Genetic Diversity and Mating Systems in a Mass-Reared Black Soldier Fly (*Hermetia illucens*) Population

by

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Declaration

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Abstract

Black soldier flies are gaining popularity as an alternative source of protein in animal feed. They have a high feed conversion ratio and can be reared on biowaste, reducing the energy input required for mass-rearing. As the number of mass-reared colonies is increasing worldwide, the importance of genetic management in commercial populations is becoming clear. This study aimed to determine the effects of domestication and mating behaviour on the genetic diversity of a mass-reared black soldier fly population. Eight microsatellite markers were used to estimate genetic diversity in two temporally separated samples of a wild black soldier fly colony ($Wild_{2015}$ and $Wild_{2018}$) and three distinct generations of a mass-reared black soldier fly colony (F_{28}, F_{48} and F_{52}). Diversity estimates decreased significantly in the mass-reared colony over time, when compared to the two wild samples. The mass-reared colony also saw an increase in relatedness over time, with a relatedness coefficient as high as 0.430 in generation F_{48}. These results indicate severe inbreeding in the mass-reared colony. Effective population sizes of between 22.6 and 59.0 in the mass-reared colony are also a cause for concern, as populations with low effective population sizes are more vulnerable to inbreeding depression and extinction. The high levels of genetic diversity observed in the two wild samples provide the potential for the wild colony to become a donor population, providing immigrants to introduce genetic diversity into the mass-reared colony. However, based on F_{ST} estimates, the two populations appear to be diverging from each other over time. Moderate differentiation was observed between $Wild_{2015}$ and F_{28} (F_{ST}=0.062; p=0.000), while great differentiation was observed between $Wild_{2018}$ and F_{52} (F_{ST}=0.161; p=0.000). To minimise the risk of outbreeding depression, the compatibility of wild individuals with the artificial environment would therefore need to be tested before immigrants are introduced into the mass-reared population. To study the mating behaviour of the black soldier fly, five mating pairs were randomly sampled *in copula* from generation F_{48} of the mass-reared colony. All candidate parents and 25 offspring from each clutch were genotyped and subjected to parentage analysis. Multiple paternity was detected in two of the five families, providing evidence for polyandry. This was a novel finding, as observation had previously led to the hypothesis that this species is monogamous. The occurrence of polyandrous mating provides evidence that adult flies can mate multiply despite being unable to replenish energy through feeding, thereby creating the potential for polygynous mating. Additionally, polyandrous mating has positive implications for the genetic management of commercial black soldier fly populations. However, these results are limited to mass-reared colonies, as the higher population densities found in captive populations increase the probability of remating. Finally, diversity estimates and inbreeding estimates were calculated for the candidate parents, offspring, and the population the parents were sourced from. Individual parent pairs showed increased levels of relatedness when compared to the source population, indicating positive assortative mating. As markers from random genomic regions were used for this study, the observed increase in relatedness may provide additional evidence for inbreeding in the mass-reared population. However, inbred populations show greater genome wide

linkage disequilibrium, meaning that mate selection for desirable traits could potentially be detected in markers not directly related to traits of interest.

Opsomming

Die gewildheid van die venstervlieg, *Hermetia illucens*, as ’n alternatiewe bron van proteïene in dierevoer is besig om toe te neem. Hulle het ’n hoë voer omsettings verhouding en kan grootgemaak word op organiese afval, wat die energie insette wat benodig word vir produksie verlaag. Die onlangse toename in kommersiële kolonies wêreldwyd is besig om die belang van genetiese bestuur in fabriek populasies na die voorgrond te bring. Die doel van hierdie studie was om die uitwerking van domestikasie en paringsgedrag op die genetiese diversiteit van ’n fabriek venstervlieg populasie te ondersoek. Agt mikrosatelliet merkers is gebruik om die genetiese diversiteit van twee temporaal-geskeide groepe van ’n wilde venstervlieg kolonie ($Wild_{2015}$ en $Wild_{2018}$) en drie diskrete generasies van ’n fabriek populasie (F_{28}, F_{48} en F_{52}) te ondersoek. In vergelyking met die wilde populasie, was daar ’n beduidende verlies van genetiese diversiteit in die fabriek populasie. Die verwantskap tussen lede van die fabriek populasie het ook mettertyd toegeneem, met ’n verwantskapskoëffisiënt van 0.430 teen generasie F_{48}. Hierdie bevindinge dui op hoë vlakke van inteling in die fabriek populasie. Verder is die effektiewe populasiegrootte in die fabriek ook ’n bron van kommer, met beraamde effektiewe populasie groottes van tussen 22.6 en 59.0 in die drie fabriek generasies. Populasies met klein effektiewe populasie groottes is meer kwesbaar vir intelingsdepressie en uitwissing. Aangesien die wilde venstervlieg kolonie hoë vlakke van genetiese diversiteit toon, sal hierdie kolonie moontlik gebruik kan word om immigrante te skenk vir die uitbreiding van diversiteit in die fabriek populasie. Volgens F_{ST} skattings, is die twee kolonies egter besig om geneties van mekaar te differensieer. Slegs matige differensiasie is waargeneem tussen $Wild_{2015}$ en F_{28} (F_{ST}=0.062; p=0.000), terwyl groot differensiasie waargeneem is tussen $Wild_{2018}$ en F_{52} (F_{ST}=0.161; p=0.000). Om die risiko van uittelingsdepressie te verlaag sal die verenigbaarheid van wilde vlieë met die kunsmatige omgewing bepaal moet word, voordat wilde immigrante aan die fabriek populasie geskenk word. Om die paringsgedrag van die venstervlieg te bestudeer, is vyf teelpare uit generasie F_{48} van die fabriek kolonie lukraak geselekteer tydens die proses van paring. Al tien potensiële ouers en 25 larwes van elke broeisel is gegenotipeer en ouerskap toetse is op die larwes uitgevoer. Veelvuldige vaderskap is in twee van die vyf families waargeneem, wat dui op poliandrie in die fabriek populasie. Hierdie was ’n nuwe bevinding, aangesien waarnemings in die verlede gedui het op monogamie in hierdie spesie. Die voorkoms van poliandriese paring wys dat volwasse vlieë wel veelvuldig kan paar, ten spyte van die feit dat hulle nie tussen parings energie kan aanvul deur te eet nie. Veelwywery is dus ook nou ’n moontlikheid in venstervlieë. Poliandrie het ook positiewe implikasies vir genetiese bestuur, aangesien veelvuldige paring ’n goeie invloed kan hê op genetiese diversiteit. Die huidige studie was egter beperk tot bevindinge in ’n kunsmatige omgewing, waar hoër populasie digthede as in die natuur kan lei tot ’n hoër waarskynlikheid van veelvuldige paring. Skattings van genetiese diversiteit en inteling vir potensiële ouers, hul nageslag en die populasie vanwaar die potensiële ouers verkry is, is gevolglik bereken. Afsonderlike ouerpare het ’n hoër mate van verwantskap getoon as individue in die algehele populasie, wat dui op positiewe assorterende paring. Aangesien

merkers uit lukrake gebiede in die genoom gebruik is vir hierdie studie, is dit moontlik dat die toename in verwantskap tussen ouerpare ’n verdere bewys is van inteling in die fabriek populasie. Ingeteelde populasies toon egter ’n groter mate van genetiese koppeling, wat kan veroorsaak dat maat seleksie vir aanloklike kenmerke waargeneem word in merkers wat nie direk verband hou met die kenmerk van belang nie.

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List of Abbreviations

% - Percent

°C – Degree Celsius

µl – Microliter

µM – Micromolar

µmolm-2s-1 – Micromole per square meter per second

A_n – Number of alleles

A_r – Allelic richness

AFLP – Amplified fragment length polymorphism

AMOVA – Analysis of molecular variance

AMP – Antimicrobial peptide

Bp – Base pair

BSF – Black soldier fly

BSFL – Black soldier fly larva

CI – Confidence interval

cm – Centimetre

CTAB – Cetyltrimethyl ammonium bromide

ddH_20 – Double-distilled water

DNA – Deoxyribonucleic acid

F_{IS} – Wright's F-statistic (individual relative to subpopulation)

F_n – Generation number

F_{ST} – Wright's F-statistic (subpopulation relative to total population)

H_o – Observed heterozygosity

HWE – Hardy-Weinberg equilibrium

I – Shannon's information index

IAM – Infinite alleles model

LD – Linkage disequilibrium

mm – Millimetre

N_e – Effective population size

ng – Nanogram

PA_r – Private allelic richness

PCoA – Principal coordinate analysis

PCR – Polymerase chain reaction

pH – Potential for Hydrogen

r – Relatedness

RAPD – Random amplified polymorphic DNA

RFPL – Restriction Fragment Length Polymorphism

SMM – Stepwise mutation model

SNP – Single nucleotide polymorphism

T_a – Annealing temperature

TPM – Two-phase model

uH_e – unbiased expected heterozygosity

v – Version

Chapter 1: Literature Review & Study Rationale

1.1 The Need for Sustainable Resources

As of 2019, there are an estimated 7.7 billion people on Earth. This number is expected to grow to a projected 9.7 billion by 2050 (United Nations Department of Economic and Social Affairs: Population Division, 2019). As food security is already a problem, especially in Africa, feeding an additional two billion people will require an extensive, multifaceted strategy (Godfray *et al*., 2010; Hall *et al*., 2017). The agricultural sector is currently faced with many challenges that need to be overcome to meet the growing demand for food- and nutrition security. These include the effects of climate change, insufficient land, a shortage of fresh water and environmental impacts, such as eutrophication (Tilman *et al.*, 2001; Falkenmark *et al.*, 2009). The fishing industry is facing its own set of complications. Although capture fishery production has remained relatively stable over the last few decades, 34.2% of species are being fished at biologically unsustainable levels (FAO, 2020). This number is on the increase, as additional factors, such as climate change, contribute to the loss of fish stocks (IPCC, 2014). Overfishing not only has a negative impact on wild fish populations, but also on coral reefs and plants that are dependent on specific fish species for survival (Correa *et al*., 2015; Zaneveld *et al*., 2016). Therefore, to compensate for the continuous rise in demand for fish, producers have turned to aquaculture. In 2018, 46% of fish production was through aquaculture (FAO, 2020). While this is a positive development, factors such as space, disease control and a need for sustainable aquafeed are limiting the potential growth of the industry. In addition to this, the aquaculture industry is currently the largest consumer of fishmeal and fish oil, still harvested from conventional capture fisheries (Hasan, 2017). It is clear that the current global dietary requirement for protein is not sustainable enough to carry the projected population growth. For this reason, the industry has started investigating alternative, more sustainable resources in the form of insects.

Although humans have likely been exploiting insects for millennia, the first known instance of cultivation occurred during the Bronze Age in China. It was at this time that the Chinese started producing silk from the domesticated silkworm, *Bombyx mori* (Barber, 1992). Various insects with medicinal value have also been used for centuries, as demonstrated by ancient Mexican civilisations (De Conconi & Moreno, 1988). In addition to this, they can be used as biological control agents, with South Africa being amongst the top ten countries with the most successful insect biological control agent introductions between 1890 and 2010 (Cock *et al.*, 2016). Finally, due to their high protein and nutrient content, around 1900 insect species are consumed by humans worldwide. Some species can even be used as substitutes for fishmeal and with a high feed conversion ratio, insect mass-rearing is more sustainable than the production of conventional terrestrial livestock and aquaculture

species (van Huis *et al*., 2013; Zielińska *et al*., 2015). One of the species that is being advocated as an alternative to fishmeal is *Hermetia illucens*, more commonly known as the black soldier fly.

1.2 *Hermetia illucens*: A Cosmopolitan Species

The black soldier fly (BSF) is a member of the order Diptera. It belongs to the Stratiomyidae family, commonly known as soldier flies. Soldier flies are characterised by a discal cell in their wing venation (figure 1.1) and 370 species within this group populate Africa. Despite the abundance of soldier flies, their taxonomy has not been resolved as only the black soldier fly is thought to be significant to forensic science (Villet, 2011). Within the subfamily Hermetiinae, *Hermetia* is the most speciose, as well as the most widespread. It contains 76 species and is distributed all over the world, with the other four genera contained to specific regions (Roháček & Hora, 2013). *Hermetia illucens*, one of the more prevalent species, is believed to have originated in South America (Walker, 1866). Its presence was first recorded in South Africa in 1915, on the coast of KwaZulu-Natal. From there, the BSF has since spread throughout South Africa, but is most prominent in the temperate and subtropical regions. This was followed by the first known colonisation of Europe in 1926, which led to the species' current distribution within tropical and sub-tropical areas across several continents. The gradual settlement of coastline areas around the world, followed by inland expansion, has led to the hypothesis that the species originally spread through maritime transport. In the absence of the food preservation methods known today, infested matter was loaded onto ships. Upon landing, the black soldier fly was then accidentally introduced into new habitats (Picker *et al.*, 2004; Marshall *et al.*, 2015). Colonisation was facilitated by the saprophagous nature of larvae, which provides them with the ability to adapt readily to foreign environments (Roháček & Hora, 2013).

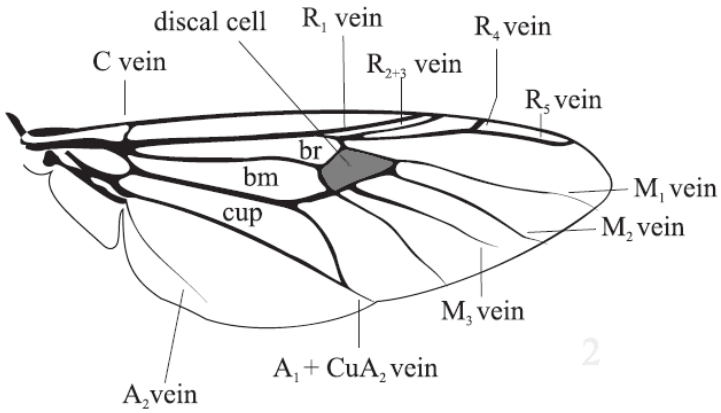

Figure 1.1: The structure of wing venation in the black soldier fly. The discal cell is indicated in grey. Adapted from Carvalho and Mello-Patiu (2008).

1.3 Life History

The five stages of black soldier fly development are: egg, larva, prepupa, pupa and adult (figure 1.2). The adult black soldier fly resembles wasps in its morphology, but only has two wings instead of four and does not have a stinger (Diclaro & Kaufman, 2009). This resemblance is intensified by

translucent areas on its abdomen, giving it the appearance of a wasp-like petiole during flight (Woodley, 2001). The length of the fly ranges between 15 and 20mm, with females generally larger than males. However, this is not a recognized sexual dimorphism, and genitalia are a more reliable indicator of sex (figure 1.3). Scanning electron microscope images have revealed that the entire body, including the wings and legs, are densely covered in hair. This gives the fly's body a velveteen appearance upon microscopic inspection. The sponge-like adult mouthpart, equally hairy, can also be observed in these images. As the adult phase of this species does not eat, surviving on larval fat reserves and water, a chewing mouthpart is not required (Oliveira *et al*., 2016). Adult flies live for approximately one week, during which their sole purpose is to mate and, in the event of females, lay eggs. This lifespan can be prolonged by access to a source of water or a humid environment. Mating can occur from two days post-emergence onwards, provided the environment is conducive. Both warm temperatures, starting at a minimum of 25°C, and full sunlight are required (Diclaro & Kaufman, 2009; Dortmans *et al*., 2017). Each male has its own territorial site that it defends until a female passes by. The male then proceeds to grab the female and return to its territory *in copula*. This behaviour is known as lekking (Tomberlin & Sheppard, 2001).

