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Rearing density effects on a commercially important insect species

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UNIVERSITY OF TARTU
Press

Department of Zoology, Institute of Ecology and Earth Sciences, Faculty of Science and Technology, University of Tartu, Estonia

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CONTENTS

LIST OF PUBLICATIONS

This thesis is the summary of the following papers, which are referred to in the thesis by their bold Roman numerals (**I–IV**).

I. **Opare, L. O.**, Holm, S., and Esperk, T. (2022). Temperature-modified density effects in the black soldier fly: Low larval density leads to large size, short development time and high fat content. Journal of Insects as Food and Feed, 8, 783–802. https://doi.org/10.3920/JIFF2021.0147

II. **Opare, L. O.**, Meister, H., Holm, S., Kaasik, A., and Esperk, T. (2023). High larval densities and high temperatures lead to a stronger immune response in the black soldier fly. Journal of Insects as Food and Feed, 9, 1177–1186. https://doi.org/10.3920/JIFF2022.0109

III. **Opare, L. O.**, Jensen A. B., Lecocq, A., Holm, S., and Esperk, T. (2024). Exposure to entomopathogenic fungus and high larval density induce a strong immune response and life-history costs in black soldier fly, a commercially important insect. Entomologia Experimentalis et Applicata, 1–10. https://doi.org/10.1111/eea.13449

IV. **Opare, L. O.**, Holm, S., and Esperk, T. (2024). Interaction effects of substrate fermentation and larval density on black soldier fly life-history traits. Journal of Insects as Food and Feed, 1–12. https://doi.org/10.1163/23524588-00001188

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Author's contribution to the studies (** denotes a high contribution, *** a leading role)

	I	II	III	IV
Original idea	**	**	**	***
Study design	***	***	***	***
Data collection	***	***	***	***
Analysis and interpretation	***	***	***	***
Manuscript writing	***	***	***	***

1. INTRODUCTION

Life history theory delves into the intricate ways organisms allocate their resources within dynamic environments to maximise their fitness (Roff, 2002; Stearns, 1992). In particular, this well-established concept seeks to understand how organisms evolve to maximise their fitness by optimising a set of life history traits when faced with various ecological challenges (Flatt & Heyland, 2011; Hutchings, 2021). In addition, research on life history evolution has been used to explore features specific to certain taxa, such as their life cycles and decisions relating to life history, sex allocation patterns and transitions from larval to the adult stage (Charlesworth, 1994; Stearns, 1992).

Principal life history traits are closely interconnected and include fecundity, body size, development time, survival, growth rate, age and size at maturity, and adult lifespan (Stearns, 1992). Although immunity is not commonly regarded as a key life history trait, its strong association with survival and fitness suggest it should be considered one. This perspective has been adopted in several recent studies (Pinzón et al., 2014; Stutchbury & Morton, 2023; Tieleman, 2018). These principal life history traits typically trade off against each other, meaning that as the value of one trait increases, the value of the other decreases (Hutchings, 2021; Roff, 2002; Stearns, 1992). This is because, due to limited resources, organisms typically cannot allocate high amounts of resources simultaneously to different life history traits (Stearns, 1989; Zera & Harshman, 2001).

Perhaps the best-known trade-off between life history traits is the trade-off between reproduction and longevity – when fertility increases, life expectancy typically decreases and vice versa (Takeshita, 2024). Other life history traits, such as body size, have also been long recognised to have extensive associations with other traits, driven by shared regulatory mechanisms and resulting in trade-offs. For instance, larger body size can lead to trade-offs with survival (Bright Ross et al., 2021; Oteyza et al., 2021; Tammaru & Teder, 2024; Teder & Kaasik, 2023), fecundity (Calvo & Molina, 2005; Gergely & Tökölyi, 2023; Lopez & Hoddle, 2014) and development time (Davidowitz & Nijhout, 2004; Davidowitz et al., 2005; Nijhout et al., 2010; Teder et al., 2014). An increase in growth rate aimed at achieving a larger body size can, in principle, shorten development time (Nijhout et al., 2010). However, high growth rates are known to have costs on their own (Fischer et al., 2005; Gotthard, 2000) and may be traded off with other life history traits (Lee et al., 2013; Lemaître et al., 2015).

Environment plays a key role in influencing optimal values of life history traits, and associations between different life history traits are highly influenced by environmental factors (Flatt & Heyland, 2011; Roff, 2002). These environmental factors, spanning both biotic and abiotic elements, include but are not limited to temperature (Issimov et al., 2020; Silva & Elliot, 2016), diet quality and quantity (Lee et al., 2006; Lopez & Hoddle, 2014), conspecific density (Sørensen & Loeschcke, 2001; Tammaru et al., 2000; Vellau & Tammaru, 2012), disease risk and level of predation (Anderson & May, 1979; Gotthard, 2000; May

& Anderson, 1979). For example, temperature plays a crucial role in shaping key life history traits such as body size (Forster et al., 2012; Verberk et al., 2021), development time (Huang et al., 2021; Tomberlin et al., 2009) and growth rate (Frazier et al., 2006). Diet quality and quantity, on the other hand, are known to influence development time and size at maturity (Morimoto et al., 2022; Parker et al., 2023; Teder et al., 2014).

Key determinants of an organism's life-history traits and fitness are conditions related to their development (Barnes, 2021; Nylin & Gotthard, 1998; West-Eberhard, 2003). Conditions that occur during the early life stages can have a long-lasting influence on organisms' fitness and life history traits (Criscuolo et al., 2008; Monaghan, 2008). Conspecific density represents a crucial biotic environmental factor that profoundly influences key life history traits and overall fitness across a diverse spectrum of organisms. The tendency to aggregate in environments of high conspecific density during the larval stage (termed larval density afterwards) emerges as a critical factor to consider, particularly among insects and other organisms characterised by high fecundity (Fantinou et al., 2008; Kawecki & Stearns, 1993; Tammaru et al., 2000). For instance, due to larval density effects, phase polyphenism occurs in locusts and other orthopterans, resulting in gregarious and solitary phases that exhibit strikingly different morphology, physiology and behaviour (Pener, 1991; Pener & Simpson, 2009; Pflüger & Bräunig, 2021).

Larval density serves as an important selection agent by influencing the life history traits of most insect species (Kawecki & Stearns, 1993; Tammaru et al., 2000). In particular, increased larval density could often lead to a darker colour and higher metabolism (Pener & Simpson, 2009; Pflüger & Bräunig, 2021), more intense intraspecific competition (Agnew et al., 2002; Schrader et al., 2015), increased cannibalism (Pervez et al., 2006; Porretta et al., 2016), increased pathogen concentration (Anderson & May, 1979; May & Anderson, 1979), reduced body mass (Esperk et al., 2007; Opare et al., 2022, 2024a; Parra Paz et al., 2015) and stronger immune response (Cotter et al., 2004; Opare et al., 2023, 2024a; Wilson & Reeson, 1998).

The effect of density on certain traits may vary qualitatively between different species or in specific environments. For example, high densities may either reduce (Esperk et al., 2007; Tammaru et al., 2000; Vellau & Tammaru, 2012) or prolong (Barragan-Fonseca et al., 2018; Dzepe et al., 2020; Opare et al., 2022) development time and either increase (Applebaum & Heifetz, 1999; Opare et al., 2022; Pener & Simpson, 2009) or decrease (Sørensen & Loeschcke, 2001; Zwaan et al., 1991) mortality. This discrepancy may arise from conspecific density acting simultaneously as a cue for a dangerous environment or depleting resources – leading to an expected increase in development rate (Teder et al., 2014; Vellau & Tammaru, 2012) – and as a stressor that inhibits larval development through direct or indirect effects (Morimoto et al., 2022; Than et al., 2020). On the other hand, most likely due to higher resource quality and quantity and less conspecific aggression, living in a low larval density environment generally leads to positive outcomes such as a higher body mass and shorter development times (Barragan-

Fonseca et al., 2018; Opare et al., 2022, 2024a; Parra Paz et al., 2015). However, low density could also have detrimental effects, such as reducing the ability to thermoregulate in colder temperatures (Rivers & Dahlem, 2022). In addition, due to the low amounts of digestive enzymes secreted at low densities, larvae may struggle to digest substrates collectively and effectively perform bioconversion of feed substrates (Green et al., 2002; Parra Paz et al., 2015; Sakaguchi & Suzuki, 2013).

The impact of larval density on life history traits, especially in conjunction with other environmental factors such as temperature, pathogens and feed substrate quality, has still received surprisingly little attention. In addition, exploring the effects of larval density on immune parameters could serve as a way to help us better understand the associations between life history traits in general. Moreover, understanding the physiological mechanisms and behavioural repertoires that insects use to cope with high and low larval densities in various environments is crucial for comprehending the evolution of their life histories. Furthermore, this knowledge could also be applied to manipulate insects for purposes such as selective breeding and optimisation in mass rearing.

Recently, insects and their life histories have attracted considerable attention due to the emergence of a new economic sector focussed on growing insects for feed and food. The demand for protein has correlated positively with the expanding global human population (Berggren et al., 2019; van Huis, 2013). As a result, global animal production has to grow remarkably to fulfil rapidly increasing demand; however, this comes with several ecological implications (van Huis, 2013, 2020). Therefore, there is a need to find alternative protein and energy sources as well as sustainable ways to mitigate these impending issues.

Edible insect species present promising solutions, offering valuable protein, frass and chitin (Rumpold & Schlüter, 2013; Tanga & Ekesi, 2024; van Huis et al., 2021). Compared to conventional livestock, edible insects exhibit superior performance in terms of feed conversion, greenhouse gas emissions and water use efficiency (van Huis, 2013). Rearing edible insects also contributes to the green economy as they can be raised on various organic side streams and food waste, a capability most conventional farm animals lack (Lalander et al., 2013, 2019; Lalander & Lopes, 2024; Lopes et al., 2023). However, the production potential for insects, both as food and feed, is likely substantially higher than what is currently being achieved (Belluco et al., 2013; van Huis, 2013). For example, fermentation – an old yet underutilised technology – could enhance the ability of insect larvae to digest the substrates high in cellulose and lignin content (Stanbury et al., 2003; Yang et al., 2001). However, our understanding of edible insects is still insufficient for optimising their mass production. This is because many crucial aspects of their physiology and life history remain largely unexplored (Berggren et al., 2019).

The life history theory framework can be utilised to optimise the mass production of commercially important insects. By specifying the trade-offs between life stage and environmental effects on life history traits, producers can focus on the traits and timing (life stage) that yield the optimal cost-benefit ratio and maximum output. Furthermore, producers can tailor their breeding and rearing practices to enhance desired traits while minimising production costs (Alonzo & Kindsvater, 2008; Stearns, 2000). Identifying the best timing for trait expression in different life stages helps maximise yield and efficiency in insect production. For example, selecting individuals with traits that promote rapid growth and efficient resource utilisation during larval stages may lead to higher biomass production within a shorter time frame. Conversely, focusing on traits associated with increased fecundity and reproductive output during adult stages may enhance overall reproductive success and yield. In essence, the life history theory framework provides producers with a systematic approach to navigating the complex interactions between life stage, environment, and life history traits in insect production.

The insect species being mass-reared most intensively are the yellow mealworm (*Tenebrio molitor*), house cricket (*Acheta domesticus*), house fly (*Musca domesticus*), and the black soldier fly (*Hermetia illucens*) (Tang et al., 2019; van Huis, 2020). According to the International Platform of Insects for Food and Feed (IPIFF), its members produce 6000 tonnes of insect food and feed annually (IPIFF, 2019). They also estimate that by the end of 2030, the amount of insect protein produced in Europe will reach a million tonnes, with the dominant mass-reared species being *Tenebrio molitor* and *Hermetia illucens* (IPIFF, 2021).

