the absence of females may reduce the energetic impacts of maintaining their sperm with these chaperone proteins.

Conversely, adult black soldier fly males 96-h-old or older preferred a warmer T_{sel} (e.g. 23.6 °C at age 168-h-old). This pattern could reflect a last attempt by males to locate females aggregated in warm environments to mature their eggs. This behavioural strategy is thought to exist in other insects, as different performances have different optimal temperatures (Sinclair *et al.*, 2012), including reproductive success (Kindle *et al.*, 2006). In addition to an increased T_{sel}, we found that older black soldier fly male also increased expression of *hsp* with age. One potential explanation for this upregulation is that increased expression of *hsp* with age may help to combat the detrimental effects of increased temperature on sperm viability, although other aspects of physiology may also be relevant to this pattern of increased expression. The increase in *hsp* expression could assist with the static sperm viability with decreased sperm count, as seen in this study. It is interesting to note, as well, that there were not significant increases in *hsp* expression in females as in males. In order to elucidate the functional relevance of increased *hsp* in males and not females, this study must be followed up with further studies of expression in specific tissues (Rout *et al.*, 2016; Scott *et al.*, 2003).

Sperm count decreased as black soldier fly adults aged (<24-h-old male with 297 sperm/individual to 168-h-old male with 167 sperm/individual), while sperm viability remained relatively consistent at ~70%. Despite being restricted to a small environment without resources, males were able to maintain viable sperm. This could potentially be due to males allocating additional resources to sperm nourishment or production that otherwise would have been used for flight and copula attempts.

Malawey *et al.* (2019) determined spermatogenesis in black soldier fly is initiated during pupation. Males emerge with a full complement of mature sperm that increases in number during the first 48 h. The data generated from the current study corroborate those earlier findings. In fact, sperm cell count peaked in 48-h-old males indicating unmated males could potentially continue to produce sperm as they age, but at low levels.

Based on results from the current study, sperm production in older flies is not able to compensate for natural cell mortality (i.e. number of sperm cells decreases as adults age). This response could be due to degeneration of stem cells, which is common in short-lived species (Dumser, 1980). Similar results have been recorded for male *D. melanogaster*. Interestingly, in males that are sexually constrained as previously described, their seminal fluid proteins accumulate over time (Sepil *et al.*, 2019). Thus, the size of the sperm package potentially does not change as the adult ages, just the sperm concentration. In such instances, low sperm counts would reduce egg fertilisation (Paoli *et al.*, 2013), but decrease the likelihood of females quickly mating again (Wedell and Cook, 1999). Unfortunately, if this is the case with the black soldier fly, such factors could explain why egg production and hatch rate are highly variable.

The impact of age and temperature on sperm viability was selected *a priori* based on studies examining adult black soldier fly biology (Booth and Sheppard, 1984; Giunti *et al.*, 2018; Sheppard *et al.*, 2002; Tomberlin and Sheppard, 2001, 2002) and larval development (Chia *et al.*, 2018; Gligorescu *et al.*, 2018; Harnden and Tomberlin, 2016; Sheppard *et al.*, 2002; Shumo *et al.*, 2019). Treatment temperatures were based on those most likely to be used in mass production of the black soldier fly (Hoc *et al.*, 2019). Therefore, determining if these temperatures were 'stressful' by impacting sperm viability provides valuable information to black soldier fly production facilities aiming to optimise fertile egg production.

The temperatures examined in the current study were not outside the species' thermal preference and did not significantly impact sperm viability. However, it should be noted the thermal tolerance data presented here were determined after the sperm viability study was completed. Nevertheless, these results do show a safe non-stressful temperature range that mass production facilities could keep black soldier fly adults at without impacting male sperm viability.

Future studies should establish the thermal preference range first and then explore the impact of temperatures outside this range to reveal the critical temperatures at which sperm viability is decreased as have been determined for other insects (Dickinson, 2018; Houston *et al.*, 2018; McAfee *et al.*, 2019). To fully investigate the relationship between temperature and sperm viability in black soldier fly, future studies should also incorporate exposure time as part of the study, as exposure to non-lethal but suboptimal temperatures for extended periods of time can have negative consequences on flies (Rusch *et al.*, 2019). In the current study, exposure duration was dictated by age (e.g. 168-h-old individuals were exposed for that amount of time to an assigned temperature). It would be interesting to determine how variable temperature exposure impacts sperm viability, as has been shown in other study systems (Dickinson, 2018; Houston *et al.*, 2018; Zizzari and Ellers, 2011).

As demonstrated in the current study, adults have preferred temperatures that shift as they age. Furthermore, the number of viable sperm decreases as adult males age. Thus, developing methods allowing adult males and females to aggregate at the appropriate time, or lek, as in the case of the black soldier fly, is critical to maximise colony fitness. Unfortunately, published methods continuously mass release adults into a cage over time, resulting in various age cohorts being commingled (Hoc *et al.*, 2019; Jucker *et al.*, 2019; Miranda *et al.*, 2019; Sheppard *et al.*, 2002; Tomberlin and Sheppard, 2002).

This strategy for colony management is potentially sub-optimal with regards to producing viable eggs optimally and consistently. Given that females are not ready to mate until two days post emergence (Tomberlin and Sheppard, 2001) and males emerge with viable sperm (Malawey *et al.*, 2019), such a relationship could result in disruptive mating, with males attempting to mate with females that are not ready or that have already mated, and males carrying reduced sperm loads by the time females are ready to mate. Future studies should examine the physiological particularities of black soldier fly males and females in order to enhance mating success and thus the collection of more fertilised eggs, ultimately improving the mass production of the black soldier fly.

Furthermore, results generated from this study are population specific. Recent evidence indicates a high degree in genetic variation across industrialised black soldier fly colonies (Ståhls *et al.*, 2020). Therefore, similar studies should be conducted across colonies to determine population plasticity in response to temperature. Such studies would allow for a meta-analysis to determine if these results are in fact population specific or general features of the species.

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Conflict of interest

The authors declare no conflict of interest.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/JIFF2020.0017.

Table S1. Primers used for sequencing genes regulating heat shock protein expression in the black soldier fly.

Table S2. Quartile (Q1, Q2, and Q3) and the interquartile range (IQR) of the temperature selection by *Hermetia illucens*.

Table S3. Quartile (Q1, Q2, and Q3) and interquartile range (IQR) of the temperature preferences by adult male *Hermetia illucens*.

Table S4. Quartile (Q1, Q2, and Q3) and interquartile range (IQR) temperature preferences by adult female *Hermetia illucens*.

Table S5. Quartile (Q1, Q2, and Q3) and interquartile range (IQR) for sperm counts (N) of adult male *Hermetia illucens*.

Table S6. Quartile (Q1, Q2, and Q3) and the interquartile range (IQR) for sperm viability (%) displayed by adult male *Hermetia illucens*.

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