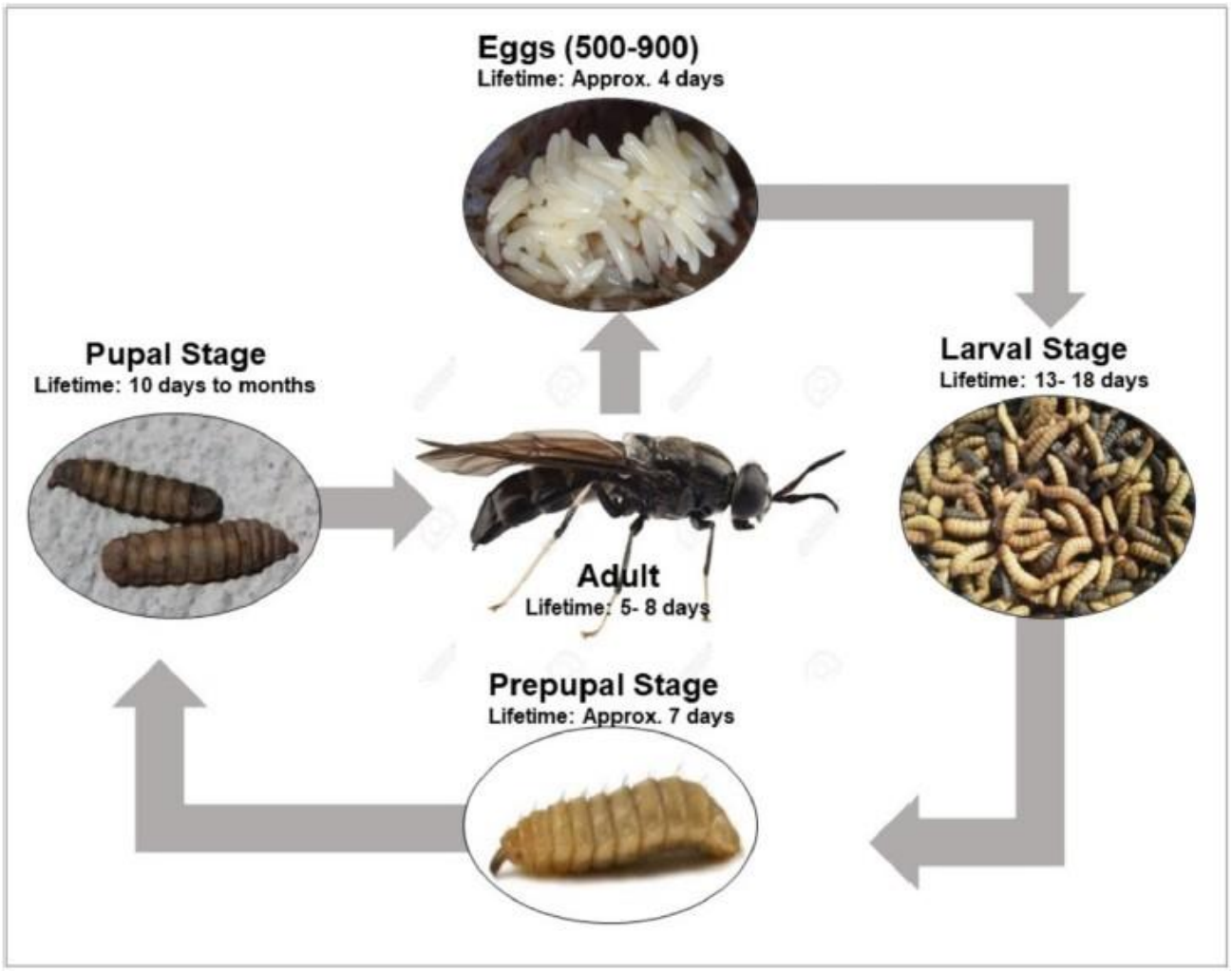

Figure 1.2: The life cycle of the black soldier fly, with estimated time spent in each stage of development included (Singh & Kumari, 2019).

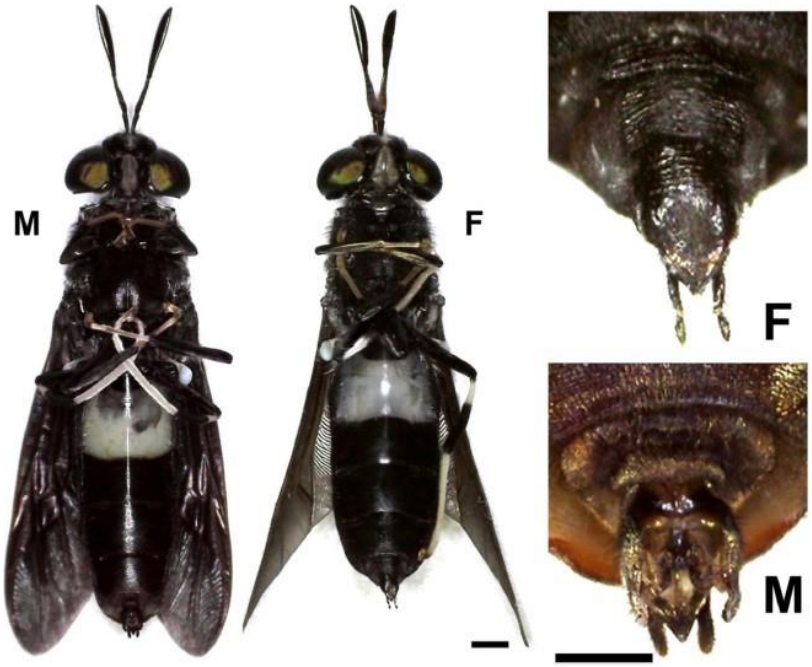

Figure 1.3: The external morphology of the black soldier fly. Full specimens are on the left, with close-up images of the genitalia on the right [Female, F; Male, M]. The scale bars represent 1mm (Oonincx *et al*., 2016).

The oviposition of eggs occurs roughly two days after mating. Females lay their eggs close to decomposing organic matter, which serves as a larval food source after hatching. In addition to this, they tend to oviposit in crevices, out of direct sunlight. This protects the eggs from both predators and dehydration. A relative humidity of at least 60% is preferred to avoid desiccation and, under controlled conditions, oviposition rarely occurs below the temperature of 26°C. Females die shortly after ovipositing, having depleted their fat reserves. The eggs then hatch after an average of four days, beginning the larval phase (Tomberlin & Sheppard, 2002; Dortmans *et al*., 2017). During this phase of development, the main goal of the insect is to accumulate and store fat reserves to sustain the pupal phase and the adult individual. There are six stages of larval development, known as instars. Larval instars can be identified by the observation of exuviae after moulting, with each instar having its own significantly different head capsule width (Kim *et al*., 2010). Similarly to adult flies, larvae are covered in hair. The mandibular-maxillary complex is highly developed for a larval phase, which is characteristic of their status as scavenger (Purkayastha *et al*., 2017). Larvae can grow up to a length of 27mm, with a width of 6mm. Within approximately 14 days, they reach the sixth instar, known as the prepupal stage (Diclaro & Kaufman, 2009). At this point, the larva changes colour and replaces its mouthpart with a hook-like structure. It then moves away from the food and younger larvae, to find a dry, safe place to pupate. During the pupal stage, protected by the puparium, the pupa undergoes metamorphosis, maturing into an imago (figure 1.4). Pupation lasts at least a week and ends in a five-minute emerging process (Barros-Cordeiro *et al*., 2014; Dortmans *et al*., 2017).

Figure 1.4: The stages of intra-puparial development. (a) Cryptocephalic pupa; (b) pharate adult; (c) imago (Barros-Cordeiro *et al.*, 2014).

1.4 Black Soldier Flies as a Valuable Resource

The black soldier fly possesses several characteristics that appeal to it being a resource for human utilisation. Larvae have the ideal fat and protein content to serve as a source of animal feed, in the form of larva meal and -oil. In addition to this, they have a higher feed conversion efficiency than conventional production animals, as well as other edible insects, such as yellow mealworms and house crickets (Oonincx *et al*., 2015). Water is utilised more efficiently than with conventional livestock, as only the adults require a small amount of additional drinking water. The primary source of water for larvae is their feed (Józefiak *et al*., 2016). The guts of BSF larvae contain a wide variety of digestive enzymes, which enables them to be polyphagous (Kim *et al*., 2011). Moreover, while their chemical composition may fluctuate slightly based on diet, their survival rate, protein and -fat content consistently remain favourable (Oonincx *et al*., 2015). Unlike house flies, which are also suitable for use in animal feed, black soldier flies are not known to be vectors of disease. This is because adult flies do not consume solid foods (Kenis *et al*., 2018). While cases of human myiasis, the infestation of the intestines with larvae, have been reported, these are extremely rare. This can only happen through the ingestion of BSF eggs, which are generally oviposited near decaying matter (Lee *et al*., 1995). Therefore, the risk of ingesting them is low. Another advantage of maintaining a black soldier fly colony, is that they can suppress the presence of house flies by up to 100%, depending on the environment (Sheppard, 1983).

Although their use in the production of animal feed is one of the more well-known applications of the black soldier fly, they are of value to humans in many other ways. In forensics, wild black soldier flies are amongst the species used to determine the post-mortem interval of decomposing bodies. The

stage of development of the insects on the body gives an indication of the amount of time that has passed since death (Pujol-Luz *et al*., 2008). Furthermore, the polyphagous nature of larvae allows them to break down biowaste that would have gone to landfills. This includes, but is not limited to, waste from the brewing industry, commercial food waste, coffee pulp and animal manure. They are even capable of feeding on human excrement, which could be utilised to improve sanitation in developing countries (Lardé, 1990; Banks *et al*., 2014; Chia, Tanga, Osuga, *et al*., 2018). While passing through the digestive tract of black soldier fly larvae, these various forms of organic waste are then processed into a nutrient rich compost that can be added to fertilisers (Salomone *et al*., 2017). Feeding BSF larvae specific types of waste may also lead to an increase in their nutritional value, such as the abundance of omega-3 fatty acids in larvae fed fish offal (St-Hilaire *et al*., 2007). To protect them from the many pathogenic microbes included in their saprophagous diet, antimicrobial peptides (AMPs) are produced within their gut. Many of these AMPs were previously unknown. Therefore, the discovery of the gene fragments encoding for these novel AMPs could lead to the production of new antibiotics. An example of this is defensin-like peptide 4 (DLP4), which was discovered in the haemolymph of *H. illucens* larvae that were immunised with Gram-positive bacteria. Analyses showed that, amongst others, DLP4 had antibacterial effects against methicillin-resistant *Staphylococcus aureus*, more commonly known as MRSA (Park *et al*., 2015; Elhag *et al*., 2017).

Additional possible applications of the BSF that have been studied include the extraction of chitin from both the larval and adult exoskeleton, and the possible production of biodiesel from larval fat (Li *et al*., 2011; Khayrova *et al*., 2019). The ideal outcome would be to combine several of these listed applications into a sustainable production system. The range of possibilities is explored in figure 1.5. Salomone *et al*. (2017) investigated a basic version of such a mass-rearing system by performing a life cycle assessment, which evaluated the environmental impact of mass-rearing black soldier flies. Their production system involved the bioconversion of organic waste to compost and dried larvae for protein and lipid extraction. The main benefit of such a system was found to be the minimal required land usage, when compared to alternative food sources. Energy usage and global warming potential are still in the process of optimisation, as this is a relatively new field. However, the authors regarded land usage as the most important variable, with available agricultural space being a limiting factor worldwide.

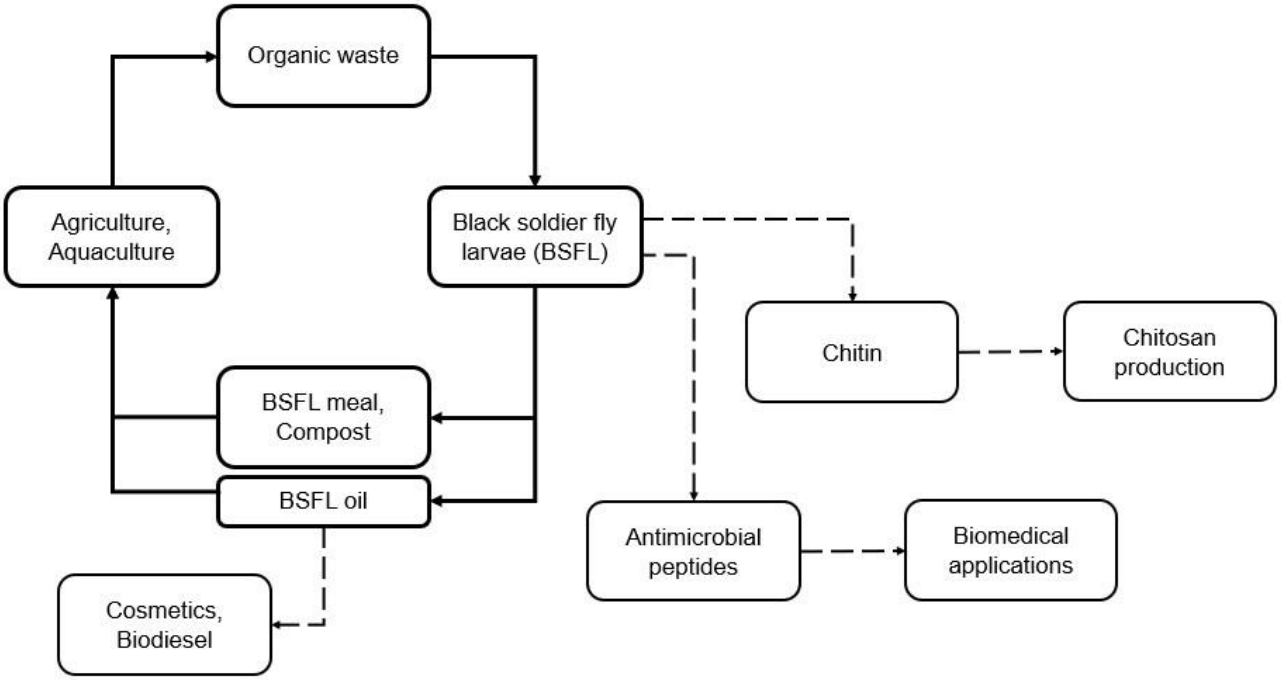

Figure 1.5: A depiction of the possible applications within a sustainable BSF production system. The main cycle, depicted by solid lines, involves the mass-rearing of black soldier fly larvae on organic waste and using the harvested BSFL meal and -oil as agricultural feed. Chitin, antimicrobial peptides and BSFL oil may also be extracted and used for other downstream applications to ensure the maximum production yield. These additional applications are indicated by dashed lines.

1.5 The Rearing Process

While the mass-rearing of black soldier flies has garnered attention in recent years, farmers have been rearing on a small scale for decades. In an environment with a constant supply of manure, such as a poultry farm, the rearing of larvae requires no additional input of energy or specialised equipment. The prepupae harvest themselves by moving away from the food source and can be fed whole to the chickens as part of their diet. A portion of the prepupae are allowed to complete their life cycle and females then oviposit their eggs close to the manure. This rearing method has the added benefit of preventing pests such as house flies from ovipositing (Sheppard *et al*., 1994).

The mass-rearing process is considerably more complicated than this basic system. The first hurdle to overcome is finding a suitable site for the mass-rearing facility. It should preferably not border on a densely populated neighbourhood, while still having a sufficient supply of electricity and water. Additionally, it needs to be readily accessible to the providers of incoming waste. It is also necessary to provide enough space for the BSF nursery, waste treatment, offices, laboratories, and hygiene facilities (Dortmans *et al*., 2017). Once the facility has been built, the next step is the creation of a production line. The continuous bioconversion of organic waste relies on a constant supply of five-day old larvae. To ensure that this demand is met, a percentage of larvae need to be retained within the nursery. Their progress needs to be tracked and optimised to produce the required output of larvae. The bioconversion larvae are reared in a separate section and prepupae that have failed to

harvest themselves need to be sieved from the waste residue. In the processing unit, prepupae are sorted into groups that are either being sold alive or require further processing. The latter group of individuals is killed through boiling, which also sanitises them. The processing of compost can be done in this unit as well (Dortmans *et al*., 2017; Salomone *et al*., 2017).

Once the production line has been established, the focus can be shifted to optimisation of the mass-rearing process. Abiotic factors such as temperature, humidity and light intensity can all contribute to larval development and adult fitness. Density is another variable that affects larval growth. Sheppard *et al.* (2002) found that a density of 2.5 larvae per cm^2 of surface area allowed uninhibited growth and development. While a broad spectrum of temperatures is tolerated in nature, the optimal mass-rearing temperature was found to be between 27 and 30°C (Tomberlin *et al*., 2009). Adult longevity is higher at 27°, due to an increased prepupal weight. Although adults live a few days shorter at 30°C, the shorter larval development time could lead to a higher turn-over rate. An optimal temperature of 30°C was supported by Chia *et al*. (2018), with a wider range of life history traits included in their analysis. Maintaining high levels of humidity also leads to better performance at all stages of BSF development. A relative humidity of 70% leads to a shorter developmental time for eggs, larvae, and pupae than lower levels of humidity. In addition to this both the hatch rate and percentage eclosion from pupae are higher, with lower larval mortality rates and increased adult longevity (Holmes *et al*., 2012). Furthermore, the composition of the pupation substrate influences the time needed for post-feeding larvae to reach pupation. The absence of a pupation substrate has been proven to slow down prepupal development, while also decreasing the rate of adult emergence. Although the presence of any substrate is beneficial, wood shavings and potting soil have been suggested as better substrates than topsoil and sand, shortening the time needed to pupate even further (Holmes *et al*., 2013). Post-eclosion, adults only need a source of water to increase longevity, but creating suitable conditions for mating is a more strenuous task (Dortmans *et al*., 2017). Temperature and relative humidity need to be at the previously discussed levels, while the majority of mating occurs at light intensities above $200\mu molm^2s^{-1}$. The density of adults per cage, as well as the time of day, are also contributing factors, with a decreased incidence of mating during the afternoon (Sheppard *et al*., 2002).

One of the aspects of BSF mass-rearing that has seen the most extensive research is larval diet. As larvae can process a wide variety of biological waste, there are many possible substrates with varying effects on their life history. The most beneficial diet appears to be food waste, producing the longest and heaviest flies (Nguyen *et al*., 2015). This is possibly due to the more complex composition of the diet, when compared to specifically plant- or animal-based substrates. The implementation of either a batch feeding system or a daily feeding system also affect larval development. While feeding the larvae daily increases larval weight, the development time is also longer (Meneguz *et al*., 2018). It would thus be sensible to evaluate the practicality of batch feeding vs. daily feeding, as well as to compare the gain in larval weight to the lag in production time. Another

consideration is the environmental impact of different feed sources. A substrate that acts favourable towards growth performance, but is detrimental to the environment, counteracts the benefits of insect mass-rearing. Smetana *et al*. (2016) studied the environmental effects of multiple possible black soldier fly diets, such as distiller's grains and municipal waste. The use of the black soldier flies as either animal feed or food for human consumption was also accounted for. Municipal waste was found to be the most environmentally friendly food source for BSF larvae intended to feed animals. This was followed by cattle manure and distiller's dried grains with solubles. These options were all more sustainable than the control ryemeal larval diet. With waste and manure diets excluded, larvae intended for human consumption proved to be a more sustainable protein supplement than whey protein, regardless of diet.