The black soldier fly (BSF) is one of the most reared species in the insects for food and feed sector, and the need to know more about its physiology has kept growing (Tomberlin & van Huis, 2020; van Huis et al., 2013,2020,2021). The BSF has received a lot of attention due to its potential for various purposes such as livestock feed (Dörper et al., 2021; Veldkamp & Vernooij, 2021), aquaculture feed (Liland et al., 2021; Mohan et al., 2022; Rawski et al., 2020), bioconversion (Arnone et al., 2022; Gao et al., 2019; Li et al., 2011), fertiliser (Setti et al., 2019; Xiao et al., 2018), and waste management (Lalander et al., 2013, 2019; Lopes et al., 2020, 2023). BSF thus holds substantial potential as a novel farm animal, and there is an increasing need to determine the environmental effects on the species to optimise its production (Berggren et al., 2019; van Huis, 2020). Several studies have explored environmental factors to optimise the BSF's key life history traits, such as prepupal, pupal and adult mass, development time, and survival. Environmental traits frequently used in studies include diet (Barragan-Fonseca et al., 2020; Cammack & Tomberlin, 2017; Gobbi et al., 2013), temperature (Chia et al., 2018; Harnden & Tomberlin, 2016; Tomberlin et al., 2009) and substrate moisture (Bekker et al., 2021; Dzepe et al., 2020). However, the effects of other important factors, such as larval density, have received less attention (but see Barragan-Fonseca et al., 2018; Parra Paz et al., 2015). Moreover, the interaction effects between various environmental factors on BSF remain largely unexplored.

The main objective of this thesis was to evaluate the effect of rearing density, as well as its interactions with temperature and diet, on the life history traits of BSF. We strived to understand how life history traits respond to varying rearing densities in different environments and how this knowledge can be exploited to optimise mass-rearing for food and feed production.

The specific aims of my thesis are:

1. To investigate larval density and temperature effects on life-history traits of BSF (**I**). Depending on the species and environment to which the larvae are exposed, larval density effects on life history traits may vary considerably. However, these effects are poorly investigated. Moreover, in most previous studies larval density is controlled only once at the beginning of the experiments. This approach may impact both the results and their interpretation, particularly if mortality depends on density. We reared BSF larvae at three different larval densities at three temperature regimes and evaluated the effects of these environmental factors on several life history traits. This would provide insights into the importance of larval density and temperature in regulating life history traits, thereby guiding informed decisions for optimising mass-rearing practices.

2. To evaluate the effects of three larval densities and three temperature treatments on the phenoloxidase activity of BSF larvae (**II**). In highly crowded environments, insect larvae are expected to invest more into their immunity to better prepare for pathogenic threats and depletion of resources, a phenomenon termed density-dependent prophylaxis. We sought to understand the underlying immune mechanisms that could help us explain the results we observed in **I** using the investment into phenoloxidase activity, as it is a frequently used measure of immunity in insect studies. We also provided a comprehensive overview of published case studies examining the associations between insect immunity and insect larval density, focusing on density-dependent prophylaxis. This would help to determine whether mass rearing at high larval densities leads to potential trade-offs with immune function. In environments of high larval density, insects have been reported to invest varying degrees into their immunity.

3. Assessing the combined effects of two strains of entomopathogenic fungus (EPF) *Beauveria bassiana* and two larval densities on the strength of immune response and other life-history traits of BSF larvae (**III**). Our aim was to determine whether an EPF-induced challenge, combined with an unfavourable larval density, would result in trade-offs with immune function. This helps us determine whether the observed increase in PO activity at a higher larval density in BSF larvae would persist or change in the presence of an additional stressor.

4. To measure the response of life history traits of BSF larvae when they are reared on fermented substrates versus standard substrates (**IV**). Fermentation offers a potential method to improve the quality of diets high in cellulose and lignin concentrations. On the other hand, larval density is recognised to interact with substrate type. By rearing BSF larvae on standard substrates (ensiled grass and brewer's spent grain) and on fermented substrates (additionally fermented ensiled grass and fermented brewer's spent grain), we aimed to test if substrate fermentation has positive effects on the life history traits of BSF larvae and if these effects depend on larval density.

2. MATERIALS AND METHODS

2.1. Study system

The black soldier fly (*Hermetia illucens* L., BSF) is a dipteran originating from the Neotropics. It is now widespread in the tropics and subtropics due to its adaptability to living in a wide range of environments (Sheppard et al., 1994). The BSF, like other dipterans, has a holometabolous life cycle consisting of egg, larval, prepupal, pupal and adult stages (Figure 1).

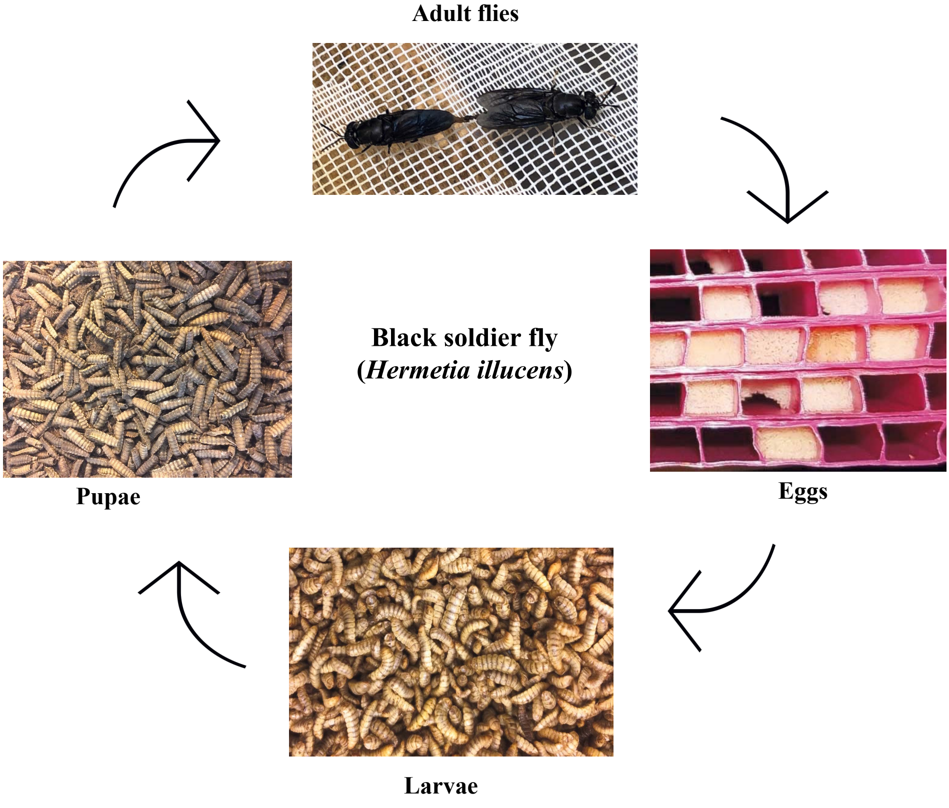

Figure 1. The life cycle of the black soldier fly (Photos: Sille Holm).

Under optimal conditions, the complete life cycle of the BSF is reported to last approximately 35 to 46 days (Chia et al., 2018; Harnden & Tomberlin, 2016). After the female BSF lays eggs, they take about 4 days to hatch (De Smet et al., 2018). The larval stage consists of six instars (Sheppard et al., 1994). The larval period lasts between 13 to 18 days or longer depending on environmental factors such as density, temperature, diet and substrate moisture (Barragan-Fonseca et al., 2018; Cammack & Tomberlin, 2017; Chia et al., 2018). The final larval instar involves emptying the gut followed by prepupation (Sheppard et al., 1994). Prepupae migrate to dark and dry areas to pupate; this period takes approximately 7 days (De Smet et al., 2018). The pupal stage typically lasts 7 to 14 days, after which adults emerge (De Smet et al., 2018). The adult BSF stage lasts about 6 days to 16 days (Chia et al., 2018; Laursen et al., 2024), and research has shown that it is possible to prolong their life by offering them water (Tomberlin & Sheppard, 2002) or sugar solution (Nakamura et al., 2016).

The BSF is also a naturally aggregating species (Shishkov et al., 2019; Shishkov & Hu, 2020), which makes it an ideal candidate for larval density experiments. Furthermore, the University of Tartu, Estonia, has had an existing colony running since 2017. The experiments for **I**, **II, and IV** were all carried out using the colony at the University of Tartu. The experiments for **III** were conducted in Denmark at the University of Copenhagen using BSF larvae originating from ENORM Biofactory A/S (Hedelundvej 15-8762 Flemming, Denmark).

2.2 Study design

2.2.1 Survival, body size, mass loss and development time and relative fat content measurements

We studied how different rearing densities, temperatures, entomopathogenic fungus exposure and substrate fermentation influence BSF life history traits **(I, II, III, IV)** (see Figures 2, 3, 4, 5 for the workflow of **I**, **II**, **III**, **IV**). The experiments involved rearing larvae at two or three different densities: 1 larva or 2 larvae/cm^2 ('low density'), 5 larvae/cm^2 (considered as 'medium density' in **I** and as 'high density' in **III**) and 10 larvae/cm^2 ('high density'). We tested these larval densities at different temperatures: 23 °C ('low temperature'), 27 °C ('medium temperature') and 30 °C ('high temperature'), with 60% relative humidity and a photoperiod regime of 12:12 (L:D) hours (**I, II**). In **I** and **II** BSF larvae were fed moistened dog food *ad libitum* using a feeding rate of 163 mg substrate/larva every day as described by Parra Paz et al. (2015). In **III** BSF larvae were fed moistened chicken feed pellets using a feeding rate of 163 mg substrate for larva per day. In **IV** BSF larvae were provided 100 g of feed substrate at the start of the study and using the feeding rate of 163 mg substrate for larva per day, additional feed was added daily. In **III** BSF larvae were treated with two isolates (strains KVL 03-122 and KVL 03-127, 10^8 conidia mL^{-1}) of the entomopathogenic fungus *Beauveria bassiana* and sterile water was used as a control. The temperature regimes used for further studies (**III, IV**) were 26 °C and 28 °C, respectively. In **I** and **II**, we aimed to address methodological issues related to rearing densities using an individual-based approach. This involved maintaining initial larval densities throughout the studies by replacing dead larvae at the end of the third larval instar.

Survival was estimated as the percentage of larvae that survived from the commencement of the study until prepupation (**I, II**). Survival was also evaluated at the end of the third larval instar of **I** and **II** after most of the larvae had moulted into their 4th instar, which we determined as described by Kim et al. (2010). After survival was estimated, individuals from the matching maintenance replicate were used to replace the larvae that had experienced mortality. Prepupal mass was measured on the day of prepupation, whilst pupal mass was measured on the second day after pupation. Adult mass and sex were recorded two days after they eclosed to ensure that their meconium had been expelled (**I, II**). Larval development time was measured from the beginning of the experiment until prepupation was observed, except in **III**, where it was calculated as the number of days from application of the fungal treatment until the formation of prepupa. Pupal development time was recorded as the number of days from pupation until adult emergence (**I, II**). Prepupae were weighed and dried to measure their relative fat content (**I**). The drying was performed in an oven (MIR-162 oven, 5-5, Sanyo Electric Co. Ltd., Osaka, Japan) for 72 h at 60 °C. Relative fat content was determined using a CEM Rapid NMR Fat Analyzer (CEM Corporation, Matthews, NC, USA).

BSF eggs were sourced from an established colony at the University of Tartu, Estonia, initiated in 2017, with approximately 500 female flies from a laboratory culture in the United Kingdom. Housed within a hand-constructed breeding cage measuring 130 cm in length, 130 cm in width, and 150 cm in height, the colony comprises around 3,000 adult BSFs all the time. These adults are of different age classes and are the offspring of several hundred females who lay eggs at different times to ensure genetic variation is maintained at all times.

The environment within the cage was meticulously controlled, with a temperature maintained at 26 ± 3 °C and a relative humidity of 40 ± 5%. To ensure the well-being of the adult BSFs, drinking water was provided daily by sprinkling it onto the cage walls. Only eggs laid by the females within 24 hours before the experiment were collected. This strict timeframe was implemented to synchronise the larval development process at the onset of the experiment, ensuring uniformity and consistency in the study's conditions **(I, II, IV)**.