Research on the effects of initial pH of larval substrate has been inconclusive. An Italian group of researchers found no significant changes in performance within a pH range of 4.0-9.5 *(Meneguz et al*., 2018). Within the same timeframe, a Chinese group found acidic substrates with a pH of 4.0 and lower to influence larval growth negatively. A pH range of 6.0-10.0 was more conducive to growth, with a pH of 8.0 having the added advantage of shorter larval development time (Ma *et al*., 2018). Many factors, such as the use of geographically separated fly strains and differing diet compositions, may explain this discrepancy. This is therefore an additional factor that would need to be explored in each unique mass-rearing environment. Both research groups, however, found that the substrate pH naturally shifts to a slightly basic pH of 8.5-9.0 towards the end of the larval phase (Ma *et al*., 2018; Meneguz *et al*., 2018).

Yet another component of dietary research involves the study of microbial content within BSF larval guts and larval substrates. The microbiome within the substrate affects the microbiome within the gastrointestinal tract. Food waste, proving to be the most beneficial substrate on many levels, has the highest impact on microbial activity (De Smet *et al*., 2018). This is thought to be due to the higher nutritional complexity, which allows a more diverse microbial community to thrive. The large variety of microorganisms is then transferred to the guts of larvae as they feed. Adding companion bacteria to larval substrates has also been shown to improve overall growth rate and larval weight, possibly as a result of bacteria aiding in the digestion of feed (De Smet *et al*., 2018). Furthermore, the assortment of microbes present in the substrate's microbiome influences the production of antimicrobial peptides within the larval gut. AMPs are produced according to the microbes present, while the addition of protein or sunflower oil to the larval diet leads to a general increase in AMP production. This can be utilised to produce specific AMPs, either for biomedical purposes or for the marketing of larva meal as a natural source of protection against specific microbes (Vogel *et al*., 2018).

Although microbial diversity in their food can be beneficial to BSF larvae, the presence of heavy metals poses possible risks. The severity of these risks varies among studies, with even the most

optimistic results indicating that the bioaccumulation of cadmium is a concern. While it may not directly affect the life history of the insects, the European Union threshold levels for use in animal feed are easily exceeded (Diener *et al*., 2015). Other studies have indicated that high concentrations of cadmium or lead may impair larval growth, in addition to the bioaccumulation of these metals at levels rendering the insects unsafe for animal consumption (Purschke *et al*., 2017). The presence of contaminants within both the larval substrate and the larval meal therefore needs to be monitored to ensure that metal concentrations remain at sufficiently low levels. Another potential threat to production is the possibility of parasitoid infestation. Several species of parasitoids have been linked to *Hermetia illucens*, with adults ovipositing on BSF pupae to provide a food source for their offspring. An uncontrolled infestation can have devastating effects on colony maintenance (Di Iorio & Turienzo, 2011; Devic & Maquart, 2015). Additionally, individuals sourced from different wild populations may not adapt uniformly to the mass-rearing environment (Simões *et al*., 2007). As the mass-rearing of black soldier flies is a relatively new industry, production can also be expensive. Optimisation of the process is still in progress and many facets of colony maintenance are yet to be explored (Józefiak *et al.*, 2016).

1.6 Genetic Implications of Domestication

The effect of the mass-rearing process and domestication on the fitness of black soldier flies is one of these variables that have not yet been determined, as the initial focus of the industry was to create the optimal rearing environment. With commercial populations now dozens of generations in, the long-term effects of mass-production are becoming an increasing concern. Domestication, explained as the genetic changes in a population purposely cultured by humans, is known to have a profound impact on mass-reared animals over time (Simões *et al*., 2007). In mammals, post-domestication changes include tamer behaviour towards humans, relaxed antipredator defences, reduced motivation for foraging, an increased production of offspring, and the disruption of natural social interactions (Mignon-Grasteau *et al*., 2005). In addition to this, domesticated populations tend to show lower levels of genetic diversity than their wild counterparts (Dixon *et al*., 2008). As only a small number of individuals is removed from the wild population to establish the mass-reared colony, a population bottleneck occurs. The new population then expands, but its genetic diversity is restricted to that of the original limited founding members. This is called the founder effect, a process known to be one of the driving forces of speciation in nature over evolutionary timescales (Gavrilets & Hastings, 1996).

After the initial population bottleneck, genetic diversity often continues to decrease in domesticated populations. As domesticated colonies are isolated from natural populations, no new genetic variation is introduced. The continued mating of individuals descended from the same original founding members causes an increase in the probability of related individuals mating over time. Commercial populations are therefore at an increased risk of becoming inbred, as observed in

various traditional livestock breeds (Udeh *et al.*, 2013; Pryce *et al*., 2014; Gholizadeh & Ghafouri-Kesbi, 2016). As genetic diversity is lost and individuals within a population become more related, the number of individuals effectively contributing to the gene pool also declines. This reduction in effective population size (N_e) leaves populations more vulnerable to the effects of genetic drift. Alleles are fixed or lost more easily, aiding the loss of diversity and homogenisation of populations, and enhancing the effects inbreeding (Ellstrand & Elam, 1993).

High levels of inbreeding could have negative implications for fitness, productivity, and adaptation to sudden environmental changes (Hughes *et al*., 2008). Reed and Frankham (2003) investigated the correlation between population-level genetic diversity and fitness in several species of plants, insects, amphibians, and mammals. It was found that the correlation was highly significant and 19% of fitness variation amongst populations could be explained by it. This reduction in fitness due to inbreeding can be explained by the inbreeding load of populations. Inbreeding load is the component of the genetic load that is solely expressed in homozygotes. As genetic diversity decreases, individuals are homozygous at more loci, increasing the expression of recessive deleterious alleles and, consequently, the inbreeding load. The decline of fitness in a population due to an increased inbreeding load is referred to as inbreeding depression (Hedrick & Garcia-Dorado, 2016). Populations experiencing inbreeding depression are less tolerant to environmental stress and highly vulnerable to extinction (Bijlsma *et al*., 2000). In the wild, gene flow amongst different colonies of the same species acts as a buffer to the detrimental effects of inbreeding. Captive-bred populations, however, lack this natural barrier to inbreeding depression (Saarinen *et al*., 2010). Maintaining sufficient levels of genetic diversity and large effective population sizes are thus crucial to the survival of domesticated populations. Many researchers have attempted to determine the minimum N_e required to avoid inbreeding depression in populations. Early estimates pointed to a threshold N_e of 50-100 necessary to sustain populations, with a minimum census population size of 1000 (Lynch *et al*., 1995; Toro *et al*., 2011). However, recent studies have found that an effective population size of 100 is not enough to avoid inbreeding depression, merely limiting the total loss of fitness to 10%. The minimum N_e required to retain the evolutionary potential for fitness indefinitely may be as high as 1 000 (Frankham *et al*., 2014).

In addition to the losses of diversity associated with demographic factors, various forms of selection also affect genetic diversity in commercial populations. As populations adapt to their new environment, natural selection for captive survival traits takes place. This may lead to selective sweeps, reducing genetic diversity in the population and potentially resulting in additional bottleneck events (Zygouridis *et al*., 2014). Artificial environments that better imitate the natural habitat maintain higher levels of diversity, while environments that differ vastly from the wild become inbred over a shorter period (Briscoe *et al*., 1992).

Furthermore, natural selection for wild survival traits becomes more relaxed. With survival instincts such as the ability to find food and evade predators less important in a captive setting, there is no longer a selective advantage for individuals that possess traits related to them. With the loss of selection pressure for these traits, they diversify in domesticated populations. This is the cause of domestication syndrome in mammals (Wilkins *et al*., 2014). Although commercial populations would not directly be affected by a loss of wild survival traits, stragglers that manage to escape captivity may survive in the wild long enough to pass these unfavourable traits on to surrounding wild populations. This could lead to the decimation of natural populations if left unchecked. Breeding schemes with the aim of releasing animals back into the wild to boost natural populations may also suffer from the effects of relaxed selection, as released animals may potentially struggle to adapt to their natural environment. Individuals could even lose their ability to reproduce in the wild entirely. Conversely, this would be beneficial to producers who do not want their genetically altered animals thriving outside of captivity (Mignon-Grasteau *et al.*, 2005; Araki *et al*., 2007).

Once populations have adapted to the captive environment, the focus shifts to the optimisation of production traits. To increase yield, animals are often selected to grow larger, be heavier, or reproduce at a higher rate. This intentional selection for improvement of production traits is referred to as artificial selection (Mignon-Grasteau *et al*., 2005). While artificial selection is often an integral part of the production process, it could lead to severe inbreeding in the long term if not managed properly. As the expression of production traits becomes more uniform to fit the needs of the producer, genetic variation within the colony is lost. This means that, with each new generation of animals, the probability of any two individuals within the population being related increases. Initially, this increase in mean relatedness is tolerated, but as the N_e decreases and deleterious alleles begin to accumulate, the population may exhibit signs of inbreeding depression. The management of these negative effects of inbreeding could lead to financial losses for the producer (Weigel, 2001). In addition to this, the improvement of production traits is finite. Response to selection will eventually plateau, at which point the cost of selection may outweigh the benefits. Domesticated colonies with less founding members are likely to reach this plateau at a faster rate than colonies with a large founding population. Populations with a small initial effective population size will thus have both an earlier onset of inbreeding depression and a less favourable response to selection. For this reason, it is important to compare the short-term economic gains of selection with the long-term effects thereof on the genetic health of the population, while also accounting for the effective size of the population (James, 1971; Simões *et al*., 2007).

Although the best method for preventing inbreeding depression is maintaining a sufficient degree of population-level genetic diversity, there are ways to protect inbred populations from a reduction in fitness. One of these is a natural process called genetic purging, which aids in protecting populations from inbreeding depression. As a population becomes more inbred, recessive deleterious alleles become exposed, leading to purifying selection against them. This form of natural selection can be

highly effective, but has the negative effect of reducing genetic diversity even more (Hedrick & Garcia-Dorado, 2016; Cvijović *et al*., 2018). A strategy that preserves fitness without reducing genetic diversity can be found in genetic rescue. Foreign individuals from different populations are introduced into the inbred population to add beneficial variation to fitness traits, through processes such as heterosis. However, the introduction of individuals that are less suited to the captive environment or that differ too vastly from the domesticated population, may lead to outbreeding depression. A thorough assessment of possible outcomes would therefore need to be done before attempting this method of fitness preservation (Tallmon *et al.*, 2004). Given the risks of both genetic purging and genetic rescue, the best way to maintain population fitness would be to avoid severe inbreeding altogether. Breeding programmes tailored to promote both selection for production traits and adequate levels of genetic diversity would be the best way forward. Such a breeding programme, however, can only be implemented successfully if the mating behaviour of the species is thoroughly understood.

1.7 Mating Systems

All animal mating systems can be divided into two main groups: monogamy and polygamy. Factors such as the distribution of resources in the environment, the ratio of sexually active males to females, and the degree of parental care needed to rear offspring contribute to the mating system of a particular species (Kvarnemo, 2018). In the case of monogamy, resources are often distributed evenly, allowing animals to disperse themselves over a wide range. Animals that need to expend large amounts of energy on the rearing of their offspring also tend to be monogamous, as the cost of finding multiple mates while raising their young becomes too high. Another variable that enforces monogamy is a short period of sexual activity. If animals are distributed over a large area and are only sexually active for a brief period, they do not have enough time to find multiple mates. Various species of birds, fish and mammals exhibit this mating behaviour (Chapman *et al*., 2004; Lambert *et al*., 2018; Gill *et al*., 2020). Strict monogamy is rare in insects, but it has been observed in some beetles and termites (Baruch *et al*., 2017; Vargo, 2019). It has also been hypothesised that the initial monogamous mating of queens is an important step in the evolution of non-reproductive workers in eusocial insects (Quiñones & Pen, 2017; Davies & Gardner, 2018), Colonies that exhibit a monogamous mating system display lower levels of genetic diversity than some of their polygamous counterparts, as only one father contributes to each set of offspring (Jaffé *et al*., 2014). Some monogamous species correct for this by engaging in extra-pair mating. Such species show monogamous behaviour, such as pairing with one partner for life and an extended period of parental care. However, when their offspring are genotyped, evidence for a second contributing father is found. Animals that appear to show monogamous behaviour while engaging in extra-pair mating are socially monogamous, but not genetically monogamous (Petrie *et al*., 1998; Huck *et al*., 2014).

Although social monogamy is not common in insects, it has been observed in beetle species that exhibit biparental care (Dillard, 2017).

Two requirements need to be met for a species to have the potential to be polygamous. Firstly, the environment needs to support the potential of polygamy. An example of this would be an uneven distribution of resources, which would allow a fraction of the population to monopolise a large portion of the available resources. As more animals are forced to aggregate at the same place to meet their needs, the potential for an individual to find multiple mates increases. The second prerequisite for polygamy to occur is the ability of a species to utilise an environmental potential for polygamy. A lesser degree of parental care leaves excess energy available for the process of finding multiple mates. In addition to this, asynchronous periods of sexual activity may skew the male to female ratio of the reproductive group within the population. This gives the less abundant sex multiple potential partners to mate with (Emlen & Oring, 1977; Kvarnemo, 2018). Although finding multiple mates requires higher energy costs than only mating once, polygamy ultimately increases the fitness of animals that mate multiply (Arnqvist & Nilsson, 2000).

The state of polygamy where one male mates with multiple females is referred to as polygyny. This mating system is often observed in situations where a small number of males defend territories containing a large number of resources. Multiple females then mate with each male to gain access to the resources within the territory. Males with optimal survival traits secure better territories and therefore attract more females. A high number of mates will lead to an increase in fitness for males, while obtaining access to the best rearing environment increases fitness for females (Venter & Willson, 1966; Fabiani *et al.*, 2004). This mating system is common in animals with a male dominant social structure (Pörschmann *et al*., 2010). Several insect species, such as the stalk-eyed fly, exhibit harem polygyny (Cotton *et al*., 2015; Griffin *et al*., 2019). While this mating system works well in environments with a clumped distribution of resources, the small number of males contributing to the gene pool leads to a low effective population size. This means that polygynous populations tend to have low levels of genetic diversity, leaving them more vulnerable to the detrimental effects of inbreeding (Ficetola *et al.*, 2010). However, the occurrence of polygyny in a predominantly polyandrous mating system can have positive population-level impacts. This can be observed in several eusocial ant species, where the presence of polygyny increases colony size (Boulay *et al*., 2014).

Populations that exhibit polyandrous mating, where one female mates with multiple males, have the opposite effect on genetic diversity. Each set of offspring contains the genetic material of multiple sires, which has positive implications for both effective population size and genetic diversity. This makes colonies more adaptable to changes in their environment and possibly more immune to disease (Tarpy, 2003; Dobelmann *et al*., 2017). Although polyandry decreases female longevity, female fitness benefits from increased fertility and fecundity. This mating system is especially

prevalent in insects (Arnqvist & Nilsson, 2000). In eusocial insects such as bees, ants and wasps, a single queen or a small group of queens mates with a larger group of reproductive males to produce the next generation (Jaffé, 2014). In some species, males and females both mate with multiple partners to varying degrees. This mating system, seen in *Drosophila melanogaster* (Flintham *et al*., 2018), is referred to as polygynandry. The multiple mating of individuals from both sexes leads to increased genetic diversity in populations exhibiting this mating system.

Genetic diversity within populations is also affected by the degree of random mating. When random mating occurs, the genotypic frequencies within a population are expected to remain constant over time. Non-random mating changes genotypic frequencies in a population, which then influences genetic diversity in the next generation (Mayo, 2008). The mating of dissimilar individuals, known as negative assortative mating, favours intermediate phenotypes. This increases heterozygosity in the next generation, which has a positive impact on genetic diversity. Conversely, when similar individuals mate, extreme phenotypes are favoured. This is known as positive assortative mating. The selection for extreme phenotypes decreases heterozygosity in following generations, reducing genetic diversity in the population (Thomas *et al*., 2008; Baniel, 2018; Serrano-Meneses *et al.*, 2018). Inbreeding occurs when individuals that share common ancestry, and are therefore genetically similar, mate and produce offspring with lower levels of heterozygosity. Inbreeding can thus be interpreted as a form of positive assortative mating, resulting in the loss of genetic diversity in closed colonies.

1.8 The Role of Genetic Markers

Genetic markers play an important part in both the calculation of genetic diversity and the determination of mating systems in insects. As insects tend to live in large colonies and have short generation times, keeping track of individuals within the population through mere observation is impossible. Traditional methods for measuring inbreeding in animals, such as tracing pedigree information, are therefore not feasible. Additionally, the inability to track individual animals during their reproductive phase makes the study of mating behaviour difficult. Genetic markers give researchers the ability to gather information that was previously inaccessible, such as the level of inbreeding within a population and the number of parents that contribute to a single clutch of eggs (Wang *et al*., 2009; Jones *et al*., 2010).

Molecular markers represent variation in DNA sequences and can include insertions, deletions, translocations, duplications, and point mutations (Singh *et al*., 2014). Markers can be divided into two groups, based on their location in the genome. Type I markers, such as allozymes, are associated with genes of known function. Type II markers, including amplified fragment length polymorphisms (AFLPs) and random amplified polymorphic DNA (RAPD), are associated with regions of no known function. Microsatellite markers, single nucleotide polymorphisms (SNPs) and

restriction fragment length polymorphisms (RFLPs) can be type I or type II markers, depending on the research question (Raza *et al*., 2016).

The RFLP was one of the first commonly used molecular markers. RFLPs are identified by digesting fragments of DNA with restriction enzymes. If an individual has a mutation in an enzyme cutting site, the enzyme will not cut, leading to a larger fragment size for individuals with the mutation than individuals without. RFLPs have the advantage of being both codominant and abundant throughout the genome, but large quantities of DNA are required for DNA digestion and the process of finding RFLPs can be time-consuming (Kumar *et al.*, 2009). With the introduction of the polymerase chain reaction (PCR) came the development of RAPD markers. As the name states, RAPDs are randomly amplified fragments of DNA. A single, non-specific primer is used to amplify multiple polymorphic loci at once. The RAPD method is quick and inexpensive, with lower DNA quantities required due to the use of PCR. However, this method has a low reproducibility and markers are dominant, masking the presence of heterozygotes (Arif & Khan, 2009; Chauhan & Rajiv, 2010). The AFLP technique combines the use of restriction enzymes and PCR. Genomic DNA is first digested using restriction enzymes, followed by PCR amplification of multiple loci at once. This type of marker has high abundance and reproducibility but is dominant and has the high DNA quantity requirement associated with restriction enzymes (Arif & Khan, 2009; Kumar *et al*., 2009).

These three molecular markers all contributed to the wealth of scientific knowledge we have today, but modern techniques have now taken their place. The two types of markers that are currently used most frequently in genetic studies are microsatellite markers and SNPs. Microsatellite markers consist of tandem repeats of short DNA segments. These segments have a high mutation rate and are codominant, which makes them ideal for detecting changes in diversity, both within and between populations. Due to the multi-allelic nature of microsatellite loci, each marker can represent a wide range of diversity, allowing for parentage assignment based on a small panel of markers (Kelkar *et al*., 2010; Toro *et al*., 2011). This type of marker has, however, been known to have issues with genotyping errors and stuttering. This is especially true for dinucleotides. Tri- and tetranucleotides, although less frequent in the genome and more strenuous to find, are used to combat this problem (Yue & Xia, 2014).

SNPs, on the contrary, have low rates of genotyping errors and the information is easily transferable between laboratories. These markers, consisting of point mutations in the genome, also have the advantage of high-throughput genotyping, shortening the period of data collection. While SNPs are abundant throughout the genome, their biallelic nature necessitates large data sets to gather the same amount of information as a small number of microsatellite markers. In addition to this, a great amount of computational power is required to analyse these data sets. Developing a panel of SNPs is also currently more expensive than the development of a microsatellite marker panel, although

the gap is closing (Hauser *et al*., 2011; Toro *et al*., 2011). Each method therefore has its positives and negatives, but both can be used to explore the genetic relationship between individuals.

1.9 Rationale, Aims and Objectives

As the global human population continues to expand, food security is becoming an increasing concern. To meet the growing demand for alternative sources of protein, the mass-rearing of insects has gained popularity worldwide. The production of *Hermetia illucens* has increased dramatically in recent years. As the black soldier fly production industry develops more advanced mass-rearing methods, research on the effects of domestication on commercial populations is lacking. With animals starting to show possible signs of inbreeding, the genetic diversity within commercial populations needs to be explored. Should the need for breeding programmes arise, whether to combat inbreeding or to select for production traits, it would be beneficial to the producers to understand the mating behaviour of this insect. In addition to this, mating systems often have an impact on genetic diversity within populations. The aim of this project was therefore to study the effects of domestication and mating systems on the genetic diversity of a mass-reared black soldier fly population.

The first objective of the study was to track the genetic diversity of a black soldier fly colony over a period of 52 generations (chapter 2). Diversity statistics were obtained for two cohorts of wild flies, representing the founding population, as well as three generational samples spread across the 52 generations. The second objective was to determine the mating system of the black soldier fly in a controlled environment (chapter 3). Tests for multiple paternity were performed on the offspring of individuals that were captured *in copula*. Estimates of genetic diversity were also used to test for assortative mating in the mass-reared population.

1.10 References

Araki, H., Cooper, B. & Blouin, M.S. 2007. Genetic effects of captive breeding cause a rapid, cumulative fitness decline in the wild. *Science*, 318(5847): 100–103.

Arif, I.A. & Khan, H.A. 2009. Molecular markers for biodiversity analysis of wildlife animals: A brief review. *Animal Biodiversity and Conservation*, 32(1): 9–17.

Arnqvist, R.A.N. & Nilsson, T. 2000. The evolution of polyandry: multiple mating and female fitness in insects. *Animal Behaviour*, 60: 145–164.

Baniel, A. 2018. Assortative mating. In J. Vonk & T. Shackelford, eds. *Encyclopedia of Animal Cognition and Behavior*. Springer International Publishing.

Banks, I.J., Gibson, W.T. & Cameron, M.M. 2014. Growth rates of black soldier fly larvae fed on fresh human faeces and their implication for improving sanitation. *Tropical Medicine and*

International Health, 19(1): 14–22.

Barber, E.J.W. 1992. *Prehistoric Textiles: The Development of Cloth in the Neolithic and Bronze Ages, with Special Reference to the Aegean*. 3rd ed. Princeton: Princeton University Press.

Barros-Cordeiro, K.B., Báo, S.N. & Pujol-Luz, J.R. 2014. Intra-puparial development of the black soldier-fly, *Hermetia illucens*. *Journal of Insect Science*, 14(83).

Baruch, O., Mendel, Z., Scharf, I. & Harari, A.R. 2017. Mating system, mate choice and parental care in a bark beetle. *Bulletin of Entomological Research*, 107(5): 611–619.

Bijlsma, R., Bundgaard, J. & Boerema, A.C. 2000. Does inbreeding affect the extinction risk of small populations?: Predictions from *Drosophila*. *Journal of Evolutionary Biology*, 13(3): 502–514.

Boulay, R., Arnan, X., Cerdá, X. & Retana, J. 2014. The ecological benefits of larger colony size may promote polygyny in ants. *Journal of Evolutionary Biology*, 27(12): 2856–2863.

Briscoe, D.A., Malpica, J.M., Robertson, A., Smith, G.J., Frankham, R., Banks, R.G. & Barker, J.S.F. 1992. Rapid loss of genetic variation in large captive populations of *Drosophila* flies: Implications for the genetic management of captive populations. *Conservation Biology*, 6(3): 416–425.

Carvalho, C.J.B. & Mello-Patiu, C.A.. 2008. Key to the adults of the most common forensic species of Diptera in South America. *Revista Brasileira de Entomologia*, 52(3): 390–406.

Chapman, D.D., Prodöhl, P.A., Gelsleichter, J., Manire, C.A. & Shivji, M.S. 2004. Predominance of genetic monogamy by females in a hammerhead shark, *Sphyrna tiburo*: Implications for shark conservation. *Molecular Ecology*, 13(7): 1965–1974.

Chauhan, T. & Rajiv, K. 2010. Molecular markers and their applications in fisheries and aquaculture. *Advances in Bioscience and Biotechnology*, 1(4): 281–291.

Chia, S.Y., Tanga, C.M., Khamis, F.M., Mohamed, S.A., Salifu, D., Sevgan, S., Fiaboe, K.K.M., Niassy, S., Van Loon, J.J.A., Dicke, M. & Ekesi, S. 2018. Threshold temperatures and thermal requirements of black soldier fly *Hermetia illucens*: Implications for mass production. *PLoS ONE*, 13(11): 1–27.

Chia, S.Y., Tanga, C.M., Osuga, I.M., Mohamed, S.A., Khamis, F.M., Salifu, D., Sevgan, S., Fiaboe, K.K.M., Niassy, S., van Loon, J.J.A., Dicke, M. & Ekesi, S. 2018. Effects of waste stream combinations from brewing industry on performance of black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae). *PeerJ*, 6(11).

Cock, M.J.W., Murphy, S.T., Kairo, M.T.K., Thompson, E., Murphy, R.J. & Francis, A.W. 2016. Trends in the classical biological control of insect pests by insects: an update of the BIOCAT database. *BioControl*, 61(4): 349–363.

De Conconi, J.R.E. & Moreno, J.M.P. 1988. The utilization of insects in the empirical medicine of ancient Mexicans. *Journal of Ethnobiology*, 8(2): 195–202.

Correa, S.B., Araujo, J.K., Penha, J.M.F., Da Cunha, C.N., Stevenson, P.R. & Anderson, J.T. 2015. Overfishing disrupts an ancient mutualism between frugivorous fishes and plants in Neotropical wetlands. *Biological Conservation*, 191: 159–167.

Cotton, A.J., Cotton, S., Small, J. & Pomiankowski, A. 2015. Male mate preference for female eyespan and fecundity in the stalk-eyed fly, *Teleopsis dalmanni*. *Behavioral Ecology*, 26(2): 376–385.

Cvijović, I., Good, B.H. & Desai, M.M. 2018. The effect of strong purifying selection on genetic diversity. *Genetics*, 209(4): 1235–1278.

Davies, N.G. & Gardner, A. 2018. Monogamy promotes altruistic sterility in insect societies. *Royal Society Open Science*, 5(172190).

Devic, E. & Maquart, P.O. 2015. *Dirhinus giffardii* (Hymenoptera: Chalcididae), parasitoid affecting Black soldier fly production systems in West Africa. *Entomologia*, 3(284): 25–27.

Diclaro, J.W.I. & Kaufman, P.E. 2009. Black soldier fly *Hermetia illucens* Linnaeus (Insecta: Diptera: Stratiomyidae). *EENY 461*: 1–3.

Diener, S., Zurbrügg, C. & Tockner, K. 2015. Bioaccumulation of heavy metals in the black soldier fly, *Hermetia illucens* and effects on its life cycle. *Journal of Insects as Food and Feed*, 1(4): 261–270.

Dillard, J.R. 2017. High rates of extra-pair paternity in a socially monogamous beetle with biparental care. *Ecological Entomology*, 42(1): 1–10.

Dixon, T.J., Coman, G.J., Arnold, S.J., Sellars, M.J., Lyons, R.E., Dierens, L., Preston, N.P. & Li, Y. 2008. Shifts in genetic diversity during domestication of Black Tiger shrimp, *Penaeus monodon*, monitored using two multiplexed microsatellite systems. *Aquaculture*, 283(1–4): 1–6.

Dobelmann, J., Loope, K.J., Wilson-Rankin, E., Quinn, O., Baty, J.W., Gruber, M.A.M. & Lester, P.J. 2017. Fitness in invasive social wasps: the role of variation in viral load, immune response and paternity in predicting nest size and reproductive output. *Oikos*, 126(8): 1208–1218.

Dortmans, B.M.A., Diener, S., Verstappen, B.. & Zurbrügg, C. 2017. *Black Soldier Fly Biowaste Processing - A Step-by-Step Guide*. P. Donahue, ed. Dübendorf, Switzerland: Eawag: Swiss Federal Institute of Aquatic Science and Technology.

Elhag, O., Zhou, D., Song, Q., Soomro, A.A., Cai, M., Zheng, L., Yu, Z. & Zhang, J. 2017. Screening, expression, purification and functional characterization of novel antimicrobial peptide genes

from *Hermetia illucens* (L.). *PLoS ONE*, 12(1): 1–15.

Ellstrand, N.C. & Elam, D.R. 1993. Population genetic conseqences of small population size: Implications for plant conservation. *Annual Review of Ecology and Systematics*, 24: 217–242.

Emlen, S.T. & Oring, L.W. 1977. Ecology, sexual selection, and the evolution of mating systems. *Science*, 197(4300): 215–223.

Fabiani, A., Galimberti, F., Sanvito, S. & Hoelzel, A.R. 2004. Extreme polygyny among southern elephant seals on Sea Lion Island , Falkland Islands. *Behavioral Ecology*, 15(6): 961–969.

Falkenmark, M., Rockström, J. & Karlberg, L. 2009. Present and future water requirements for feeding humanity. *Food Security*, 1: 59–69.

FAO. 2020. *The state of world fisheries and aquaculture 2020*.

Ficetola, G.F., Padoa-Schioppa, E., Wang, J. & Garner, T.W.J. 2010. Polygyny, census and effective population size in the threatened frog, *Rana latastei*. *Animal Conservation*, 13(Supplementary 1): 82–89.

Flintham, E.O., Yoshida, T., Smith, S., Pavlou, H.J., Goodwin, S.F., Carazo, P. & Wigby, S. 2018. Interactions between the sexual identity of the nervous system and the social environment mediate lifespan in *Drosophila melanogaster*. *Proceedings of the Royal Society B: Biological Sciences*, 285(1892).

Frankham, R., Bradshaw, C.J.A. & Brook, B.W. 2014. Genetics in conservation management: Revised recommendations for the 50/500 rules, Red List criteria and population viability analyses. *Biological Conservation*, 170: 56–63.

Gavrilets, S. & Hastings, A. 1996. Founder effect speciation: A theoretical reassessment. *American Naturalist*, 147(3): 466–491.

Gholizadeh, M. & Ghafouri-Kesbi, F. 2016. Inbreeding depression in growth traits of Baluchi sheep. *Small Ruminant Research*, 144: 184–190.

Gill, L.F., Van Schaik, J., Von Bayern, A.M.P. & Gahr, M.L. 2020. Genetic monogamy despite frequent extrapair copulations in ‘strictly monogamous’ wild jackdaws. *Behavioral Ecology*, 31(1): 247–260.

Godfray, H.C.J., Beddington, J.R., Crute, I.R., Haddad, L., Lawrence, D., Muir, J.F., Pretty, J., Robinson, S., Thomas, S.M. & Toulmin, C. 2010. Food Security: The Challenge of Feeding 9 Billion People. *Science*, 327(February): 812–819.

Griffin, M.J., Holwell, G.I. & Symonds, M.R.E. 2019. Insect harem polygyny: when is a harem not a harem? *Behavioral Ecology and Sociobiology*, 73(4): 1–11.

Hall, C., Dawson, T.P., Macdiarmid, J.I., Matthews, R.B. & Smith, P. 2017. The impact of population growth and climate change on food security in Africa: looking ahead to 2050. *International Journal of Agricultural Sustainability*, 15(2): 124–135.

Hasan, M.R. 2017. Feeding Global Aquaculture Growth. *FAO Aquaculture Newsletter*, (56): II,III.

Hauser, L., Baird, M., Hilborn, R., Seeb, L.W. & Seeb, J.E. 2011. An empirical comparison of SNPs and microsatellites for parentage and kinship assignment in a wild sockeye salmon. *Molecular Ecology Resources*, 11(Supplement 1): 150–161.

Hedrick, P.W. & Garcia-Dorado, A. 2016. Understanding inbreeding depression, purging, and genetic rescue. *Trends in Ecology and Evolution*, 31(12): 940–952.

Holmes, L.A., Vanlaerhoven, S.L. & Tomberlin, J.K. 2012. Relative humidity effects on the life history of *Hermetia illucens* (Diptera: Stratiomyidae). *Environmental Entomology*, 41(4): 971–978.

Holmes, L.A., Vanlaerhoven, S.L. & Tomberlin, J.K. 2013. Substrate effects on pupation and adult emergence of *Hermetia illucens* (Diptera: Stratiomyidae). *Environmental Entomology*, 42(2): 370–374.

Huck, M., Fernandez-Duque, E., Babb, P. & Schurr, T. 2014. Correlates of genetic monogamy in socially monogamous mammals: Insights from Azara's owl monkeys. *Proceedings of the Royal Society B: Biological Sciences*, 281: 20140195.

Hughes, A.R., Inouye, B.D., Johnson, M.T.J., Underwood, N. & Vellend, M. 2008. Ecological consequences of genetic diversity. *Ecology Letters*, 11(6): 609–623.

Van Huis, A., Van Itterbeeck, J., Klunder, H., Mertens, E., Halloran, A., Muir, G. & Vantomme, P. 2013. *Edible Insects: Future Prospects for Food and Feed Security*. Rome: Food and Agriculture Organization of the United Nations.

Di Iorio, O. & Turienzo, P. 2011. *A Preliminary Bibliographic Survey of the Insects Found in Poultry Houses from the Neotropical Region, with Remarks on Selected Taxa Shared with Native Birds' Nests*. Auckland: Magnolia Press.

IPCC. 2014. *Climate Change 2014: Impacts, Adaptation, and Vulnerability. Part B: Regional Aspects. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. V. R. Barros, C. B. Field, D. J. Dokken, M. D. Mastrandrea, K. J. Mach, T. E. Bilir, M. Chatterjee, K. L. Elbi, Y. O. Estrada, R. . Genova, B. Girma, E. S. Kissel, A. N. Levy, S. MacCracken, P. R. Mastrandrea, & L. L. White, eds. Cambridge, United Kingdom and Newyork, NY, USA: Cambridge University Press.

Jaffé, R. 2014. An updated guide to study polyandry in social insects. *Sociobiology*, 61(1): 1–8.

Jaffé, R., Pioker-hara, F.C., Santos, C.F., Santiago, L.R., Alves, D.A., Kleinert, A. de M.P., Francoy, T.M., Arias, M.C. & Imperatriz-fonseca, V.L. 2014. Monogamy in large bee societies : a stingless paradox. *Naturwissenschaften*, 101: 261–264.