2.2.2 Phenoloxidase activity measurements

To evaluate the larval density and temperature effect on immunity, phenoloxidase (PO) activity – a trait closely related to insects' immune response and often used in insect immunity studies (Cerenius & Söderhäll, 2004; González-Santoyo & Córdoba-Aguilar, 2012) – was estimated. PO was measured using haemolymph samples obtained from 5th-instar larvae (**II**).

In **II**, to measure phenoloxidase (PO) activity, 5th-instar larvae were pierced in the abdomen with a sterile insulin needle. A 3 µl sample of haemolymph was extracted and placed into Eppendorf tubes containing 10 µl of phosphate-buffered saline, then stored in a –80 °C freezer until analysis. To obtain a clear supernatant, the frozen haemolymph samples were thawed and centrifuged at 9,000 × g for 10 minutes at 4 °C. Subsequently, 3 µl of the supernatant was mixed with 200 µl of 3 mmol/L L-Dopa (L-3,4-dihydroxyphenylalanine; #333786, Sigma, St Louis, MO, USA). PO activity was measured using a spectrophotometer (Enspire, Perkin-Elmer, Waltham, MA, USA) at 30 °C and 490 nm for 90 minutes, with readings taken at 1-minute intervals. The slope of the absorbance curve from 10 to 40 minutes, where the slope was linear, was used to estimate PO activity (Freitak et al., 2019; Meister et al., 2017).

In **III**, we collected haemolymph samples from 5th-instar larvae 24 hours after treatment exposure. For each replicate, we collected haemolymph from five larvae (constituting one sample) and pooled these to obtain a total volume of 10 µL per replicate. In total, we collected haemolymph from 15 larvae (three samples) for each of the six treatments (two densities × three treatments; two fungus strains and a control). The haemolymph was extracted by puncturing the abdomen of the larvae with a sterile insulin needle and then collecting the haemolymph with a pipette. The pooled haemolymph samples (10 µL) were added to 1.5-mL Eppendorf tubes containing 30 µL of phosphate-buffered saline (PBS) and stored at −80 °C. Subsequently, the frozen haemolymph samples were thawed and centrifuged at 3000 g for 10 minutes at 4 °C to obtain a clear supernatant (Laughton & Siva-Jothy, 2011). We then pipetted 10 µL of the supernatant, along with 110 µL of ddH2O, 30 µL of PBS buffer, and 30 µL of L-DOPA (16 mM L-3,4-dihydroxyphenylalanine; Sigma, St Louis, MO, USA) into a 96-well plate (Shi et al., 2014). Each sample was replicated three times. Finally, we measured PO activity using a Spectra Max 190 photo spectrometer with Softmax Pro v.7.1 software at 30 °C and 490 nm for 90 minutes, taking readings at 1-minute intervals. The slope of the reaction curve from 10 to 60 minutes was used to measure PO activity, based on enzyme activity (Vmax; Laughton & Siva-Jothy, 2011).

2.2.3 Literature review

We comprehensively reviewed published literature on density-dependent prophylaxis in insects to provide a detailed background to evaluate our results **(II)**. Our literature search used a central literature database (Web of Science) using the search phrase combination: larval densit* immunit* insect*.

2.2.4 Data analysis

We sought to explain BSF's life history and immune traits dependence on larval density and temperature (**I**, **II**), larval density and entomopathogenic fungus exposure (**III**), and larval density and substrate fermentation **(IV)**. For this, we constructed linear mixed-effect models (LMMs) incorporating the container (**I, II, III**) and replicates (**IV**) as random effects. To estimate larval survival differences between the treatments, binomial models were built using the R package pbkrtest (Halekoh & Højsgaard, 2014). The R packages emmeans (Lenth, 2023), lme4 (Bates et al., 2015) and lmerTest (Kuznetsova et al., 2017) were used to build the GLMMs in analysing prepupal mass, pupal mass, adult mass, larval development time, pupal development time, adult longevity, fat content and PO activity. Denominator degrees of freedom were calculated using the Satterthwaithe method. Data analyses were performed using R statistical software (R Core Team, 2024) in RStudio (Posit team, 2024), SAS 9.4 (SAS Institute Inc., Cary, NC, USA) (**II**) and Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) (**II**). For the further details of data analysis, see **I–IV**

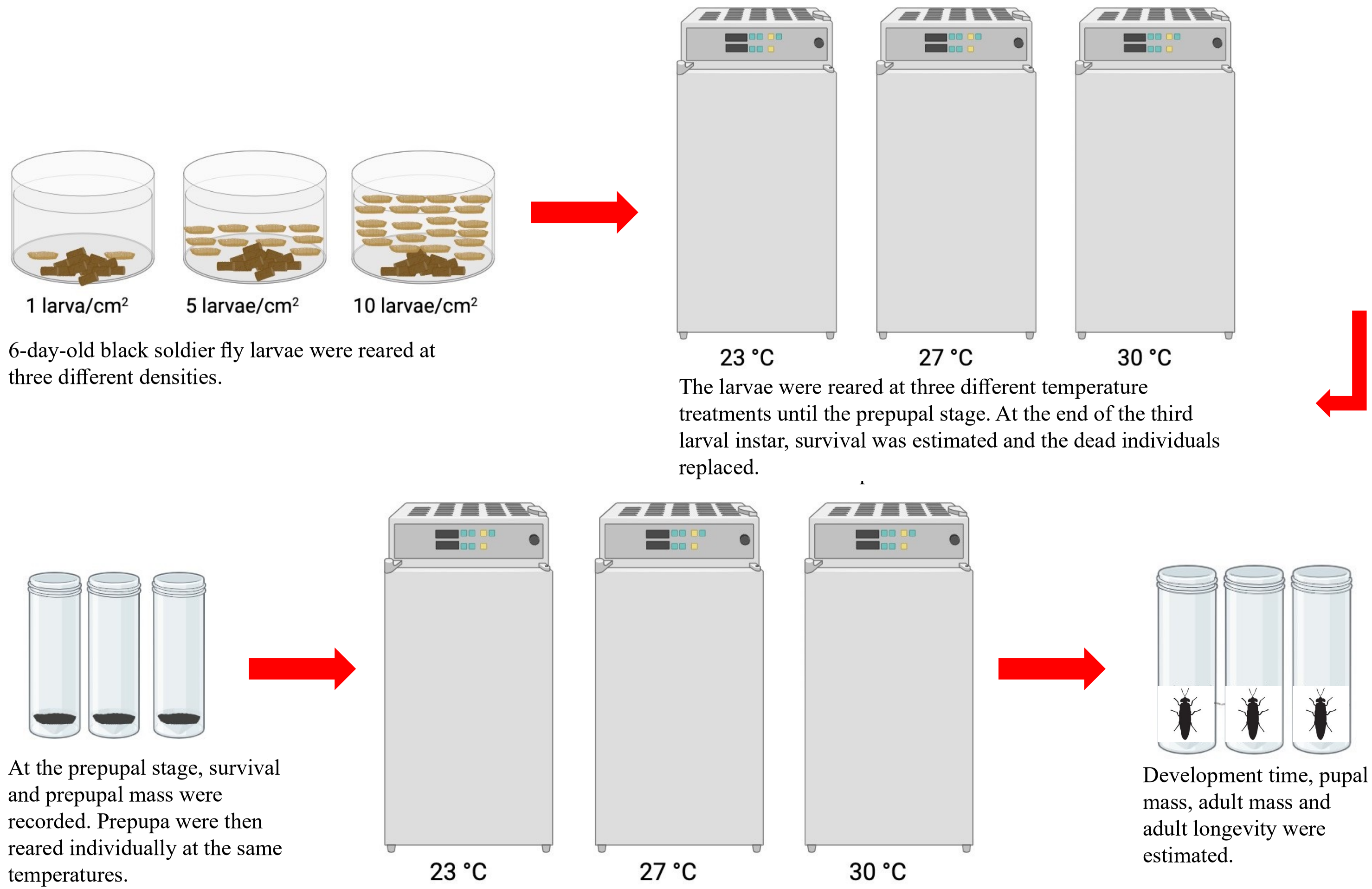

Figure 2. Workflow of **I**: a study exploring the effect of three larval densities (1, 5 and 10 larvae/cm^2) and three temperatures (23, 27 and 30 °C) on life history traits of black soldier fly. The image was created with Biorender (www.biorender.com).

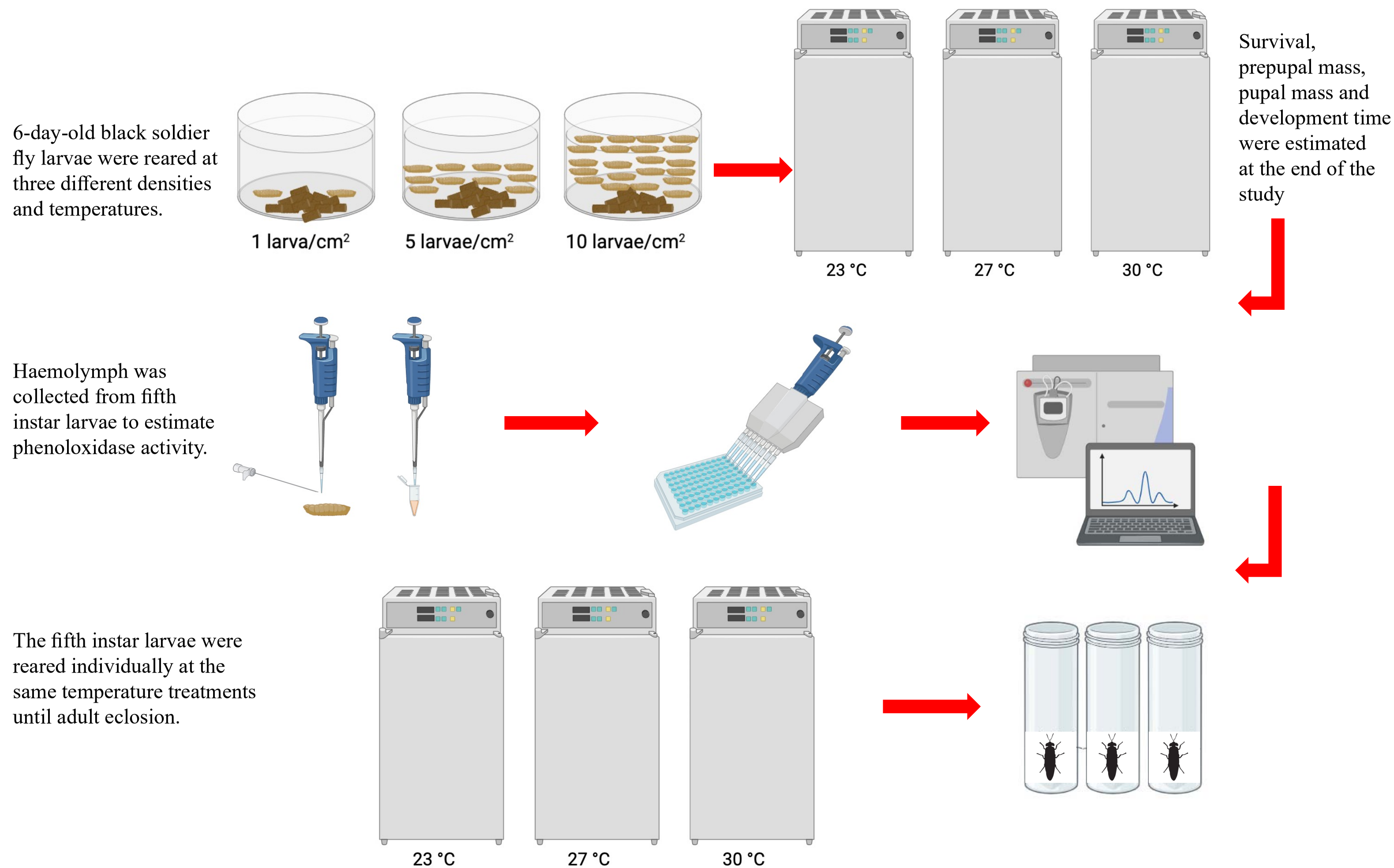

Figure 3. Workflow of **II**: a study exploring the effect of three larval densities (1, 5 and 10 larvae/cm^2) and three temperatures (23, 27 and 30 °C) on phenoloxidase activity of black soldier fly larvae. The image was created with Biorender.