James, J.W. 1971. The founder effect and response to artificial selection. *Genetical Research*, 16(3): 241–250.

Jones, A.G., Small, C.M., Paczolt, K.A. & Ratterman, N.L. 2010. A practical guide to methods of parentage analysis. *Molecular Ecology Resources*, 10(1): 6–30.

Józefiak, D., Józefiak, A., Kierończyk, B., Rawski, M., Świątkiewicz, S., Długosz, J. & Engberg, R.M. 2016. Insects - A natural nutrient source for poultry - A review. *Annals of Animal Science*, 16(2): 297–313.

Kelkar, Y.D., Strubczewski, N., Hile, S.E., Chiaromonte, F., Eckert, K.A. & Makova, K.D. 2010. What is a microsatellite : A computational and experimental definition based upon repeat mutational behavior at A/T and GT/AC repeats. *Genome Biology and Evolution*, 2: 620–635.

Kenis, M., Bouwassi, B., Boafo, H., Devic, E., Han, R., Koko, G., Koné, N., Maciel-Vergara, G., Nacambo, S., Pomalegni, S.C.B., Roffeis, M., Wakefield, M., Zhu, F. & Fitches, E. 2018. Small-scale fly larvae production for animal feed. In A. Halloran, R. Flore, P. Vantomme, & N. Roos, eds. *Edible Insects in Sustainable Food Systems*. Cham: Springer: 239–261.

Khayrova, A., Lopatin, S. & Varlamov, V. 2019. Black soldier fly *Hermetia illucens* as a novel source of chitin and chitosan. *International Journal of Sciences*, 8(04): 81–86.

Kim, W., Bae, S., Park, H., Park, K., Lee, S., Choi, Y., Han, S. & Koh, Y. 2010. The larval age and mouth morphology of the black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae). *International Journal of Industrial Entomology*, 21(2): 185–187.

Kim, W., Bae, S., Park, K., Lee, S., Choi, Y., Han, S. & Koh, Y. 2011. Biochemical characterization of digestive enzymes in the black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae). *Journal of Asia-Pacific Entomology*, 14(1): 11–14.

Kumar, P., Gupta, V.K., Misra, A.K., Modi, D.R. & Pandey, B.K. 2009. Potential of molecular markers in plant biotechnology. *Plant Omics Journal*, 2(4): 141–162.

Kvarnemo, C. 2018. Why do some animals mate with one partner rather than many? A review of causes and consequences of monogamy. *Biological Reviews*, 93(4): 1795–1812.

Lambert, C.T., Sabol, A.C. & Solomon, N.G. 2018. Genetic monogamy in socially monogamous mammals is primarily predicted by multiple life history factors: A meta-analysis. *Frontiers in Ecology and Evolution*, 6(SEP): 1–11.

Lardé, G. 1990. Recycling of coffee pulp by *Hermetia illucens* (Diptera: Stratiomyidae) larvae. *Biological Wastes*, 33(4): 307–310.

Lee, H.L., Chandrawathani, P., Wong, W.Y., Tharam, S. & Lim, W.Y. 1995. A case of human enteric myiasis due to larvae of *Hermetia illucens* (Family: Stratiomyiadae): First report in Malaysia. *The Malaysian journal of pathology*, 17(2): 109–111.

Li, Q., Zheng, L., Qiu, N., Cai, H., Tomberlin, J.K. & Yu, Z. 2011. Bioconversion of dairy manure by black soldier fly (Diptera: Stratiomyidae) for biodiesel and sugar production. *Waste Management*, 31(6): 1316–1320.

Lynch, M., Conery, J. & Burger, R. 1995. Mutation accumulation and the extinction of small populations. *The American Naturalist*, 146(4): 489–518.

Ma, J., Lei, Y., Rehman, K.U., Yu, Z., Zhang, J., Li, W., Li, Q., Tomberlin, J.K. & Zheng, L. 2018. Dynamic effects of initial pH of substrate on biological growth and metamorphosis of black soldier fly (Diptera: Stratiomyidae). *Environmental Entomology*, 47(1): 159–165.

Marshall, S.A., Woodley, N.E. & Hauser, M. 2015. The historical spread of the black soldier fly, *Hermetia illucens* (L.) (Diptera, Stratiomyidae, Hermetiinae), and its establishment in Canada. *Journal of the Entomological Society of Ontario*, 146: 51–54.

Mayo, O. 2008. A century of Hardy-Weinberg equilibrium. *Twin Research and Human Genetics*, 11(3): 249–256.

Meneguz, M., Gasco, L. & Tomberlin, J.K. 2018. Impact of pH and feeding system on black soldier fly (*Hermetia illucens*, L; Diptera: Stratiomyidae) larval development. *PLoS ONE*, 13(8): 1–15.

Mignon-Grasteau, S., Boissy, A., Bouix, J., Faure, J.M., Fisher, A.D., Hinch, G.N., Jensen, P., Le Neindre, P., Mormède, P., Prunet, P., Vandeputte, M. & Beaumont, C. 2005. Genetics of adaptation and domestication in livestock. *Livestock Production Science*, 93(1): 3–14.

Nguyen, T.T.X., Tomberlin, J.K. & Vanlaerhoven, S. 2015. Ability of black soldier fly (Diptera: Stratiomyidae) larvae to recycle food waste. *Environmental Entomology*, 44(2): 406–410.

Oliveira, F., Doelle, K. & Smith, R. 2016. External morphology of *Hermetia illucens* Stratiomyidae: Diptera (L.1758) based on electron microscopy. *Annual Research & Review in Biology*, 9(5): 1–10.

Oonincx, D.G.A.B., Van Broekhoven, S., Van Huis, A. & Van Loon, J.J.A. 2015. Feed conversion, survival and development, and composition of four insect species on diets composed of food by-products. *PLoS ONE*, 10(12): 1–20.

Oonincx, D.G.A.B., Volk, N., Diehl, J.J.E., Van Loon, J.J.A. & Belušič, G. 2016. Photoreceptor

spectral sensitivity of the compound eyes of black soldier fly (*Hermetia illucens*) informing the design of LED-based illumination to enhance indoor reproduction. *Journal of Insect Physiology*, 95(October): 133–139.

Park, S.I., Kim, J.W. & Yoe, S.M. 2015. Purification and characterization of a novel antibacterial peptide from black soldier fly (*Hermetia illucens*) larvae. *Developmental and Comparative Immunology*, 52(1): 98–106.

Petrie, M., Doums, C., Moller, P. & Kingdom, U. 1998. The degree of extra-pair paternity increases with genetic variability. *Proceedings of the National Academy of Sciences of the United States of America*, 95(16): 9390–5.

Picker, M., Griffiths, C. & Weaving, A. 2004. *Field Guide to Insects of South Africa*. 2nd ed. Cape Town: Penguin Random House South Africa.

Pörschmann, U., Trillmich, F., Mueller, B. & Wolf, J.B.W. 2010. Male reproductive success and its behavioural correlates in a polygynous mammal, the Galápagos sea lion (*Zalophus wollebaeki*). *Molecular Ecology*, 19(12): 2574–2586.

Pryce, J.E., Haile-Mariam, M., Goddard, M.E. & Hayes, B.J. 2014. Identification of genomic regions associated with inbreeding depression in Holstein and Jersey dairy cattle. *Genetics Selection Evolution*, 46(1): 1–14.

Pujol-Luz, J.R., Francez, P.A.D.C., Ururahy-Rodrigues, A. & Constantino, R. 2008. The black soldier-fly, *Hermetia illucens* (Diptera, Stratiomyidae), used to estimate the postmortem interval in a case in Amapá State, Brazil. *Journal of Forensic Sciences*, 53(2): 476–478.

Purkayastha, D., Sarkar, S., Roy, P. & Kazmi, A. 2017. Isolation and morphological study of ecologically-important insect "*Hermetia illucens*" collected from Roorkee compost plant. *Pollution*, 3(3): 453–459.

Purschke, B., Scheibelberger, R., Axmann, S., Adler, A. & Jäger, H. 2017. Impact of substrate contamination with mycotoxins, heavy metals and pesticides on the growth performance and composition of black soldier fly larvae (*Hermetia illucens*) for use in the feed and food value chain. *Food Additives and Contaminants - Part A*, 34(8): 1410–1420.

Quiñones, A.E. & Pen, I. 2017. A unified model of Hymenopteran preadaptations that trigger the evolutionary transition to eusociality. *Nature Communications*, 8(15920).

Raza, S., Shoaib, M.W. & Mubeen, H. 2016. Genetic Markers : Importance , uses and applications. *International Journal of Scientific and Research Publications*, 6(3): 221–223.

Reed, D.H. & Frankham, R. 2003. Correlation between fitness and genetic diversity. *Conservation Biology*, 17(1): 230–237.

Roháček, J. & Hora, M. 2013. A northernmost European record of the alien black soldier fly *Hermetia illucens* (Linnaeus, 1758) (Diptera: Stratiomyidae). *Casopis Slezskeho Zemskeho Muzea (A)*, 62(2): 101–106.

Saarinen, E. V., Austin, J.D. & Daniels, J.C. 2010. Genetic estimates of contemporary effective population size in an endangered butterfly indicate a possible role for genetic compensation. *Evolutionary Applications*, 3(1): 28–39.

Salomone, R., Saija, G., Mondello, G., Giannetto, A., Fasulo, S. & Savastano, D. 2017. Environmental impact of food waste bioconversion by insects: Application of life cycle assessment to process using *Hermetia illucens*. *Journal of Cleaner Production*, 140(2): 890–905.

Serrano-Meneses, M.A., López-García, K. & Carrillo-Muñoz, A.I. 2018. Assortative mating by size in the American rubyspot damselfly (*Hetaerina americana*). *Journal of Insect Behavior*, 31(6): 585–598.

Sheppard, C. 1983. House fly and lesser fly control utilizing the black soldier fly in manure management systems for caged laying hens. *Environmental Entomology*, 12(5): 1439–1442.

Sheppard, D.C., Newton, G.L., Thompson, S.A. & Savage, S. 1994. A value added manure management system using the black soldier fly. *Bioresource Technology*, 50: 275–279.

Sheppard, D.C., Tomberlin, J.K., Joyce, J.A., Kiser, B.C. & Sumner, S.M. 2002. Rearing methods for the black soldier fly (Diptera: Stratiomyidae). *Journal of Medical Entomology*, 39(4): 695–698.

Simões, P., Rose, M.R., Duarte, A., Gonçalves, R. & Matos, M. 2007. Evolutionary domestication in *Drosophila subobscura*. *Journal of Evolutionary Biology*, 20(2): 758–766.

Singh, A. & Kumari, K. 2019. An inclusive approach for organic waste treatment and valorisation using black soldier fly larvae : A review. *Journal of Environmental Management*, 251(April): 1–13.

Singh, U., Deb, R., Alyethodi, R.R., Alex, R., Kumar, S., Chakraborty, S., Dhama, K. & Sharma, A. 2014. Molecular markers and their applications in cattle genetic research: A review. *Biomarkers and Genomic Medicine*, 6(2): 49–58.

De Smet, J., Wynants, E., Cos, P. & Van Campenhout, L. 2018. Microbial community dynamics during rearing of black soldier fly larvae (*Hermetia illucens*) and impact on exploitation potential. *Applied and Environmental Microbiology*, 84(9): 1–17.

Smetana, S., Palanisamy, M., Mathys, A. & Heinz, V. 2016. Sustainability of insect use for feed and food: Life Cycle Assessment perspective. *Journal of Cleaner Production*, 137(November): 741–

751.

St-Hilaire, S., Cranfill, K., Mcguire, M.A., Mosley, E.E., Tomberlin, J.K., Newton, L., Sealey, W., Sheppard, C. & Irving, S. 2007. Fish offal recycling by the black soldier fly produces a foodstuff high in omega-3 fatty acids. *Journal of the World Aquaculture Society*, 38(2): 309–313.

Tallmon, D.A., Luikart, G. & Waples, R.S. 2004. The alluring simplicity and complex reality of genetic rescue. *Trends in Ecology and Evolution*, 19(9): 489–496.

Tarpy, D.R. 2003. Genetic diversity within honeybee colonies prevents severe infections and promotes colony growth. *Proceedings of the Royal Society B: Biological Sciences*, 270(1510): 99–103.

Thomas, J.W., Cáceres, M., Lowman, J.J., Morehouse, C.B., Short, M.E., Baldwin, E.L., Maney, D.L. & Martin, C.L. 2008. The chromosomal polymorphism linked to variation in social behavior in the white-throated sparrow (*Zonotrichia albicollis*) is a complex rearrangement and suppressor of recombination. *Genetics*, 179(3): 1455–1468.

Tilman, D., Fargione, J., Wolff, B., D'Antonio, C., Dobson, A., Howarth, R., Schindler, D., Schlesinger, W.H., Simberloff, D. & Swackhamer, D. 2001. Forecasting agriculturally driven global environmental change. *Science*, 292(5515): 281–284.

Tomberlin, J.K., Adler, P.H. & Myers, H.M. 2009. Development of the black soldier fly (Diptera: Stratiomyidae) in relation to temperature. *Environmental Entomology*, 38(3): 930–934.

Tomberlin, J.K. & Sheppard, D.C. 2002. Factors influencing mating and oviposition of black soldier flies (Diptera: Stratiomyidae) in a colony. *Journal of Entomological Science*, 37(4): 345–352.

Tomberlin, J.K. & Sheppard, D.C. 2001. Lekking behavior of the black soldier fly (Diptera: Stratiomyidae). *The Florida Entomologist*, 84(4): 729–730.

Toro, M.A., Meuwissen, T.H.E., Fernández, J., Shaat, I. & Mäki-Tanila, A. 2011. Assessing the genetic diversity in small farm animal populations. *Animal*, 5(11): 1669–1683.

Udeh, I., Ogbu, C.C. & Oleforuh-Okoleh, V.U. 2013. Comparative egg production and mortality of two commercial egg strains, indigenous chicken and their inbred progenies. *Journal of Agricultural Science*, 5(9): 148–155.

United Nations Department of Economic and Social Affairs: Population Division. 2019. *World Population Prospects 2019: Highlights (ST/ESA/SER.A/423)*. New York.

Vargo, E.L. 2019. Diversity of termite breeding systems. *Insects*, 10(2): 1–12.

Venter, J. & Willson, M.F. 1966. The influence of habitats on mating systems of North American passerine birds. *Ecological Society of America*, 47(1): 143–147.

Villet, M.H. 2011. African carrion ecosystems and their insect communities in relation to forensic entomology. *Pest Technology*, 5(1): 1–15.

Vogel, H., Müller, A., Heckel, D.G., Gutzeit, H. & Vilcinskas, A. 2018. Nutritional immunology: Diversification and diet-dependent expression of antimicrobial peptides in the black soldier fly *Hermetia illucens*. *Developmental and Comparative Immunology*, 78: 141–148.

Walker, F. 1866. Synopsis of the Diptera of the Eastern Archipelago discovered by Mr. Wallace, and noticed in the 'Journal of the Linnean Society'. *The Journal of the Linnean Society of London, Zoology*, 9(33): 1–30.

Wang, M., Barkley, N. & Jenkins, T. 2009. Microsatellite markers in plants and insects. Part I: Applications of biotechnology. *Genes, Genomes and Genomics*, 3(1): 54–67.

Weigel, K.A. 2001. Controlling inbreeding in modern breeding programs. *Journal of Dairy Science*, 84: E177–E184.

Wilkins, A.S., Wrangham, R.W. & Tecumseh Fitch, W. 2014. The 'domestication syndrome' in mammals: A unified explanation based on neural crest cell behavior and genetics. *Genetics*, 197(3): 795–808.

Woodley, N.E. 2001. *A World Catalog of the Stratiomyidae (Insecta: Diptera)*. F. C. Thompson, ed. Leiden: Backhuys Publishers.

Yue, G.H. & Xia, J.H. 2014. Practical considerations of molecular parentage analysis in fish. *Journal of the World Aquaculture Society*, 45(2): 89–103.

Zaneveld, J.R., Burkepile, D.E., Shantz, A.A., Pritchard, C.E., McMinds, R., Payet, J.P., Welsh, R., Correa, A.M.S., Lemoine, N.P., Rosales, S., Fuchs, C., Maynard, J.A. & Thurber, R.V. 2016. Overfishing and nutrient pollution interact with temperature to disrupt coral reefs down to microbial scales. *Nature Communications*, 7(May): 1–12.

Zielińska, E., Baraniak, B., Karaś, M., Rybczyńska, K. & Jakubczyk, A. 2015. Selected species of edible insects as a source of nutrient composition. *Food Research International*, 77: 460–466.

Zygouridis, N.E., Argov, Y., Nemny-Lavy, E., Augustinos, A.A., Nestel, D. & Mathiopoulos, K.D. 2014. Genetic changes during laboratory domestication of an olive fly SIT strain. *Journal of Applied Entomology*, 138(6): 423–432.

Chapter 2: Genetic Diversity in a Mass-Reared Black Soldier Fly Population

Abstract

The commercial mass-rearing of black soldier flies is a relatively new field. The effects of domestication on this species are therefore poorly understood. This chapter aimed to study the genetic effects of mass-rearing to assist factories in maintaining healthy levels of genetic diversity in their black soldier fly colonies. The genetic diversity within three distinct generations of a mass-reared black soldier fly population: F_{28}, F_{48} and F_{52}, was studied using eight species-specific microsatellite markers. The results were then compared to two temporally spaced samples of a wild population: $Wild_{2015}$ and $Wild_{2018}$. A significant decrease in all genetic diversity estimates was observed in the factory population over time. The mean pairwise relatedness reached a maximum of 0.430 in generation 48, compared to -0.148 and -0.036 for the two wild cohorts, respectively. All five sample groups were estimated to have low effective population sizes, with the highest N_e of 59.0 observed in F_{28} and the lowest N_e of 22.1 observed in $Wild_{2015}$. However, effective population size may have been underestimated in the wild population due to limitations of the LD method. Evidence for recent population expansions was found in F_{28} and F_{48}, potentially due to population augmentation or increased larval production. Differentiation between the wild- and mass-reared populations was found to be greater than differentiation within each colony (F_{SC}=0.055; p=0.000 and F_{CT}=0.108; p=0.000), Differentiation between the factory population and the wild population increased over time, due to temporal structure in the wild population and the effects of genetic drift in small populations. The low differentiation between the factory cohorts, when compared to the increasing differentiation from wild sample groups, may have negative implications for genetic management. Genetic rescue requires the successful reproduction of immigrants in the artificial environment and the compatibility of wild flies with inbred flies would need to be tested before immigrants are introduced into the factory population.

2.1 Introduction

As the mass production of black soldier flies is in its infancy, current research on mass-reared colonies is focused on the creation of the optimal mass-rearing environment. The impact of domestication on the genetic health of commercial colonies is therefore not known. Genetically diverse populations have been shown to exhibit greater productivity and fitness than their less diverse counterparts (Mattila & Seeley, 2007). Genetic diversity also improves resistance to diseases and parasites, both of which are common issues in commercial populations (Ekroth *et al*., 2019). Furthermore, diverse populations respond better to environmental stressors and disturbances (Hughes *et al.*, 2008). The benefits of genetic diversity are thus clear, but maintaining genetic diversity in commercial colonies can be difficult.

During the process of domestication, the founder effect causes an initial population bottleneck (Gavrilets & Hastings, 1996). This is then followed by further potential losses in genetic diversity, leading to an increased probability of inbreeding in commercial populations over time. The effects of genetic drift in small populations and artificial selection for production traits may further increase the risk of inbreeding in mass-reared colonies (Ellstrand & Elam, 1993; Mignon-Grasteau *et al*., 2005). This problem is compounded in insects, as their short generation time accelerates the processes and consequences of inbreeding. Briscoe *et al*. (1992) studied the effects of captivity on genetic variation in *Drosophila melanogaster* and found that up to 62% of population genetic diversity could be lost in as little as 26 generations, and up to 86% could be lost in 56 generations. With an average generation time of around two weeks, captive *Drosophila melanogaster* populations could therefore lose up to 86% of their genetic diversity in two and a half years.

As the degree of inbreeding becomes more severe, more loci become homozygous and the potential for a loss of fitness increases (Hughes *et al*., 2008). The expression of recessive deleterious alleles increases, which in turn increases the inbreeding load of the population. This loss of fitness as a result of inbreeding load is called inbreeding depression (Hedrick & Garcia-dorado, 2016). Populations suffering from inbreeding depression are at a higher risk for extinction, especially when exposed to environmental stressors (Bijlsma *et al*., 2000). Inbred populations also show decreased productivity and have reduced response to selection (James, 1971; Hughes *et al*., 2008).

Populations suffering from severe inbreeding often evade inbreeding depression through the process of genetic purging. While this is effective in the short term, purifying selection decreases genetic diversity even more (Hedrick & Garcia-dorado, 2016; Cvijović *et al*., 2018). Genetic rescue is a method of improving fitness in a commercial population while also reintroducing genetic diversity. This is done by introducing immigrants from donor populations into the inbred population. Genetic rescue has shown great success in the past, even when using two inbred lines of *Drosophila melanogaster* to augment each other (Heber *et al*., 2012; Whiteley *et al*., 2015). However, success is dependent on the genetic similarity between immigrants and the inbred population. The

introduction of individuals that are too differentiated from the commercial colony may lead to a decrease in offspring fitness, by breaking up coadapted gene complexes or introducing maladaptive alleles. This occurrence is referred to as outbreeding depression (Tallmon *et al*., 2004; Kronenberger *et al*., 2018).

The negative effects of inbreeding on fitness, as well as the risks associated with reintroducing diversity into a population, stress the importance of maintaining a healthy level of genetic diversity in commercial BSF colonies. As mass-rearing facilities approach their 20th, 40th and 50th generations of black soldier flies, their focus is starting to shift from environmental optimisation to the long-term maintenance and genetic improvement of colonies. The aim of this chapter was to study the domestication effects of commercial mass-rearing, in the long term, on the genetic diversity of a factory population of black soldier fly. Samples were collected from a wild BSF population, as well as from three distinct generations of a mass-reared black soldier fly colony, spanning across 52 generations. The genetic diversity within each group was studied, in addition to the estimation of effective population size and tests for recent population bottlenecks. The degree of differentiation between all groups was also estimated.

2.2 Methods and Materials

2.2.1 Sample Collection and DNA Preparation

To represent the founding population of the mass-reared colony, two samples of 30 adult flies each were randomly selected from a wild black soldier fly population in Durban, South Africa (29.8587° S, 31.0218° E) in 2015 and 2018, respectively. These cohorts will be referred to as $Wild_{2015}$ and $Wild_{2018}$. In addition to this, 30 adult flies per generation were collected from generations F_{28}, F_{48} and F_{52} of the factory population. Each fly was stored individually in a tube containing 90% ethanol, at -20°C. The data for cohorts $Wild_{2015}$ and F_{28} were obtained from Rozane Badenhorst (Badenhorst, 2017). Therefore, the following DNA extraction and amplification steps only pertain to $Wild_{2018}$, F_{48} and F_{52}. For DNA preparation, parts of the head, thorax and legs of individuals were removed using a sterile surgical scalpel. The extraction of genomic DNA was performed using a modified version of the cetyltrimethyl ammonium bromide (CTAB) method, with adjustments such as the addition of proteinase K to accommodate the extraction of DNA from insects (Saghai-Maroof *et al.*, 1984; Wang & Wang, 2012). After extraction, the quantity and quality of isolated genomic DNA was evaluated using a NanoDropTM ND 1,000 spectrophotometer (Thermo Fisher Scientific). Working dilutions with a final concentration of 20ng/µl were prepared and stored at -20°C.

2.2.2 Genotyping

Ten microsatellite loci previously developed by Rhode *et al*. (2020) were amplified in three multiplex PCR reactions (table 2.1; table A.1). A final reaction volume of 10µl included KAPA2G™ Fast Multiplex PCR Mix, 20ng of genomic DNA and 0.8µM of each fluorescently labelled primer.

Reactions started with an initial denaturing step at 95°C for 5 minutes. This was followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at the annealing temperature (T_a) for 30 seconds, and an extension step at 72°C for 90 seconds. Reactions then concluded with a final extension at 72°C for 30 seconds. Successful amplification was confirmed through visualisation via 1.5% agarose gel electrophoresis. Samples were then diluted with double-distilled water at a ratio of 3dd$H_2$0: 1DNA before being sent to the Central Analytical Facilities at Stellenbosch University for capillary electrophoresis. Alleles were scored using GeneMapper v5.0.3 (Applied Biosystems).

Table 2.1: The ten microsatellite loci, developed by Rhode *et al*. (2020), used to amplify genomic DNA. *Hill_29* and *Hill_30* failed to amplify and were discarded.

Multiplex 1	**Multiplex 2**	**Multiplex 3**
Hill_17	Hill_41	Hill_25
Hill_29	Hill_23	Hill_33
Hill_30	Hill_14	
	Hill_42	
	Hill_6	

2.2.3 Genetic Data Analysis

Input files for relevant software were first created using Microsatellite Toolkit v3.1 (Park, 2001). Micro-checker v2.2.3 (Van Oosterhout *et al*., 2004) was used to check for null alleles, stuttering and allelic drop out at the remaining eight loci (1 000 randomisations, 95% confidence interval). The Brookfield 1 method was used to estimate null allele frequencies (Brookfield, 1996). Exact tests were performed on Genepop on the web v4.7 (Raymond & Rousset, 1995; Rousset, 2008) to test for the conformation of loci to Hardy-Weinberg equilibrium (HWE). Genetic diversity statistics such as the number of alleles (A_n), observed and unbiased expected heterozygosity (H_o and uH_e, respectively), Shannon's information index (I), and per locus F_{IS} were calculated using GenAlEx v6.503 (Peakall & Smouse, 2012). The mean F_{IS} value for each group was determined by calculating the average of per locus F_{IS} estimates. The allelic richness (A_r) and private allelic richness (PA_r) of each group were determined, implementing the rarefraction technique to correct for sampling bias. This was done in HP-Rare v1.1 (Kalinowski, 2005). Next, a Kruskal-Wallis test was conducted to test for significant differences in diversity estimates between the five cohorts ($p<0.01$). The mean relatedness (r) within each group was also calculated in GenAlEx (1 000 permutations, 95% confidence interval), using the Queller and Goodnight method (Queller & Goodnight, 1989).

The effective population size of each generation was calculated using the heterozygote excess and linkage disequilibrium (LD) methods in NeEstimator v2.01, with a 95% confidence interval (Do *et al*., 2014). A random mating LD model was assumed and a minimum allele frequency of 0.02 was

selected for both methods. A Wilcoxon signed rank test for heterozygote excess was performed in Bottleneck v1.2.02 (Piry *et al*., 1999) to test for evidence of a recent population bottleneck within each group. The analysis was composed of 1 000 iterations, at a 5% nominal level. The two-phase model (TPM), which incorporates both the infinite alleles model (IAM) and the stepwise mutation model (SMM), was used. The TPM consisted of 30% IAM and 70% SMM, with a variance of 30. Wilcoxon tests were also conducted under the IAM and the SMM, for comparison.

To determine the level of differentiation between the wild population and the factory population, as well as the different generations within the factory population, Arlequin v3.5.2.2 (Excoffier & Lischer, 2010) was used to calculate pairwise F_{ST}-estimates between the five cohorts (10 000 permutations; $p<0.05$). A hierarchical analysis of molecular variance (AMOVA; 10 000 permutations; $p<0.05$) was also performed in Arlequin. Samples were separated into two groups: a wild group containing $Wild_{2015}$ and $Wild_{2018}$ and a factory group containing generations F_{28}, F_{48} and F_{52} of the factory population. The differentiation amongst populations was visualised through a principal coordinate analysis (PCoA; covariance-standardized method), which was performed in GenAlEx.

2.3 Results

2.3.1 Genetic Diversity

Two of the microsatellite markers, *Hill_29* and *Hill_30*, failed to amplify and were excluded from subsequent analyses. Possible null alleles were detected in varying degrees throughout all cohorts, due to a homozygote excess across loci. This homozygote excess was also found to be responsible for the deviations of several loci from Hardy-Weinberg equilibrium ($p<0.01$, table A.2). Factory generation F_{52} was monomorphic at locus *Hill_41*, leaving the exact test unable to be performed for this locus. Additionally, evidence for possible stuttering was found for *Hill_42* in several of the cohorts, both wild and mass-reared. No evidence for large allele dropout was found. Analyses performed with *Hill_41* and *Hill_42* excluded yielded similar results to analyses with both markers included. *Hill_41* and *Hill_42* were thus included in all analyses.

There was a clear decrease in all genetic diversity estimates over the course of the 52 generations within the factory colony (figure 2.1; table A2). The decrease in number of alleles ($p=0.000$), allelic richness ($p=0.000$), private allelic richness ($p=0.001$), observed and unbiased expected heterozygosity ($p=0.008$; $p=0.004$), were all shown to be significant through a Kruskal-Wallis statistical test ($p<0.01$). $Wild_{2018}$ showed evidence of a novel allele in *Hill_23*. As this is a tetranucleotide marker, alleles include lengths of 409 and 413 base pairs, respectively. However, a 411 base pair allele was found to be present in both homozygote and heterozygote form. The 409 and 413 alleles were also present in this sample and allele 411 was not found in any other cohort.

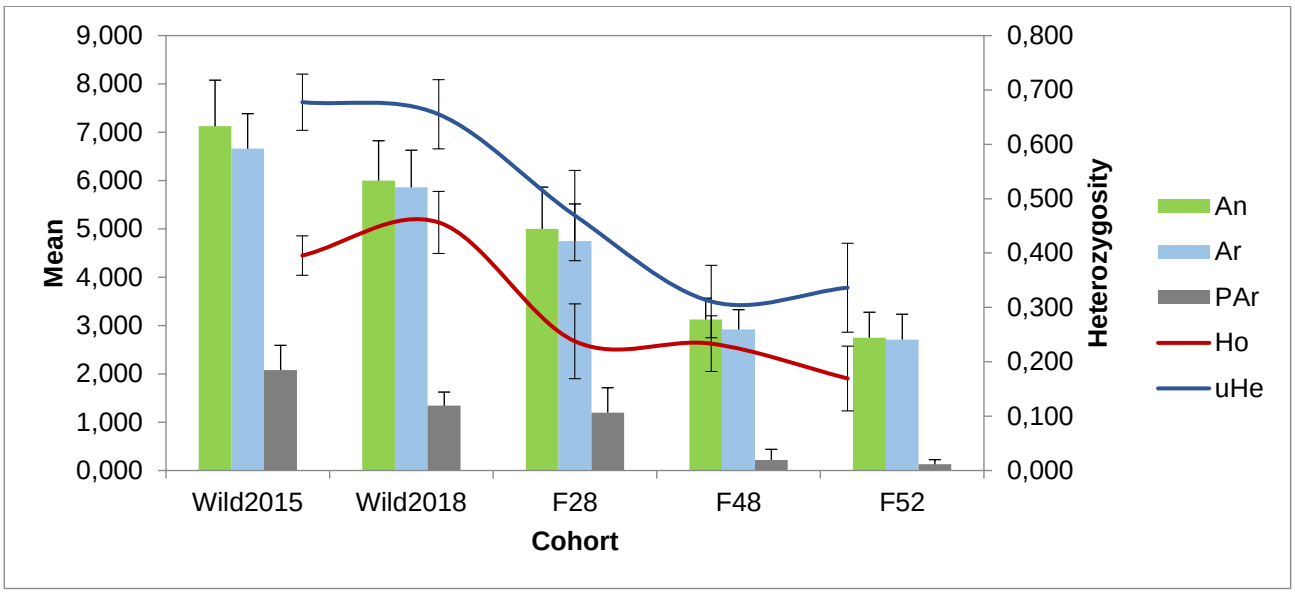

Figure 2.1: Genetic diversity statistics over the course of 52 generations within a mass-reared colony, as well as within two temporally spaced samples of a single wild population. The number of alleles (A_n), allelic richness (A_r), private allelic richness (PA_r), observed heterozygosity (H_o) and unbiased expected heterozygosity (uH_e) of each cohort are included. Standard error is represented by error bars.

2.3.2 Relatedness and Inbreeding

Both wild samples showed non-significant levels of relatedness, with negative mean relatedness coefficients. In contrast, all factory samples had mean relatedness coefficients significantly greater than zero. The relatedness coefficients in this group ranged from 0.147 in F_{28} to 0.430 in F_{48} (figure 2.2). Despite this, no significant difference in inbreeding coefficients (F_{IS}) was found between the populations (p=0.496). Inbreeding coefficients in the factory population were only slightly higher than in the wild population, with the lowest F_{IS} of 0.239 found in F_{48} (table 2.2).

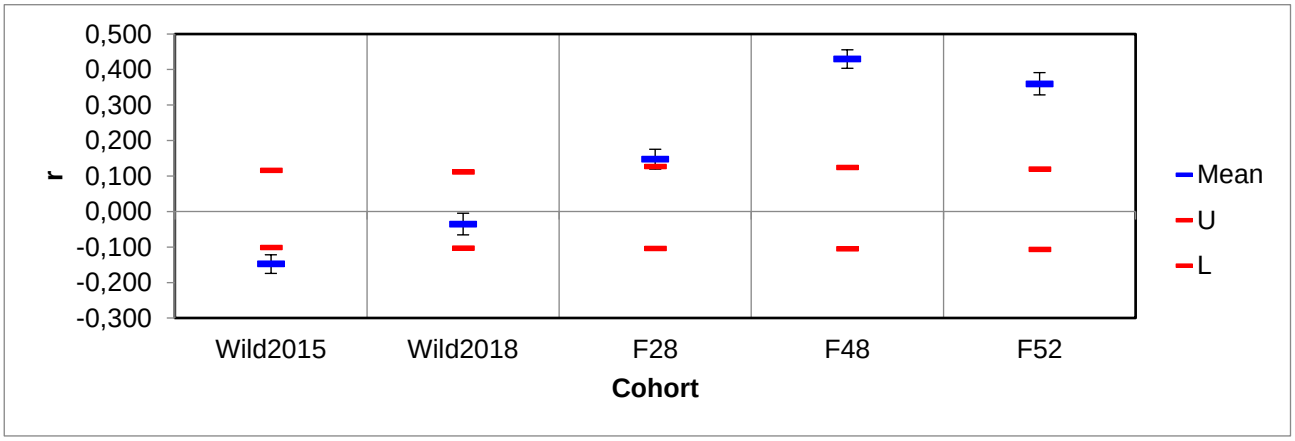

Figure 2.2: The mean pairwise relatedness (r) within each of the five sample groups. Red bars indicate the upper and lower 95% confidence intervals for the null hypothesis of no difference between the different groups. Error bars indicate standard error.