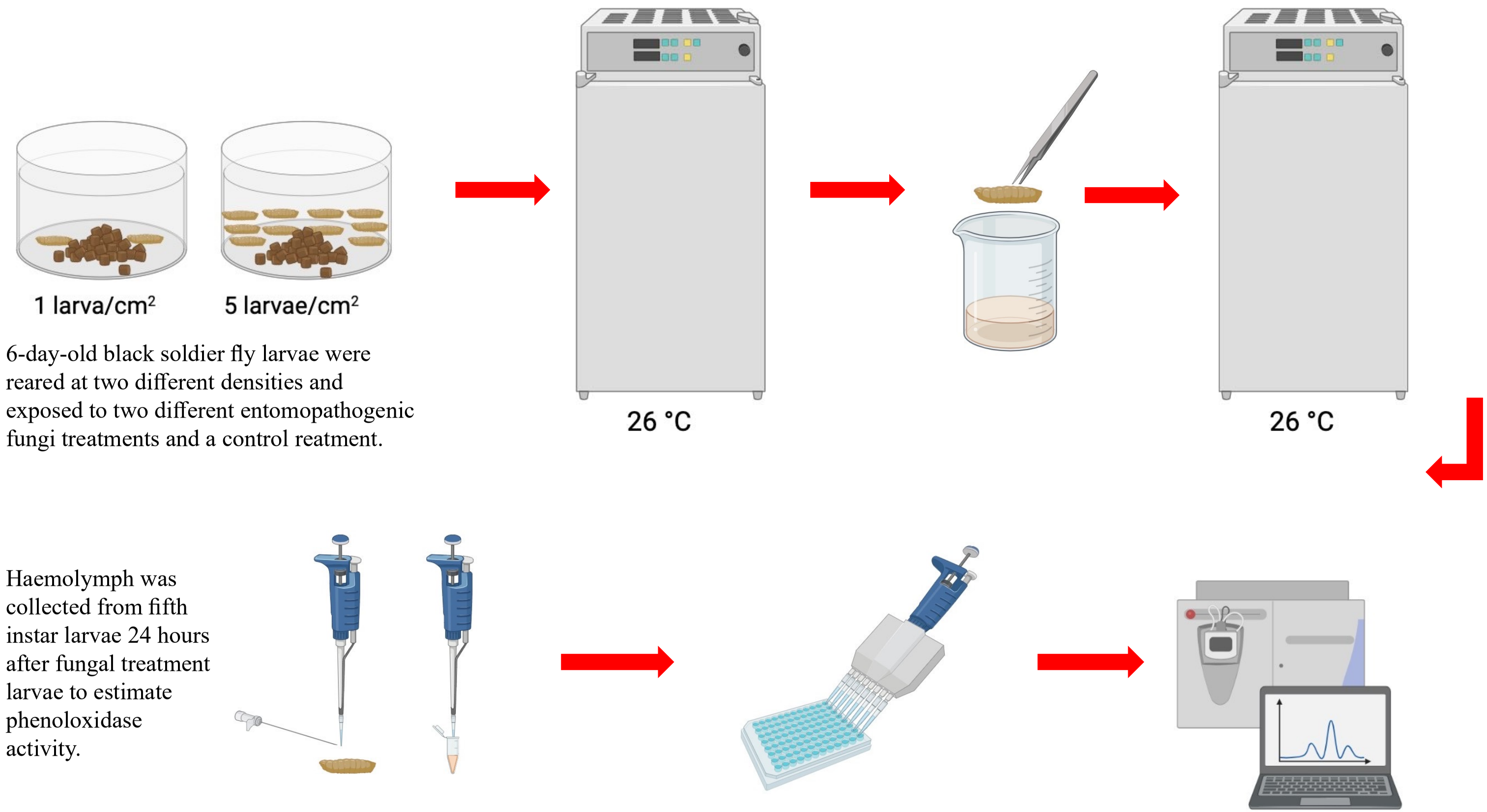

Figure 4. Workflow of **III**: a study exploring the effect of two larval densities (1 and 5 larvae/cm^2) two entomopathogenic fungus (*Beauveria bassiana*) strains and a control treatment on life history traits and phenoloxidase activity of black soldier fly. The image was created with Biorender.

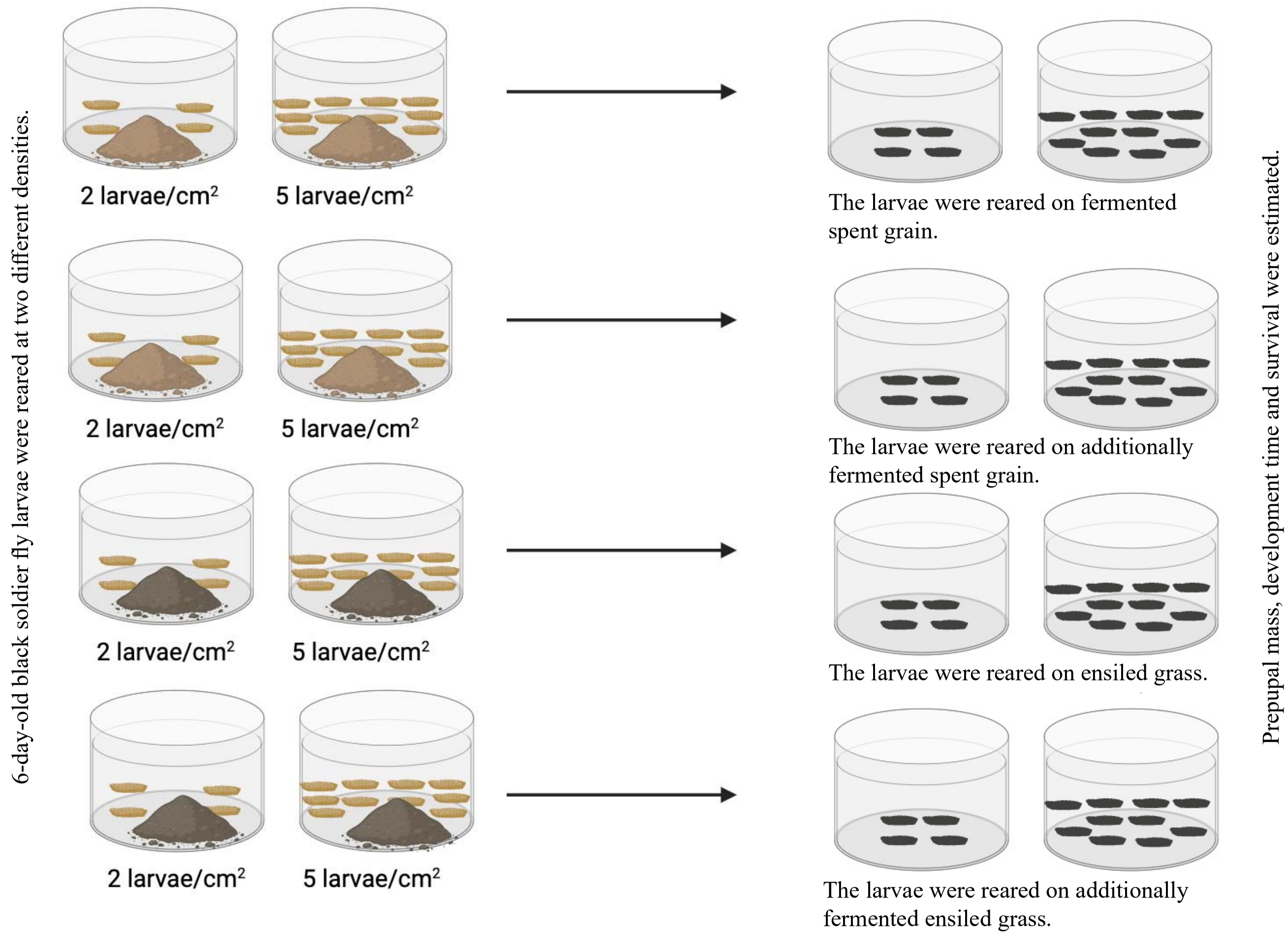

Figure 5. Workflow of **IV**: a study exploring the effect of two larval densities (2 and 5 larvae/cm^2) and fermented substrates (fermented spent grain (FSG) and additionally fermented ensiled grass (AFEG)) versus standard substrates (ensiled grass (EG) and spent grain (SG)) on black soldier fly life history traits. The image was created with Biorender.

3. RESULTS

3.1 Larval density and temperature influence on life history traits (I)

Our results reveal that rearing BSF at low larval density (1 larva/cm^2) results in a higher prepupal mass than at medium (5 larvae/cm^2) and at high larval density (10 larvae/cm^2) ($F_{2, 693}$ = 136.8, $P < 0.0001$; Figure 6). Prepupal mass was also influenced by temperature ($F_{2,693}$ = 117.6, $P < 0.0001$; Figure 6). There was a significant interaction between larval density and temperature ($F_{4,693}$ = 121.6, $P < 0.0001$). Specifically, we observed higher prepupal masses of larvae from the low temperature treatment (23 °C) compared to other thermal treatments when the individuals were reared at high and low densities but not at medium density (Figure 6).

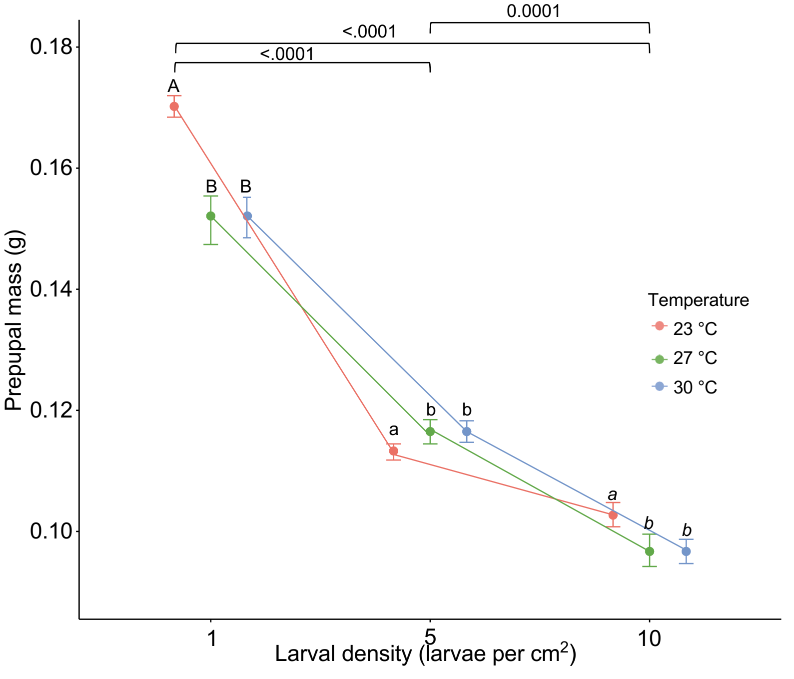

Figure 6. Visualised interactions between larval density and temperature on the prepupal mass of black soldier fly. Estimated marginal means at the same larval density followed by the same lowercase, uppercase or italic letters are not significantly different ($P > 0.05$). Estimated marginal means were estimated using R studio version 4.3.3, package emmeans (Lenth, 2023). Error bars indicate standard error.

Rearing BSF at low larval density also resulted in higher adult mass compared to those reared at medium and high density ($F_{2,693}$ = 4722.5, P < 0.0001; Figure 7). Adult mass was also influenced by temperature – individuals reared at low temperature achieved lower adult masses than those reared at higher temperatures ($F_{2,693}$ = 6.9, P < 0.0001; Figure 7). There was a significant interaction between larval density and temperature ($F_{4,693}$ = 3.4, P = 0.0049). In particular, we observed lowest differences in adult mass between the larvae from the low temperature treatment and other thermal treatments when the individuals were reared at the highest larval density (Figure 7).

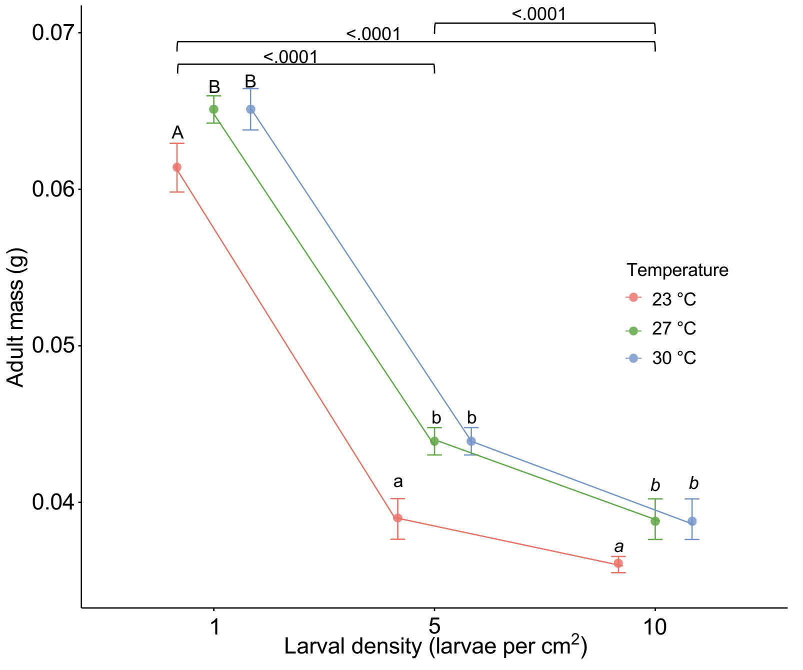

Figure 7. Visualised interactions between larval density and temperature on the adult mass of black soldier fly. Estimated marginal means at the same larval density followed by the same lowercase, uppercase or italic letters are not significantly different (P > 0.05). Estimated marginal means were estimated using R studio version 4.3.3, package emmeans (Lenth, 2023). Error bars indicate standard error.

Rearing BSF at low larval density resulted in shorter development times than rearing them at medium and high density ($F_{2,693} = 43.3$, $P < 0.0001$; Figure 8). Development time was also influenced by temperature – larvae reared at low temperature had longer development time than those from other thermal treatments ($F_{2,693} = 6.9$, $P < 0.0001$; Figure 8). There was a significant interaction between larval density and temperature ($F_{4,693} = 3.4$, $P = 0.0049$). In particular, the development time difference between the larvae reared at low temperature treatment compared to those from other thermal treatments was the lowest at high density (Figure 8).

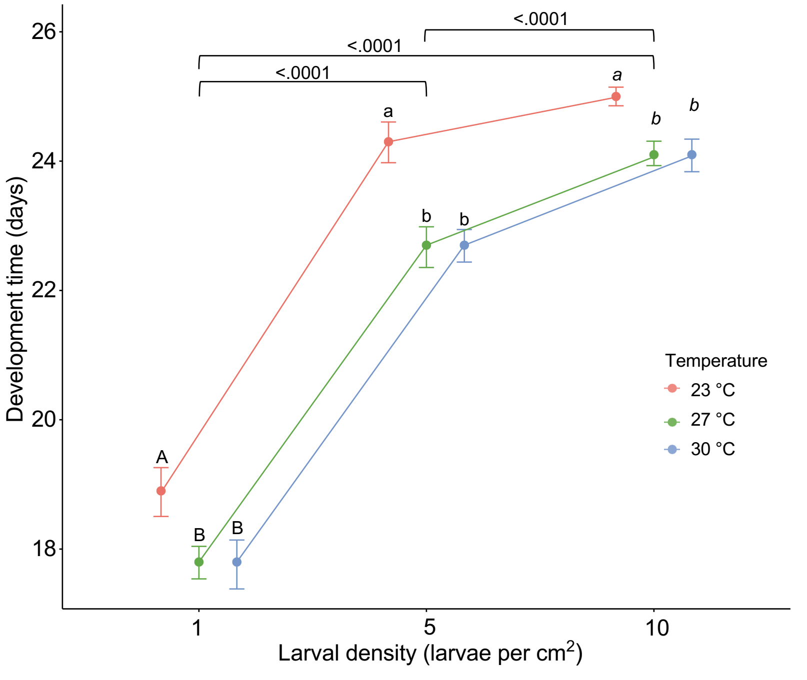

Figure 8. Visualised interactions between larval density and temperature on development time of black soldier fly larvae. Estimated marginal means at the same larval density followed by the same lowercase, uppercase or italic letters are not significantly different ($P > 0.05$). For density treatment comparisons P-values are presented. Estimated marginal means were estimated using R studio version 4.3.3, package emmeans (Lenth, 2023). Error bars indicate standard error.

Our results also revealed that rearing BSF at low larval density results in a higher survival than at medium and high density both from the start of the experiment until the 4th larval instar ($df = 2$, $P < 0.0001$) and from the 4th larval instar until prepupation ($df = 2$, $P < 0.0001$). There was also a significant interaction between larval density and temperature ($F_{4,198} = 11.9$, $P < 0.0001$). Specifically, survival was higher at low and medium larval density when BSF larvae were reared at low and medium temperatures. Fat content in prepupae was also influenced by larval density ($F_{2,198} = 157$, $P < 0.0001$). In particular, BSF larvae had higher fat content at low larval density compared to the medium and high density treatments.

3.2 Larval density and temperature influence on phenoloxidase activity (II)

Our results showed that larval density influences the PO activity of BSF larvae ($F_{2,406} = 3.7$, $P = 0.027$; Figure 9). In particular, PO activity increased at higher larval densities (5 larvae/cm^2 and 10 larvae/cm^2) compared to the low larval density (1 larva/cm^2). Temperature also influenced the PO activity ($F_{2,263} = 6.8$, $P = 0.001$; Figure 9), with higher temperatures (27 °C and 30 °C) inducing higher PO activity compared to the low temperature (23 °C).

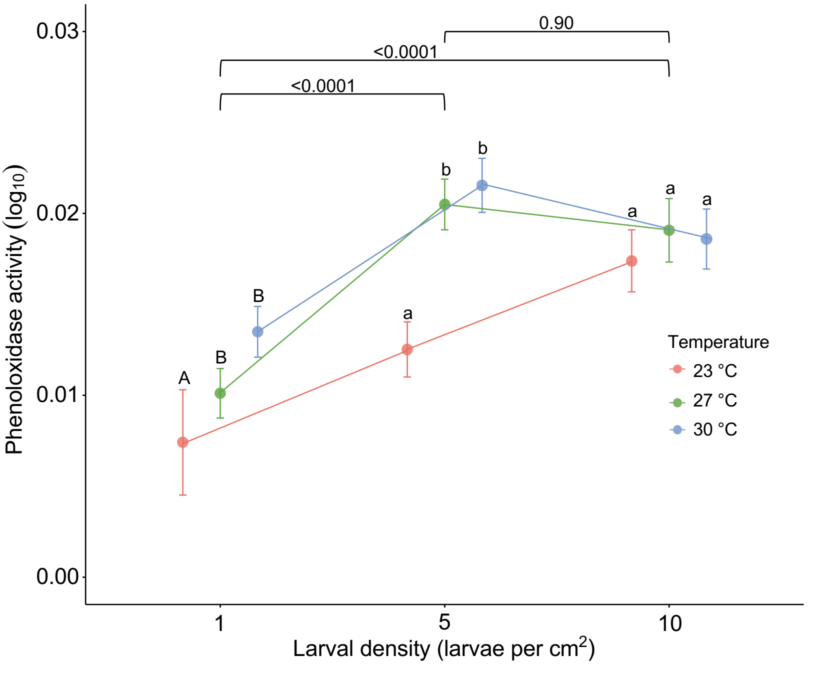

Figure 9. Visualised interactions between larval density and temperature on phenoloxidase activity of black soldier fly larvae. Estimated marginal means at the same larval density followed by the same lowercase, uppercase or italic letters are not significantly different ($P > 0.05$). For density treatment comparisons P-values are presented Estimated marginal means were estimated using R studio version 4.3.3, package emmeans (Lenth, 2023). Error bars indicate standard error.

3.3 Entomopathogenic fungus exposure and larval density effects on black soldier fly larvae life history traits and phenoloxidase activity (III)

Our results showed that larval density ($F_{1,307} = 5094.4$, $P < 0.0001$) and fungal treatment ($F_{2,297} = 73.4$, $P < 0.0001$) both significantly influenced prepupal mass (Figure 10). We also observed a significant interaction between larval density and fungal treatment ($F_{2,307} = 73.4$, P = < 0.0001). More specifically, at low density (1 larva/cm^2), control larvae had higher prepupal masses than fungal-treated larvae, while larvae treated with the fungal strain KVL 03-127 exhibited higher prepupal masses than control larvae at higher density (5 larvae/cm^2).

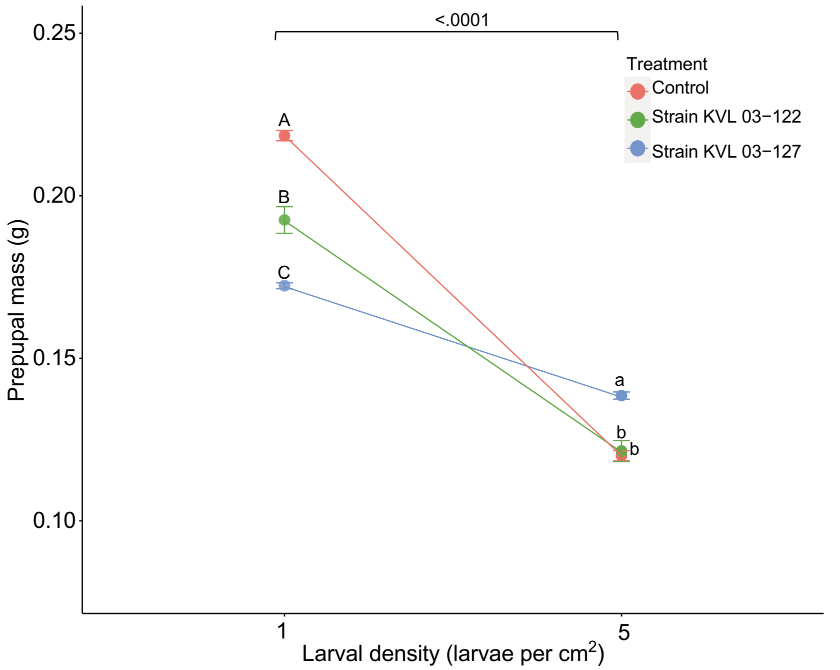

Figure 10. Visualised interactions between larval density and exposure to two different strains of entomopathogenic fungus (*Beauveria bassiana*) on the prepupal mass of black soldier fly. Estimated marginal means at the same larval density followed by the same lowercase, uppercase, or lowercase letters are not significantly different (P > 0.05). For density treatments comparison P-value is presented. Estimated marginal means were estimated using R studio version 4.3.3, package emmeans (Lenth, 2023). Error bars indicate standard error.

Larval density ($F_{1,307} = 498$, $P < 0.0001$) and fungal treatment ($F_{2,306} = 24.8$, $P = 0.0009$) both significantly influenced the development time of BSF larvae. More specifically, larvae reared at low larval density had a shorter development time than those reared at higher density and development time of control larvae was shorter compared to fungal-infected larvae (Figure 11). We also observed a significant interaction between larval density and fungal treatment ($F_{2,307} = 3.3$, $P = 0.039$). In particular, the difference in development time between the control and larvae treated with fungal strain KVL 03-127 was higher at low density than at higher density (Figure 11).