Table 2.2: Mean population F_{IS} estimates for each of the five groups. Standard errors are included in the table.

	$Wild_{2015}$	**$Wild_{2018}$**	**F_{28}**	**F_{48}**	**F_{52}**
F_{IS}	0.398	0.303	0.429	0.239	0.410
Standard error	±0.049	±0.038	±0.149	±0.141	±0.140

2.3.3 Effective Population Size

The heterozygote excess method estimated the effective population size of all populations to be infinite (table 2.3). The F_{28} and F_{48} generations of the factory cohort were calculated to have the highest N_e according to the linkage disequilibrium method, with estimates of 59.0 and 56.7, respectively. $Wild_{2015}$ had the lowest estimated N_e, with a value of 22.1. However, the 95% confidence intervals of all sample groups overlapped. The results for the Wilcoxon signed rank test varied greatly between the three models (table 2.4). Under the IAM, no significant heterozygote deficiency was found, while the $Wild_{2018}$ population was found to have an excess of heterozygotes ($p<0.05$), indicating a possible recent population bottleneck. In contrast, three cohorts had a significant heterozygote deficiency ($p<0.05$) under the SMM. This could be indicative of a recent population expansion. The two-phase model, as a combination of both previous mutational models, found a heterozygote deficiency in only two of the sample groups (F_{28} and F_{48}) with no significant heterozygote excess found. F_{28} and F_{48} were therefore the two generations most likely to have experienced recent population expansions.

Table 2.3: The estimated effective population size of each of the five cohorts, as calculated through the heterozygote excess and linkage disequilibrium methods. The parametric 95% confidence interval of each calculation is included.

Method	**$Wild_{2015}$**	**$Wild_{2018}$**	**F_{28}**	**F_{48}**	**F_{52}**
Heterozygote excess	∞	∞	∞	∞	∞
Parametric 95% CI	∞	∞	∞	∞	∞
Linkage disequilibrium	22.1	30.0	59.0	56.7	22.6
Parametric 95% CI	14.7-36.6	16.4-82.4	21.7-∞	14.9-∞	5.3-∞

Table 2.4: The results of a Wilcoxon signed rank test for heterozygote excess and deficiency, based on one of three mutational models. Results under the infinite alleles model (IAM), the stepwise mutation model (SMM) and the two-phase model (TPM) are displayed.

Mutation Model	$Wild_{2015}$	$Wild_{2018}$	F_{28}	F_{48}	F_{52}
IAM					
H_e excess	0.191	0.004**	0.680	0.727	0.148
H_e deficiency	0.844	0.998	0.371	0.320	0.945
SMM					
H_e excess	0.902	0.986	0.998	0.986	0.813
H_e deficiency	0.125	0.020*	0.004**	0.020*	0.234
TPM					
H_e excess	0.727	0.371	0.986	0.980	0.406
H_e deficiency	0.320	0.680	0.020*	0.027*	0.656

*Statistical significance at the 5% nominal level

**Statistical significance at the 1% nominal level

2.3.4 Population Differentiation

Significant differentiation was found between all groups ($p<0.05$), except for F_{48} and F_{52} ($F_{ST}=0.003$; $p=0.447$). Low to moderate differentiation was found between factory samples, with moderate differentiation observed between the two wild samples. The three factory cohorts all showed moderate differentiation from $Wild_{2015}$ and great differentiation from $Wild_{2018}$ (table 2.5). This was supported in the PCoA (figure 2.3), where factory samples clustered to the left and wild samples to the right. $Wild_{2015}$ had a broad distribution, overlapping with all groups. $Wild_{2018}$ showed little overlap with the factory cohorts, mainly clustering with $Wild_{2015}$. The AMOVA revealed significant variation both between the two groups and between samples within the groups, with $F_{ST}=0.163$ ($p=0.000$), $F_{SC}=0.055$ ($p=0.000$) and $F_{CT}=0.108$ ($p=0.000$; table 2.6).

Table 2.5: Pairwise F_{ST}-values for the five sample groups. F_{ST}-values are below the diagonal and p-values are above the diagonal.

	$Wild_{2015}$	$Wild_{2018}$	F_{28}	F_{48}	F_{52}
$Wild_{2015}$	-	0.000**	0.000**	0.000**	0.000**
$Wild_{2018}$	0.062**	-	0.000**	0.000**	0.000**
F_{28}	0.087**	0.132**	-	0.000**	0.008**
F_{48}	0.117**	0.171**	0.071**	-	0.447
F_{52}	0.097**	0.161**	0.044**	0.003	-

*Statistical significance at the 5% nominal level

**Statistical significance at the 1% nominal level

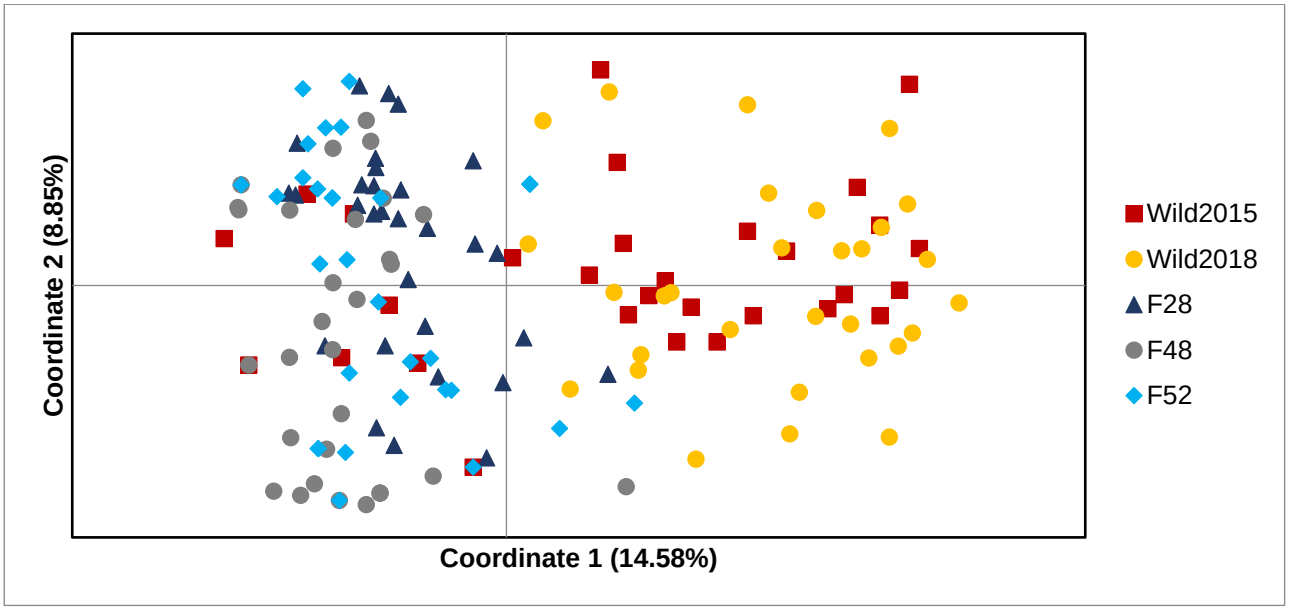

Figure 2.3: Principal coordinate analysis (PCoA) plot representing every individual from each of the five sample populations. Members of the population $Wild_{2015}$ are shown in red, $Wild_{2018}$ in yellow, F_{28} in dark blue, F_{48} in grey and F52 in light blue.

Table 2.6: Results of a hierarchical AMOVA, based on data from eight microsatellite loci. Wild samples and factory samples were grouped separately.

Source of variation	Sum of squares	Variance components	% of variation
Among groups	42.823	0.251	10.802
Among samples within groups	27.414	0.114	4.918
Within groups	542.18	1.957	84.280
Total	612.417	2.322	
F_{ST}: 0.163	***P*:** 0.000**		
F_{SC}: 0.055	***P*:** 0.000**		
F_{CT}: 0.108	***P*:** 0.000**		

**Statistical significance at the 1% nominal level

2.4 Discussion

2.4.1 Genetic Diversity and Effective Population Size

The mass-reared black soldier fly population showed a significant decline in all genetic diversity estimates over time, when compared to the wild population that the colony originated from (figure 2.1). This is a common occurrence within domesticated animal populations, especially when no supplementation with individuals from outside the population takes place (Bruford *et al.*, 2003). In insects, this problem is intensified by short generation times, with allozyme heterozygosity in captive *Drosophila melanogaster* colonies declining by up to 86% in as little as 56 generations (Briscoe *et*

al., 1992). Mean relatedness within the factory population also increased over time, reaching a maximum relatedness coefficient of 0.430 in generation F_{48}. This number is alarmingly close to the coefficient of 0.5 associated with full siblings (Taylor, 2015), indicating severe inbreeding in the factory colony. Inbreeding coefficients, however, did not support this trend. F_{IS} estimates did not change significantly over time and generation F_{48}, the cohort with the greatest degree of mean relatedness, also had the lowest mean F_{IS} (F_{IS}=0.239). Additionally, all cohorts had relatively high F_{IS} values (table 2.2), despite the wild cohorts both exhibiting high levels of genetic diversity and negative relatedness between individuals.

Two factors may have contributed to this discrepancy between relatedness and F_{IS}, the first being the high level of relatedness in generation F_{48}. Expected heterozygosity estimators have previously been found to underestimate heterozygosity in samples containing related individuals (Harris & DeGiorgio, 2017). As F_{48} had the highest mean relatedness of 0.430, the expected heterozygosity in this generation may have been underestimated. This would have reduced the difference between expected and observed heterozygosity, as seen in figure 2.1. F_{IS} is calculated based on estimates of heterozygosity, so a smaller discrepancy between uH_e and H_o would have deflated F_{IS} estimates for this cohort. Further, an unrelated study performed using $Wild_{2015}$ found that this cohort was potentially sampled from two differentiated populations (Rhode *et al*., 2020). As in this study, $Wild_{2015}$ was found to have a negative mean relatedness and a low observed heterozygosity in relation to expected heterozygosity. This reduction in heterozygosity as a result of subpopulation structure is known as the Wahlund effect (Wahlund, 1928). Further supporting this hypothesis, the F1 progeny of a colony founded by individuals from $Wild_{2015}$ showed an increase in observed heterozygosity, indicating an isolate breaking event. The low heterozygosity observed in $Wild_{2015}$ compared to its expected heterozygosity could also have affected inbreeding estimates, inflating the mean F_{IS} for this sample.

The test for recent population bottlenecks found a significant deficiency in heterozygosity in generations F_{28} and F_{48} of the factory population, which may provide evidence for recent population expansions (table 2.4). To increase production output, the factory may have had to dedicate a larger portion of individuals to production of offspring. This in turn may have led to an increased effective population size, as well as the observed pattern of population expansion. Furthermore, the mass-reared colony may have been augmented with immigrant flies to help meet these increased production demands. Augmentation is, however, associated with increased genetic diversity in populations, which was not observed in the factory population (Kronenberger *et al*., 2018).

While the heterozygote excess method found all groups to have an infinite N_e, the linkage disequilibrium method yielded more realistic results. Interestingly, F_{28} and F_{48} were estimated to have the largest effective population sizes, which fits the narrative of recent population expansions (table 2.3). With an N_e of 59.0 and 56.7, respectively, their effective population sizes were found to be

nearly twice as large as the two wild samples. However, there was an overlap in the 95% confidence intervals of all groups. Larger effective population sizes in F_{28} and F_{48} could be attributed to the stability of the factory environment when compared to the wild. In the mass-rearing environment, adult flies are kept in cages that are controlled for temperature, humidity, light intensity and even population density (Sheppard *et al*., 2002; Dortmans *et al*., 2017). Additionally, each stage of the life cycle is reared separately, in conditions that induce growth and survivability (Holmes *et al*., 2012; Chia *et al*., 2018). This creates the optimal environment for reproduction, ensuring that as many flies as possible contribute to the following generation. In contrast, natural black soldier fly populations are subject to unstable external conditions. The fluctuation of abiotic factors throughout the day, as well as across seasons, creates a less favourable environment.

Colony size is also limited by the availability of natural resources for the rearing of larvae (Tomberlin & Sheppard, 2001). Long distance migration is not common in black soldier flies, but localised migration by females in search of oviposition sites may result in the mixture of genetically differentiated populations, local population extinctions and the founding of new colonies (Okeyo *et al*., 2017; Park *et al*., 2017). This may lead to cryptic population structure in wild black soldier fly populations. As linkage disequilibrium is influenced by genetic structure in populations, the LD method for estimating N_e tends to underestimate N_e in samples containing mixtures of differentiated populations (Heath *et al*., 2002; Wang *et al*., 2016). Furthermore, the LD method does not account for overlapping generations. Factory flies are reared in discrete generations, but wild populations are likely to have a more continuous distribution. This could lead to the inflation of linkage disequilibrium through a two-locus Wahlund effect, which would lower the estimated effective population size (Neel *et al.*, 2013; Waples *et al*., 2014). The true N_e for $Wild_{2015}$ and $Wild_{2018}$ may therefore be greater than predicted by the LD-based estimation.

Even though F_{28} and F_{48} were estimated to have the largest effective population sizes, the N_e for generation F_{52} was estimated to be as low as 22.6. While this number is comparable to that of the wild samples, the high degree of inbreeding exhibited in F_{52} in combination with such a low N_e is a cause for concern. Furthermore, even the highest estimated effective population sizes barely fell within Toro *et al.*'s (2011) required N_e of 50-100 to protect populations from inbreeding depression. They also fell short of more conservative estimates, which suggest that populations with effective population sizes lower than 500 are at risk of fitness loss (Frankham *et al*., 2014). The factory population may therefore be under serious threat of extinction if new genetic diversity is not introduced. As the wild population appears to be in good genetic health, it could be considered as a potential source of immigrants for augmentation (Tallmon *et al*., 2004).

2.4.2 Population Differentiation

Low to moderate differentiation was found between the two wild samples, providing evidence for temporal structure within this population (table 2.5). Temporal structure has previously been found in other fly species and may be caused by a wide range of factors (Okeyo *et al*., 2017). Firstly, the small effective population size of the wild population would have left it more susceptible to random changes in allele frequencies over time, known as genetic drift (Whitlock, 2000). Genetic drift can cause major disruptions to allele frequencies in a short period of time, as seen in *Aedes albopictus* (Maynard *et al*., 2017). Furthermore, a sample of only thirty individuals out of potentially thousands within the wild black soldier fly colony was selected. The differentiation found between the two temporal samples may be an indicator of great genetic diversity within the wild population as a whole, which may have been overlooked due to a sampling bias (Sánchez-Montes *et al*., 2017). A novel mutation was also found in $Wild_{2018}$, which may have increased differentiation between the two wild samples. Although moderate differentiation was found between the two wild groups, they still clustered together as a distinct population in the PCoA plot (figure 2.3)

Results of the AMOVA indicated greater differentiation between the wild and factory groups than between generations within each group (F_{SC}=0.055; p=0.000 and F_{CT}=0.108; p=0.000). Differentiation between the two populations also increased over time (table 2.5). The lowest differentiation was found between the earliest samples of each population, $Wild_{2015}$ and F_{28} (F_{ST}=0.087; p=0.000), while the greatest differentiation was found between $Wild_{2018}$ and F_{48} (F_{ST}=0.171; p=0.000). Similar patterns of differentiation have been observed with the founding of captive colonies in other fly species (Nowak *et al*., 2007; Santos *et al*., 2012). While the factory population showed increased differentiation from both wild samples over time, the presence of temporal structure within the wild population appears to be accelerating differentiation between the two colonies. In the factory colony, generation F_{28} displayed low to moderate differentiation from the latter generations (F_{ST}=0.071; p=0.000 and F_{ST}=0.044; p=0.008, respectively). As in the wild population, differentiation between different factory generations could be attributed to the effects of genetic drift in a small population (Whitlock, 2000; Maynard *et al*., 2017).

The increasing levels of differentiation between the factory and wild populations may have negative implications for the eventual reintroduction of genetic diversity into the factory colony. One of the risks of genetic rescue is that the introduction of individuals that differ too much from the inbred population may cause a reduction in fitness instead of the desired increase (Tallmon *et al*., 2004; Kronenberger *et al.*, 2018). If the wild population therefore becomes too differentiated from the mass-reared colony over time, the wild flies may not be able to adapt to the artificial environment, leading to outbreeding depression (Barmentlo *et al.*, 2018). To avoid this worst-case scenario, the success of population augmentation should be tested on a small subset of the factory colony, as has previously been done in *Drosophila* (Heber *et al*., 2012).

2.5 Conclusions

A lack of genetic management in the mass-reared black soldier fly population has led to significantly low levels of genetic diversity, leaving it vulnerable to the negative effects of inbreeding. It would be beneficial to the genetic health of the population to attempt genetic rescue through augmentation with wild immigrants. However, temporal structure in the wild population and genetic drift have led to an increase in differentiation between the wild- and mass-reared colonies over time. Greater differentiation between donor and receptor populations increases the risk of outbreeding depression, as highly differentiated immigrants may disrupt adaptations to the artificial environment. It would therefore be in the best interest of the factory population to test the effects of genetic rescue on a controlled group before introducing immigrants into the population.

2.6 References

Badenhorst, R. 2017. *Genetic Diversity in a Commercial Black Soldier Fly ,* Hermetia illucens *(Diptera : Stratiomydiae), population*. Stellenbosch University.