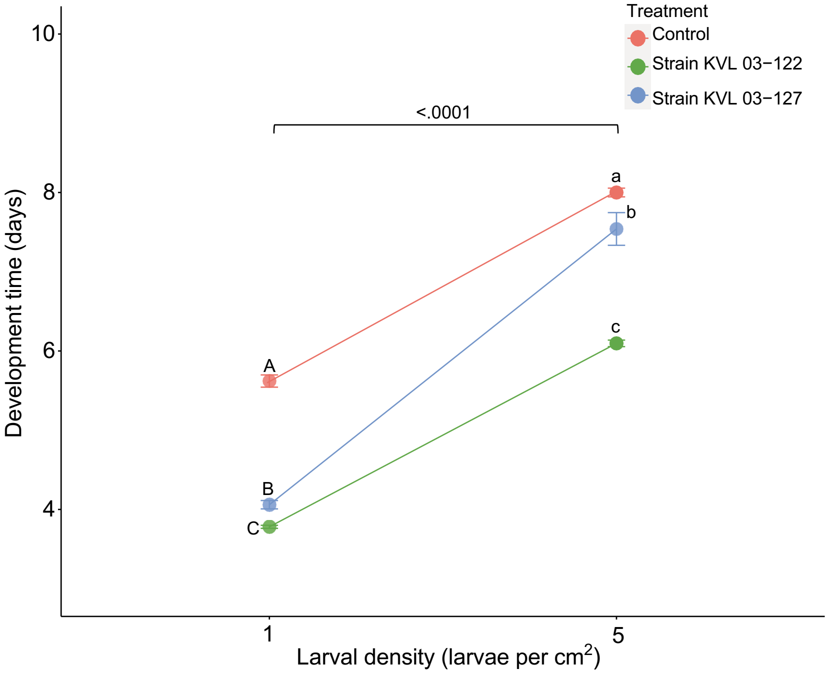

Figure 11. Visualised interactions between larval density and exposure to two different strains of entomopathogenic fungus (*Beauveria bassiana*) on development time of black soldier fly larvae. Estimated marginal means at the same larval density followed by the same lowercase, uppercase, or lowercase letters are not significantly different ($P > 0.05$). For density treatments comparison P-value is presented. Estimated marginal means were estimated using R studio version 4.3.3, package emmeans (Lenth, 2023). Error bars indicate standard error.

Larval density ($F_{1,15} = 182.2$, $P < 0.0001$) and fungal treatment ($F_{2,20} = 29.7$, $P < 0.0001$) both significantly influenced the PO activity of BSF larvae with fungal-infected larvae having higher PO activity than control treatment larvae (Figure 12). We also observed a significant interaction between larval density and fungal treatment ($F_{2,19} = 4.4$, $P = 0.028$). In particular, while larvae from the control treatment and those treated with KVL 03-122 had higher PO activity at higher than at low larval density, there was no difference in PO activity between the two density treatments in larvae treated with a *B. bassiana* strain KVL 03-127 (Figure 12).

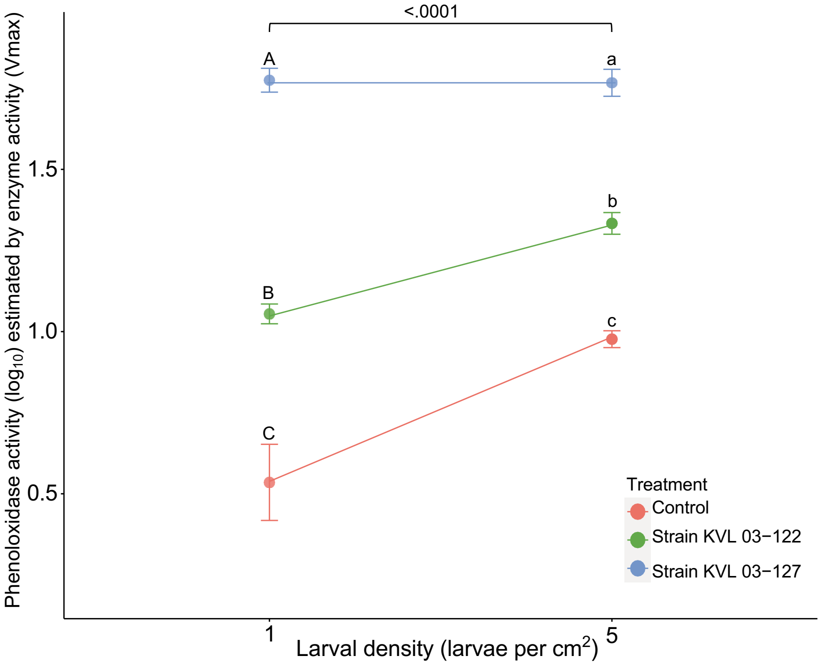

Figure 12. Visualised interactions between larval density and exposure to two different strains of entomopathogenic fungus (*Beauveria bassiana*) on phenoloxidase activity (estimated as enzyme activity) of black soldier fly larvae. Estimated marginal means at the same larval density followed by the same lowercase, uppercase, or lowercase letters are not significantly different ($P > 0.05$). For density treatments comparison P-value is presented. Estimated marginal means were estimated using R studio version 4.3.3, package emmeans (Lenth, 2023). Error bars indicate standard error.

3.4 Larval density and substrate fermentation effects on life history traits of the black soldier fly (IV)

Our results indicated a significant effect of feed substrate ($F_{3,1445} = 21.09$, $P < 0.0001$) and larval density ($F_{3,1435} = 117.97$, $P < 0.0001$) on prepupal mass, and the effect varied depending on the substrate and larval density (significant interaction term: $F_{3,1466} = 54.91$, $P = 0.0027$). More specifically, the positive effect of fermentation on prepupal mass was more prominent at lower (2 larvae/cm^2) than at higher (5 larvae/cm^2) larval density (Figure 13).

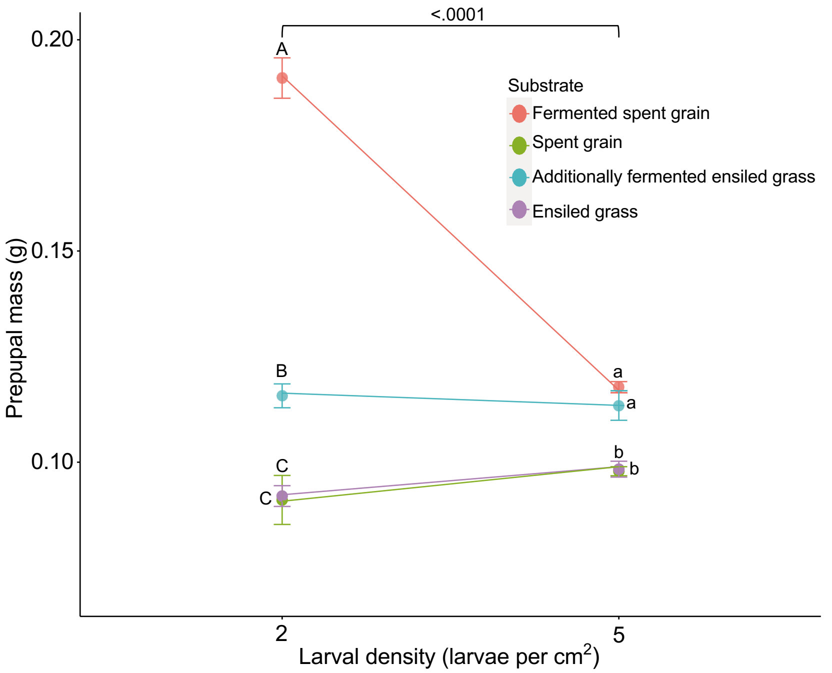

Figure 13. Visualised interactions between larval density and substrate fermentation on the prepupal mass of black soldier fly. Estimated marginal means at the same larval density followed by the same lowercase, uppercase, or lowercase letters are not significantly different ($P > 0.05$). For density treatments comparison P-value is presented. Estimated marginal means were estimated using R studio version 4.3.3, package emmeans (Lenth, 2023). Error bars indicate standard error.

Our results indicated that only substrate ($F_{3,1443}$ = 464.1, P = 0.021) and not larval density ($F_{1,1450}$ = 122.5, P = 0.73) had a significant effect on development time (Figure 14). In particular, development time was shorter on spent grain treatments (spent grain and fermented spent grain) than on ensiled grass treatments (ensiled grass, additionally fermented ensiled grass) (Figure 14).

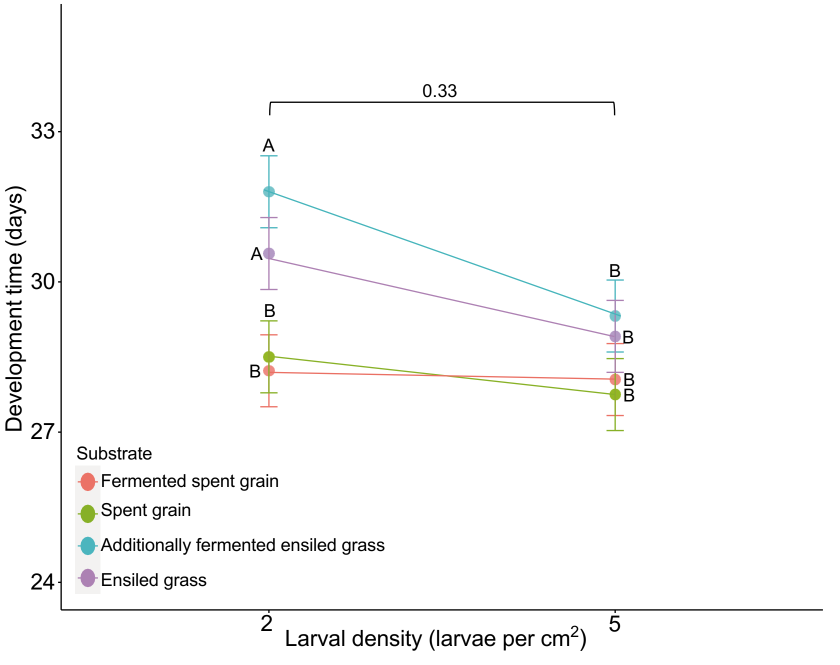

Figure 14. Visualised interactions between larval density and substrate fermentation exposure on the development time of black soldier fly larvae. Estimated marginal means at the same larval density followed by the same lowercase, uppercase, or lowercase letters are not significantly different (P > 0.05). For density treatments comparison P-value is presented. Estimated marginal means were estimated using R studio version 4.3.3, package emmeans (Lenth, 2023). Error bars indicate standard error.

Our results indicated that both feed substrate ($F_{3,36} = 49.1$, $P = 0.0001$) and larval density ($F_{1,33} = 12.7$, $P = 0.0089$) had a significant effect on larval survival. Moreover, there was a significant interaction effect observed between larval density and substrate on larval survival ($F_{3,64} = 56.7$, $P = 0.0001$). In particular, spent grain treatments (both fermented and unfermented) increased larval survival at low density, but had a decreased survival at high density when compared to ensiled grass treatments (ensiled grass and additionally fermented ensiled grass) (Figure 15).

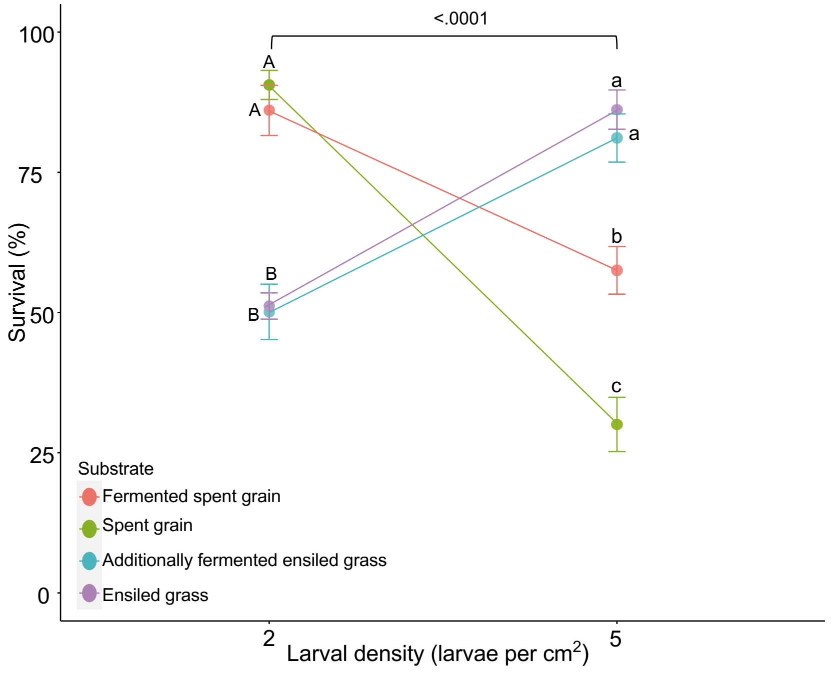

Figure 15. Visualised interactions between larval density and substrate fermentation exposure on larval survival of black soldier fly larvae. Estimated marginal means at the same larval density followed by the same lowercase, uppercase, or lowercase letters are not significantly different ($P > 0.05$). For density treatments comparison P-value is presented. Estimated marginal means were estimated using R studio version 4.3.3, package emmeans (Lenth, 2023). Error bars indicate standard error.

4. DISCUSSION

Life history traits are influenced and modified by several environmental factors, some of which are abiotic and some biotic in nature. In the case of the BSF, its life history traits are well known to be significantly influenced by temperature (Harnden & Tomberlin, 2016; Tomberlin et al., 2009), moisture (Bekker et al., 2021; Dzepe et al., 2020) and diet quality (Barragan-Fonseca et al., 2020; Gobbi et al., 2013; Oonincx et al., 2015). Due to the phenotypic plasticity of life history traits, it is possible to increase the performance of BSF larvae by finding the optimal values for these various environmental factors.

Larval density is an important biotic factor influencing fitness and life history trait expression, but it remains underexplored in edible insects (Mueller et al., 1991; Rivers & Dahlem, 2022). In recent years, several developments have been aimed at effectively incorporating commercially important insect species into food and feed production (van Huis, 2020). Studies like this thesis are crucial in determining the appropriate rearing density range for BSF larvae to maximise insect production and ultimately implement it on an industrial scale. In **I–IV** we found evidence that larval density significantly influences BSF life history. The effect of larval density on life history traits depended significantly on temperature, exposure to entomopathogenic fungus and feed substrate type (**I–IV**). In **I** and **II**, we aimed to reduce methodological issues associated with rearing densities using an individual-based approach where dead larvae were replaced at the end of the third larval instar, thus maintaining the initial larval densities throughout the studies. We also addressed the immunity-larval density relationship in dipterans (**II**, **III**), with our studies, in our knowledge, being the only ones with a commercially important dipteran species at its focus.

All the studied traits pointed towards low larval density being the optimal environment and high larval density being a suboptimal environment for BSF larvae. Traits such as survival, prepupal mass, pupal mass, adult mass, larval development time, adult longevity and fat content all pointed towards this direction and are consistent with similar studies found in the literature (Barragan-Fonseca et al., 2018; Guillaume et al., 2023; Parra Paz et al., 2015). Based on our studies, low larval density leads to higher survival, larger body mass and shorter development time in BSF larvae (Figures 6–8), and this could be exploited by those interested in commercially raising this species as the effects were qualitatively consistent across all the thermal treatments applied in our study. PO activity and development time on low-quality substrates showed an opposite pattern (PO activity higher and development time shorter at high densities). This suggests that conditions of optimal density might differ depending on the environment and the trait in question. Alternatively, or additionally, the values of certain traits (e.g. immunity) might be enhanced in high-density environments, which are more hazardous, at the expense of other life history traits.

The hypothesis of density-dependent prophylaxis is an ecological concept that refers to the phenomenon where individuals in densely crowded conditions invest more in their immune defences compared to those in less densely populated environments (Cotter et al., 2004; Wilson & Cotter, 2009). This increased investment in immunity is thought to correspond to the higher risk of disease transmission in crowded conditions (Wilson & Cotter, 2009; Wilson & Reeson, 1998). We found evidence that BSF larvae exhibit density-dependent prophylaxis, i.e. they can elevate their PO activity in environments containing high numbers of conspecifics (**II–III**). Our results are supported by several previous studies that found high PO activities at high larval densities in various insect species (Kong et al., 2018; Murzagulov et al., 2013; Ruiz-González et al., 2009). This finding suggests that high-rearing densities benefit from a stronger immune response but entail higher costs for other life history traits. The prolonged developmental period and small prepupal size of individuals reared at high larval densities may be the costs of investing more resources into immunity.

The results of the literature review exploring larval density and immune trait relationship pointed towards larval density being an important biotic factor for several orders of insects (**II**). In different studies, larval density shows positive, negative and quadratic relationships with different immune traits. Furthermore, the relationship between larval density and immune traits differs between and within insect orders. For example, the immune traits of the insects in the order Diptera (other species than BSF) showed no relationship with larval density, whilst those from the Lepidoptera showed positive, quadratic and negative relationships with larval density. In addition, within the order Lepidoptera, all the immune traits of the species *Pieris napi* exhibited a negative relationship with density, whilst all the immune traits of the species *Spodoptera littoralis* showed a positive relationship with density. The restricted number of case studies (22), with most studies conducted in one insect order (Lepidoptera), prevented us from fully evaluating the extent to which the hypothesis of density-dependent prophylaxis holds true across insects.

We found strong thermal effects on our studied life history traits (**I**, **II**). This phenomenon was also observed by Harnden & Tomberlin (2016) and Tomberlin et al. (2009). In addition, our results indicate that temperature modulates the effects of larval density on life history traits at varying degrees depending on the studied larval density. Therefore, in commercial rearing, one must consider several environmental factors and account for them concurrently. We observed a significant relationship between temperature and PO activity, with larvae reared at higher temperatures having higher PO activity (**II**). A potential explanation for this finding is that BSF larvae invest more into their immune function at temperatures that fall within their optimal thermal range. An alternative and perhaps more likely explanation is that their response to higher temperatures is adaptive and in accordance with a perceived high risk of pathogenic threats at those temperatures (Haine et al., 2008; Linder et al., 2008; Silva & Elliot, 2016). We conclude that at high larval densities and high temperatures, BSF larvae exhibit a stronger immune investment (PO activity), which enhances their performance in environments with a high risk of pathogen infection.

Environmental interactions on BSF larvae, in general, remain largely underexplored. One predominant but still understudied aspect is the influence of pathogens on larval density effects (Eilenberg et al., 2018; Vogel et al., 2022). In exploring these interaction effects, we assessed the effect of entomopathogenic fungus (B*eauveria bassiana*) and larval density on immune function (PO activity) and life history traits (**III**). Our results revealed that the fungal treatment caused an increased PO activity at both low and high densities. The control treatments showed increased PO activity as larval density increased, supporting our results (**II)** that BSF larvae exhibit density-dependent prophylaxis. The fungal treatment significantly influenced prepupal mass, and development time, with control larvae performing better than fungal-treated ones. The control treatments' results are consistent with **I** (regarding the prepupal mass and development time). However, this study (**III**) also found that at high-density, BSF larvae from the control treatments had lower prepupal mass than BSF larvae which received treatment of one from the two fungal strains. An explanation for this could be related to the pathogen stress and high conspecific density, so that individuals may overcompensate by therapeutic feeding and increase their body size when food stress is absent (Lee et al., 2006, 2008; Povey et al., 2009). We demonstrated the complexity of larval density and EPF interaction effects on the immune investment of BSF larvae (**III)**. BSF larvae can maintain their elevated PO activity even when larval density is coupled with EPF stress at both low and high densities, but at the cost of an extended development time and at low density, also with the cost of reduced body mass.

Feed substrate quality has been established as a key factor influencing BSF life history traits. High-quality substrates are responsible for increased body mass and shorter development times, while low-quality substrates are responsible for decreased body mass and longer development (Barragan-Fonseca et al., 2020; Cammack & Tomberlin, 2017; Oonincx et al., 2015). Low-quality diets are usually highly fibrous and nutritionally lacking (Palma et al., 2019). We explored the effect of substrate fermentation on BSF larval performance (**IV**). Our results revealed that larval density and substrate fermentation significantly interact. In addition, fermenting low-quality substrates before feeding them to larvae may have a positive impact on their growth and development. An explanation for this could be that fermentation reduces the particle size of substrates, making them more accessible to the BSF larvae's mouthparts. This phenomenon was also observed by Norgren et al. (2023) when their results revealed that fermentation made pulp and paper bio-sludge easier for BSF larvae to convert into their biomass.

In exploring the phenotypic plasticity of life history traits concerning rearing density, this thesis sought to understand the relationship between rearing density and other biotic and abiotic factors. In line with our expectations, we revealed that the relationship between rearing density and other environmental factors is not as straightforward as it may seem. It is important to note that all our studies were conducted in the laboratory, and our results reflect what is optimal for this species in laboratory conditions and not necessarily in the wild. Despite this reservation, this thesis sheds light on the nature of density-dependent relationships. It contributes to the existing literature by establishing a foundation that can be further developed due to the methodology employed in the thesis.

From a commercial rearing perspective, this thesis suggests that maintaining low larval density combined with medium rearing temperatures creates an optimal environment for BSF larvae. This combination results in higher survival rates, larger body size, shorter development times, and a higher fat content. In addition, BSF larvae reared at high densities exhibit elevated immune response that comes with fitness costs, including reduced body size and longer development time, and these traits should be avoided if they are of interest to the producer. Furthermore, our findings suggest that fermentation and additional fermentation can improve the capacity of BSF larvae to feed on high-fibre substrates, though the effectiveness depends on the specific substrate and environmental factors like larval density. It is essential to acknowledge that these factors interact in complex ways, where changing one parameter can significantly affect another. Therefore, a nuanced approach that accounts for the interplay between environmental factors, substrate characteristics, and production costs is vital for optimising BSF production.

5. CONCLUSIONS

The following conclusions can be drawn from my thesis:

- We observed a better performance of most life history traits of black soldier fly (BSF) at low larval densities on high-quality substrates compared to high larval densities. It is, therefore, possible to use larval density and temperature to manipulate the life history traits to suit and optimise different mass-rearing requirements.

- BSF larvae exhibit density-dependent prophylaxis. At higher larval densities and temperatures, they invest more resources into their immune function, which may lead to an extended development period and a reduction in their final body mass.

- Exposure to entomopathogenic fungus (B*eauveria bassiana*) and larval density significantly interact in a way that fungal infection and larval density influence the expression of life history traits and PO activity. BSF larvae can upregulate their immune investment in the presence of multiple stressors, and they appear to engage in therapeutic feeding to mitigate the costs involved.

- BSF larvae perform better on fermented low-quality substrates at high larval density than at low larval density. Substrate fermentation may, therefore, be important to improve the performance of BSF larvae on low-quality substrates.

- Interactions between larval density and other biotic and abiotic factors are frequent and remain largely unexplored: my studies shed important light on some interesting and key relationships; however, much remains to be done in this field.

SUMMARY

Using insects as food and feed has lately been regarded as a promising and sustainable alternative to standard food and feed production. The primary driver behind this is the finding that edible insects have largely comparable and similar nutritional profiles to traditional farm animals. Importantly, however, they possess higher feed conversion ratios and are more sustainable in terms of energy and water use, making the production of insects more efficient compared to traditional farm animals. Due to this industry's short history, several aspects of mass-rearing insects remain unexplored. Specifically, knowledge gaps hinder the development of optimal rearing techniques, including a lack of detailed understanding of the life histories, physiologies, and behaviour of insect species suitable for food and feed.

Life history theory aims to provide insights into the multifaceted aspects of an organism's life cycle that also encompasses its phenotypic characteristics and environmental context. It explores the diversity of adaptive strategies and how organisms use them to maximise their fitness. Due to limited resources, organisms cannot typically invest in all life-history traits (traits closely related to fitness) simultaneously. As a result, life history traits are often traded off against each other – investing more in one means investing less in another. Additionally, the optimal values of life history traits strongly depend on environmental factors and their interplay.

Larval density is a key biotic factor that impacts most dipterans and many other insects due to their high fecundity and natural aggregation behaviour during the larval period. Living in environments with increased larval density can profoundly affect life history traits, including, increased metabolism, reduced (or sometimes prolonged) development time, reduced body mass, as well as an increase in intraspecific competition, pathogen concentration and mortality. Larval density influences various life history traits to different degrees, and determining optimal larval densities can notably enhance individual performance.

The black soldier fly (BSF) is a dipteran species with high potential for use in food and feed production. The BSF has garnered significant attention for its diverse applications, including but not limited to its potential as livestock feed and aquaculture feed, as well as its use in bioconversion, production of fertilisers and waste management. Recognised for its vast potential, there is a growing imperative to evaluate the environmental implications of its rearing in order to optimise production methods. One way to maximise the potential of this species is to understand and harness the potential of its naturally aggregating behaviour to help obtain its optimum in mass-rearing conditions.

This thesis explores how different larval densities affect life history traits (including immune traits) in response to temperature, entomopathogenic fungi and substrate fermentation. The aim was to determine the potential optimal larval density for rearing BSF larvae and to investigate how it may interact with other environmental factors. Our results showed that BSF larval survival decreased as

larval density increased. In addition, prepupal, pupal and adult mass, fat content and development time all indicated superior performance at lower (1 larva or 2 larvae/cm^2) than at higher larval densities (5 and 10 larvae/cm^2). Importantly, the effect of larval density depended on the temperature. The optimal conditions for rearing BSF larvae appear to be low larval density and a medium temperature of 27 °C. However, at high larval densities, phenoloxidase activity in BSF larvae was found to increase, suggesting that they are better prepared for pathogen attacks at a cost of smaller size and extended development time.

Furthermore, we showed that the entomopathogenic fungus *Beauveria bassiana* can significantly influence the prepupal mass and the development time of BSF larvae at both low and high larval density. The phenoloxidase activity of the larvae was found to be higher in fungal treatments compared to the control treatments in high larval densities. This suggests that the larvae can still invest in immune function in situations with multiple stressors. Regarding prepupal mass, we observed that when reared at higher density, the larvae treated with a certain fungus strain (*B. bassiana* strain KVL 03-127) were heavier than larvae from the control treatment, suggesting that they may indulge in therapeutic feeding to offset the cost associated with mounting an immune response.

Lastly, we found that the effect of larval density on BSF life history traits depends significantly on substrate fermentation. We found that fermentation could partly improve the highly fibrous substrates for BSF larvae. Moreover, substrates with high fibre could also partially mitigate the negative impacts of high density. This could be due to the larvae being able to digest their food collectively due to their large numbers.

The results of this thesis suggest that BSF life history traits are significantly influenced by larval density. The effect, however, significantly depends on environmental factors such as temperature, entomopathogenic fungus and substrate fermentation. This thesis underscores the importance of managing and regulating the rearing density in economically motivated endeavours. Additionally, our discovery of the interplay of different environmental factors in affecting BSF life history underscores the need to take into account multiple environmental variables simultaneously, recognising that altering one factor may produce either favourable or adverse consequences on others.

From a commercial rearing perspective, this thesis recommends maintaining low larval density and medium rearing temperatures for optimal BSF larval development. This approach leads to higher survival rates, larger body sizes, shorter development times, and higher fat content. Conversely, high larval densities should be avoided as they trigger an increased immune response, resulting in reduced body size and longer development times, which are undesirable outcomes for producers. Additionally, fermentation can enhance the larvae's ability to feed on high-fibre substrates, though its effectiveness varies with the substrate and environmental conditions like larval density.

SUMMARY IN ESTONIAN

Asustustiheduse mõjud majanduslikult olulise putukaliigi elukäigule

Putukate kasutamine toidu ja söödana on viimasel ajal pälvinud tähelepanu kui paljutõotav ja jätkusuutlik alternatiiv tavapärasele toidu ja sööda tootmisele. Söödavad putukad on toiteväärtuse poolest enamasti võrreldavad traditsiooniliste farmiloomadega. Samas on neil kõrgem sööda konversioonimäär ning putukate kasvatamine on tavapäraste põllumajandusloomade kasvatamisest oluliselt vee- ja energiasäästlikum, mis teeb nende tootmise traditsiooniliste farmiloomade kasvatusest tõhusamaks. Selle tööstuse lühikese ajaloo tõttu on putukate mass-tootmise mitmed aspektid veel vähe uuritud. Optimaalsete kasvatustehnikate väljatöötamist takistavad muuhulgas toidu ja sööda tootmiseks sobivate putuka-liikide elutsüklite ning füsioloogiliste ja käitumuslike eripärade ebapiisav tundmine.

Elukäiguteooria uurib kohastumisstrateegiate mitmekesisust ja seda, kuidas organismid neid muutuvates keskkonnatingimustes kohasuse maksimeerimiseks kasutavad. Piiratud ressursside tõttu ei saa organismid tavaliselt investeerida kõi-gisse elukäiguomadustesse (omadused, mis on tihedalt seotud kohasusega) sama-võrra. Seetõttu on elukäiguomadused sageli üksteisega lõivsuhetes – investeerides rohkem ühte, saab vähem investeerida teistesse. Lisaks sõltuvad elukäigu-omadused tugevalt keskkonnateguritest ja nende koosmõjudest.

Liigikaaslaste asustustihedus kasvukeskkonnas on keskkonnategur, millel on potentsiaalselt eriti tugev mõju putukatele ja teistele kõrge viljakusega ning looduses koonduvatele organismidele. Suurenenud asustustihedusega keskkondades elamine võib oluliselt mõjutada elukäiguomadusi ning põhjustada muuhulgas värvuse muutusi, ainevahetuse intensiivistumist, arenguaja lühenemist (või mõnikord hoopis pikenemist), kehamassi vähenemist ning liigisisese konkurentsi, patogeenide kontsentratsiooni ja suremuse suurenemist. Asustustihedus mõjutab erinevaid elukäiguomadusi erineval määral ning optimaalne asustustihedus võib märkimisväärselt suurendada kohasusust ja elukäiguomaduste väärtusi.

Hermetia illucens (BSF) on kahetiivaliste seltsi ja ogakärblaste sugukonda kuuluv putukaliik, millel on suur potentsiaal toidu ja sööda tootmiseks. BSF on muuhulgas pälvinud tähelepanu perspektiivi tõttu söödana loomakasvatuses ja vesiviljeluses, biotransformatsioonil, väetiste tootmisel ja jäätmekäitluses. Tootmispotentsiaali realiseerimiseks ja tootmise optimeerimiseks on aga oluline mõista ja rakendada elukäiguomaduste vaheliste seoste eripärasid ning neid mõjutavate keskkonnategurite üksik- ja koosmõjusid. Üks viis selle vastsestaadiumis vabatahtlikult koonduva liigi rakendusliku potentsiaali realiseerimiseks on mõista ja arvestada tootmisel vastsete koondumiskäitumisega seotud aspekte.

Selles väitekirjas uurin, kuidas asustustihedus vastseperioodil mõjutab elukäigu tunnuseid (sealhulgas immuunreaktsiooni) vastusena temperatuurile, entomopatogeensetele seentele ja substraadi fermentatsioonile. Eesmärk oli määrata kindlaks BSF optimaalne asustustihedus ja uurida, kuidas asustustihedus suhestub teiste keskkonnateguritega.

Tulemused näitasid, et kõrgetel vastsete asustustihedustel (5 ja 10 vakla/cm^2) väheneb BSF vastsete elumus, eelnukkude, nukkude ja valmikute mass ning eelnukkude rasvasisaldus, samuti pikenes vastseperiood madala asustustihedusmenetlusega (1 või 2 vakla/cm^2) võrreldes. Olulise leiuna selgus, et asustustiheduse mõju sõltus temperatuurist. Katsetulemustele toetudes saab BSF optimaalseks kasvukeskkonnaks pidada madalat vastsete asustustihedust ja kasvutemperatuuri 27 °C ümbruses. Kõrgetel asustustihedustel täheldati siiski vastsete fenooloksüdaasi aktiivsuse suurenemist, mis on seotud tugevama immuunvastusega ja parema valmisolekuga patogeenide rünnakuteks. Teisalt kaasnesid tugevama immuunvastusega kõrgetel asustustihedustel väiksemad kehamõõtmed ja pikem arenguaeg, mis ei ole tootmisaspektist soovitavad tulemid.

Lisaks selgus, et entomopatogeensel seentel liigist *Beauveria bassiana* võib olla oluline mõju BSF eelnukkude ja nukkude massile ning arenguajale nii madalal kui kõrgel asustustihedusel. Fenooloksüdaasi aktiivsus oli seentega nakatatud vastsetel kõrgem kui nakatamata vastsetel. See tulemus viitab sellele, et BSF vastsed suudavad märkimisväärselt investeerida immuunfunktsiooni ka mitme stressori (kõrge asustustihedus, patogeenid) samaaegsel esinemisel. Täheldasime, et kõrgema asustustiheduse korral saavutasid teatud seenetüvega (*B. bassiana* tüvi KVL 03-127) nakatatud vastsed eelnukuna suurema massi kui nakatamata vastsed. Selline tulemus viitab asjaolule, et kõrgel asustustihedusel võib esineda konsumeerimise suurenemist kompenseerimaks immuunvastuse tekitamisega seotud kulusid.

Lõpuks leidsime, et asustustiheduse mõju BSF elukäiguomadustele sõltub substraadi tüübist ja fermenteeritusest. Selgus, et fermentatsioon võib osaliselt parandada kiudainerikaste substraatide seedimist BSF vastsetes. Lisaks võivad kiuainerikkad substraadid osaliselt leevendada kõrge asustustiheduse negatiivseid mõjusid. See võib olla tingitud sellest, et raskesti seeditavatel substraatidel võimaldab kõrge asustustiheduse korral liigikaaslaste suur arv toitu paremini lagundada ja seeläbi toidu seedimist parandada.

Väitekirja tulemused viitavad sellele, et BSF elukäiguomadused sõltuvad oluliselt asustustihedusest. Asustustiheduse mõju aga sõltub teistest keskkonnateguritest – temperatuurist, entomopatogeensetest seentest ja substraadi fermentatsioonist. Seega tuleb putukate kasvatamisel arvestada korraga mitme keskkonnateguriga, sest ühe teguri muutmisega võivad kaasneda muutused teiste tegurite mõjus ning selliste koosmõjude suund ja tugevus võivad erinevate elukäiguomaduste puhul olla erinevad.

Ogakärblase *Hermetia illucens* toiduks ja söödaks kasvatamisel on selle väitekirja tulemuste alusel optimaalne kasutada vastsete madalat asustustihedust ja keskmisi kasvatustemperatuure. See lähenemine toob kaasa kõrgema elumuse, suuremad kehamõõtmed, lühema vastseperioodi ja eelnukkude suurema rasvasisalduse. Seevastu kõrge asustustihedus viib küll vastsete tugevama immuunvastuseni, kuid selle hinnaks on väiksem kehamass ja pikemad arenguperioodid, mis ei ole tootjatele majanduslikult optimaalne lahendus. Lisaks võib fermentatsioon parandada BSF vastsete võimet toituda kõrge kiudainesisaldusega substraatidest, kuid selle tõhusus varieerub substraadist ja asustustihedusest sõltuvalt.

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Conference presentations

Opare L. O., Meister H., Holm S., Kaasik A., Esperk T., Life history traits and immune investment in the black soldier fly are influenced by larval density. XII European Congress of Entomology, Crete Greece. *Oral presentation*

Opare L. O., Meister H., Holm S., Kaasik A., Esperk T., To maintain social distance or not? Quantifying the effects of larval density on black soldier fly larvae life-history and immunity. INSECTA 2022. Justus Liebig University, Giessen, Germany. *Oral presentation*

Opare L. O., Meister H., Holm S., Kaasik A., Esperk T., Larval density effects on key life history traits and immunity in the black soldier fly. Annual Conference of the Institute of Ecology and Earth Sciences, 2022. *Oral presentation*

Opare L. O., Meister H., Holm S., Esperk T., Larval density effects on black soldier fly (*Hermetia illucens*) life history traits. XXII European congress of lepidopterology, Laulasmaa, Estonia. *Poster Presentation*

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Opare, L. O., Holm, S., and Esperk, T. (2022). Temperature-modified density effects in the black soldier fly: Low larval density leads to large size, short development time and high fat content. Journal of Insects as Food and Feed, 8, 783–802. https://doi.org/10.3920/JIFF2021.0147

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