Barmentlo, S.H., Meirmans, P.G., Luijten, S.H., Triest, L. & Oostermeijer, J.G.B. 2018. Outbreeding depression and breeding system evolution in small, remnant populations of *Primula vulgaris*: consequences for genetic rescue. *Conservation Genetics*, 19(3): 545–554.

Bijlsma, R., Bundgaard, J. & Boerema, A.C. 2000. Does inbreeding affect the extinction risk of small populations?: Predictions from *Drosophila*. *Journal of Evolutionary Biology*, 13(3): 502–514.

Briscoe, D.A., Malpica, J.M., Robertson, A., Smith, G.J., Frankham, R., Banks, G. & Barker, J.S.F. 1992. Rapid loss of genetic variation in large captive populations of *Drosophila* flies : Implications for the genetic management of captive populations. *Conservation Biology*, 6(3): 416–425.

Brookfield, J.F.Y. 1996. A simple new method for estimating null allele frequency from heterozygote deficiency. *Molecular Ecology Notes*, 5: 453–455.

Bruford, M.W., Bradley, D.G. & Luikart, G. 2003. DNA markers reveal the complexity of livestock domestication. *Nature Reviews Genetics*, 4(11): 900–910.

Chia, S.Y., Tanga, C.M., Khamis, F.M., Mohamed, S.A., Salifu, D., Sevgan, S., Fiaboe, K.K.M., Niassy, S., Van Loon, J.J.A., Dicke, M. & Ekesi, S. 2018. Threshold temperatures and thermal requirements of black soldier fly *Hermetia illucens*: Implications for mass production. *PLoS ONE*, 13(11): 1–27.

Cvijović, I., Good, B.H. & Desai, M.M. 2018. The effect of strong purifying selection on genetic diversity. *Genetics*, 209(4): 1235–1278.

Do, C., Waples, R.S., Peel, D., Macbeth, G.M., Tillett, B.J. & Ovenden, J.R. 2014. NeEstimator v2: re-implementation of software for the estimation of contemporary effective population size (Ne) from genetic data. *Molecular Ecology Resources*, 14(1): 209–214.

Dortmans, B.M.A., Diener, S., Verstappen, B.. & Zurbrügg, C. 2017. *Black Soldier Fly Biowaste Processing - A Step-by-Step Guide*. P. Donahue, ed. Dübendorf, Switzerland: Eawag: Swiss Federal Institute of Aquatic Science and Technology.

Ekroth, A.K.E., Rafaluk-Mohr, C. & King, K.C. 2019. Host genetic diversity limits parasite success beyond agricultural systems: A meta-analysis. *Proceedings of the Royal Society B: Biological Sciences*, 286(1911).

Ellstrand, N.C. & Elam, D.R. 1993. Population genetic conseqences of small population size: Implications for plant conservation. *Annual Review of Ecology and Systematics*, 24: 217–242.

Excoffier, L. & Lischer, H.E.L. 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, 10(3): 564–567.

Frankham, R., Bradshaw, C.J.A. & Brook, B.W. 2014. Genetics in conservation management: Revised recommendations for the 50/500 rules, Red List criteria and population viability analyses. *Biological Conservation*, 170: 56–63.

Gavrilets, S. & Hastings, A. 1996. Founder effect speciation: A theoretical reassessment. *American Naturalist*, 147(3): 466–491.

Hall, C., Dawson, T.P., Macdiarmid, J.I., Matthews, R.B. & Smith, P. 2017. The impact of population growth and climate change on food security in Africa: looking ahead to 2050. *International Journal of Agricultural Sustainability*, 15(2): 124–135.

Harris, A.M. & DeGiorgio, M. 2017. An unbiased estimator of gene diversity with improved variance for samples containing related and inbred individuals of any ploidy. *G3: Genes, Genomes, Genetics*, 7(2): 671–691.

Heath, D.D., Busch, C., Kelly, J. & Atagi, D.Y. 2002. Temporal change in genetic structure and effective population size in steelhead trout (*Oncorhynchus mykiss*). *Molecular Ecology*, 11: 197–214.

Heber, S., Briskie, J. V. & Apiolaza, L.A. 2012. A test of the 'genetic rescue' technique using bottlenecked donor populations of *Drosophila melanogaster*. *PLoS ONE*, 7(8): e43113.

Hedrick, P.W. & Garcia-dorado, A. 2016. Understanding inbreeding depression , purging , and genetic rescue. *Trends in Ecology & Evolution*, 31(12): 940–952.

Holmes, L.A., Vanlaerhoven, S.L. & Tomberlin, J.K. 2012. Relative humidity effects on the life history of *Hermetia illucens* (Diptera: Stratiomyidae). *Environmental Entomology*, 41(4): 971–978.

Hughes, A.R., Inouye, B.D., Johnson, M.T.J., Underwood, N. & Vellend, M. 2008. Ecological consequences of genetic diversity. *Ecology Letters*, 11(6): 609–623.

Van Huis, A. & Oonincx, D.G.A.B. 2017. The environmental sustainability of insects as food and feed. A review. *Agronomy for Sustainable Development*, 37(5).

James, J.W. 1971. The founder effect and response to artificial selection. *Genetical Research*, 16(3): 241–250.

Kalinowski, S.T. 2005. HP-RARE 1.0: A computer program for performing rarefaction on measures of allelic richness. *Molecular Ecology Notes*, 5(1): 187–189.

Kim, W., Bae, S., Park, K., Lee, S., Choi, Y., Han, S. & Koh, Y. 2011. Biochemical characterization of digestive enzymes in the black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae). *Journal of Asia-Pacific Entomology*, 14(1): 11–14.

Kronenberger, J.A., Gerberich, J.C., Fitzpatrick, S.W., Broder, E.D., Angeloni, L.M. & Funk, W.C. 2018. An experimental test of alternative population augmentation scenarios. *Conservation Biology*, 32(4): 838–848.

Mattila, H.R. & Seeley, T.D. 2007. Genetic diversity in honey bee colonies enhances productivity and fitness. *Science*, 317(5836): 362–364.

Maynard, A.J., Ambrose, L., Cooper, R.D., Chow, W.K., Davis, J.B., Muzari, M.O., Van den Hurk, A.F., Hall-Mendelin, S., Hasty, J.M., Burkot, T.R., Bangs, M.J., Reimer, L.J., Butafa, C., Lobo, N.F., Syafruddin, D., Maung Maung, Y.N., Ahmad, R. & Beebe, N.W. 2017. Tiger on the prowl: Invasion history and spatio-temporal genetic structure of the Asian tiger mosquito *Aedes albopictus* (Skuse 1894) in the Indo-Pacific. *PLoS Neglected Tropical Diseases*, 11(4): 1–27.

Mignon-Grasteau, S., Boissy, A., Bouix, J., Faure, J.M., Fisher, A.D., Hinch, G.N., Jensen, P., Le Neindre, P., Mormède, P., Prunet, P., Vandeputte, M. & Beaumont, C. 2005. Genetics of adaptation and domestication in livestock. *Livestock Production Science*, 93(1): 3–14.

Neel, M.C., McKelvey, K., Ryman, N., Lloyd, M.W., Short Bull, R., Allendorf, F.W., Schwartz, M.K. & Waples, R.S. 2013. Estimation of effective population size in continuously distributed populations: There goes the neighborhood. *Heredity*, 111(3): 189–199.

Nowak, C., Vogt, C., Diogo, J.B. & Schwenk, K. 2007. Genetic impoverishment in laboratory cultures of the test organism *Chironomus riparius*. *Environmental Toxicology and Chemistry*, 26(5): 1018–1022.

Okeyo, W.A., Saarman, N.P., Mengual, M., Dion, K., Bateta, R., Mireji, P.O., Okoth, S., Ouma, J.O., Ouma, C., Ochieng, J., Murilla, G., Aksoy, S. & Caccone, A. 2017. Temporal genetic differentiation in *Glossina pallidipes* tsetse fly populations in Kenya. *Parasites and Vectors*, 10(1): 1–13.

Oonincx, D.G.A.B., Van Broekhoven, S., Van Huis, A. & Van Loon, J.J.A. 2015. Feed conversion, survival and development, and composition of four insect species on diets composed of food by-products. *PLoS ONE*, 10(12): 1–20.

Van Oosterhout, C., Hutchinson, W.F., Wills, D.P.M. & Shipley, P. 2004. Micro-Checker: Software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, 4(3): 535–538.

Park, S., Choi, H., Choi, J. & Jeong, G. 2017. Population structure of the exotic black soldier Fly, *Hermetia illucens* (Diptera: Stratiomyidae) in Korea. *Korean Journal of Environment and Ecology*, 31(6): 520–528.

Park, S.D.E. 2001. Microsatellite Toolkit. http://animalgenomics.ucd.ie/sdepark/ms-toolkit.

Peakall, R. & Smouse, P.E. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research. *Bioinformatics (Oxford, England)*, 28(19): 2537–2539.

Piry, S., Luikart, G. & Cornuet, J.M. 1999. Bottleneck: a computer program for detecting recent reduction in the effective population size using allele frequency data. *Journal of Heredity*, 90(47): 502–503.

Queller, D.C. & Goodnight, K.F. 1989. Estimating relatedness using genetic markers. *Evolution*, 43(2): 258–275.

Raymond, M. & Rousset, F. 1995. Genepop (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, 86: 248–249.

Rhode, C., Badenhorst, R., Hull, K.L., Greenwood, M.P., Bester-van der Merwe, A.E., Andere, A.A., Picard, C.J. & Richards, C. 2020. Genetic and phenotypic consequences of early domestication in black soldier flies (*Hermetia illucens*). *Animal Genetics*, 51(5): 752–762.

Rousset, F. 2008. Genepop'007: A complete re-implementation of the Genepop software for Windows and Linux. *Molecular Ecology Resources*, 8(1): 103–106.

Saghai-Maroof, M.A., Soliman, K.M., Jorgensen, R.A. & Allard, R.W. 1984. Ribosomal DNA spacer-length polymorphisms in barley: mendelian inheritance, chromosomal location, and population dynamics. *Proceedings of the National Academy of Sciences*, 81(24): 8014–8018.

Sánchez-Montes, G., Ariño, A.H., Vizmanos, J.L., Wang, J. & Martínez-Solano, Í. 2017. Effects of

sample size and full sibs on genetic diversity characterization: A case study of three syntopic Iberian pond-breeding amphibians. *Journal of Heredity*, 108(5): 535–543.

Santos, J., Pascual, M., Simões, P., Fragata, I., Lima, M., Kellen, B., Santos, M., Marques, A., Rose, M.R. & Matos, M. 2012. From nature to the laboratory: The impact of founder effects on adaptation. *Journal of Evolutionary Biology*, 25(12): 2607–2622.

Sheppard, D.C., Tomberlin, J.K., Joyce, J.A., Kiser, B.C. & Sumner, S.M. 2002. Rearing methods for the black soldier fly (Diptera: Stratiomyidae). *Journal of Medical Entomology*, 39(4): 695–698.

Tallmon, D.A., Luikart, G. & Waples, R.S. 2004. The alluring simplicity and complex reality of genetic rescue. *Trends in Ecology and Evolution*, 19(9): 489–496.

Taylor, H.R. 2015. The use and abuse of genetic marker-based estimates of relatedness and inbreeding. *Ecology and Evolution*, 5(15): 3140–3150.

Tomberlin, J.K. & Sheppard, D.C. 2001. Lekking behavior of the black soldier fly (Diptera: Stratiomyidae). *The Florida Entomologist*, 84(4): 729–730.

Toro, M.A., Meuwissen, T.H.E., Fernández, J., Shaat, I. & Mäki-Tanila, A. 2011. Assessing the genetic diversity in small farm animal populations. *Animal*, 5(11): 1669–1683.

Wahlund, S. 1928. Zusammensetzung von populationen und korrelationserscheinungen vom standpunkt der vererbungslehre aus betrachtet. *Hereditas*, 11: 65–106.

Wang, J., Santiago, E. & Caballero, A. 2016. Prediction and estimation of effective population size. *Heredity*, 117(4): 193–206.

Wang, Q. & Wang, X. 2012. Comparison of methods for DNA extraction from a single chironomid for PCR analysis. *Pakistan Journal of Zoology*, 44(2): 421–426.

Waples, R.S., Antao, T. & Luikart, G. 2014. Effects of overlapping generations on linkage disequilibrium estimates of effective population size. *Genetics*, 197(2): 769–780.

Whiteley, A.R., Fitzpatrick, S.W., Funk, W.C. & Tallmon, D.A. 2015. Genetic rescue to the rescue. *Trends in Ecology and Evolution*, 30(1): 42–49.

Whitlock, M.C. 2000. Fixation of new alleles and the extinction of small populations: Drift load, beneficial alleles, and sexual selection. *Evolution*, 54(6): 1855–1861.

Chapter 3: Mating Systems in the Black Soldier Fly and Their Effects on Genetic Diversity

Abstract

Genetic diversity in animals is influenced by three factors: population- and evolutionary history, environmental heterogeneity, and mating systems. Mating systems affect genetic diversity based on the relative contribution of different individuals to the following generation. Non-random mating may promote the increase of either homozygosity or heterozygosity in populations, based on the type of mate selection. The aim of this chapter was to assess the genetic and social mating system in the black soldier fly. This was done by testing for monandry and random mating in five sets of candidate parents and a sample of each family's offspring, using a panel of five microsatellite markers. Genetic diversity estimates were calculated and compared between the candidate parents, their offspring, and the population they were sourced from. Parentage analysis was performed to test for multiple paternity in offspring. Increased relatedness within parent pairs when compared to the general population provided evidence for positive assortative mating, either due to mate selection for desirable traits or inbreeding. Multiple paternity was found in two of the five families tested, revealing the presence of polyandry in this species. Polyandrous mating could have positive implications for genetic management and breeding programmes. This study did not look at the potential for polygyny or mating systems in the wild and could not provide definitive answers as to the reason for positive assortative mating in the commercial population. Future studies on these topics would further improve our understanding of black soldier fly mating systems.

3.1 Introduction

The mass production of black soldier flies for various biotechnological applications has seen a major increase in recent years. It is important to maintain genetic diversity within these newly established commercial colonies, as genetic diversity contributes to overall population fitness, as well as providing a genetic buffer to respond to environmental stressors (Hughes *et al*., 2008). Genetic diversity within a population is dependent on three major factors: the evolutionary history and demographic dynamics of the organism, the heterogeneity of its environment, and its mating system (Booy *et al.*, 2000). Mass-rearing environments are often controlled to ensure maximum production, leaving little room for development of environmental heterogeneity. The preservation of genetic diversity in mass-reared colonies thus relies on understanding the micro-evolutionary and demographic history of the black soldier fly population, as well as its mating system.

There are four different potential mating systems, each having a unique influence on genetic diversity. First is monogamy, where each animal only mates with one other individual. This mating system is rare in insects, but there are species of termites and beetles that exhibit monogamy (Baruch *et al*., 2017; Vargo, 2019). Monogamous populations tend to exhibit lower levels of genetic diversity, as well as smaller effective population sizes, than highly polygamous populations. In some monogamous species where males and females partner for life, animals participate in extra-pair mating, leading to an increase in genetic diversity within the population. (Jaffé *et al*., 2014; Winternitz *et al*., 2015; Kvarnemo, 2018).

The three remaining mating systems are all variations of polygamy: polygyny, polyandry and polygynandry. Polygyny refers to the mating of one male with multiple females. This mating system is common in animals with a male dominant social structure, such as sea lions (Pörschmann *et al*., 2010). It has also been observed in several species of flies, including the stalk-eyed fly and the olive fly (Cotton *et al*., 2015; Gerofotis *et al*., 2015). Due to small effective population sizes, polygynous populations are more likely to be genetically homogenous and susceptible to the adverse effects of inbreeding and loss of diversity *(*Ficetola *et al.*, 2010; Kvarnemo, 2018). Although polygyny may have negative implications for populations exhibiting female monogamy, it can be beneficial to eusocial insect colonies. This can be seen in ant colonies, where the occurrence of polygyny leads to increased colony size (Boulay *et al*., 2014). The next mating system, polyandry, occurs in populations where females mate with multiple males. Many insect species use this mating system (Arnqvist & Nilsson, 2000). Eusocial insects, such as bees and ants, display an extreme form polyandry, where one queen mates with multiple males to produce offspring (Mattila & Seeley, 2007; Jaffé, 2014). Polyandry is typically associated with an increased heterozygosity in offspring, when compared to both monogamy and polygyny (Taylor *et al*., 2014). In many populations, both polygyny and polyandry occur in varying degrees. The mating of both sexes with multiple mates, as seen in

Drosophila melanogaster, is known as polygynandry (Flintham *et al*., 2018). As all individuals have the potential to mate multiply, this mating system poses the most benefits for genetic diversity.

Black soldier flies exhibit lekking behaviour, a system where males aggregate at sites known as leks, each defending their own small territory. Females then visit these lekking sites for the sole purpose of mating (Tomberlin & Sheppard, 2001). Fly species that show lekking behaviour include melon flies, Mediterranean fruit flies and sandflies (Jones & Hamilton, 1998; Shelly *et al*., 2012; Mir & Mir, 2016). Lekking has also been observed in other species from the genus *Hermetia* (Alcock, 1990). As lekking behaviour can help males to mate with multiple females, lekking insects often exhibit polygynous mating behaviour. Evidence for polyandry has also been found in selected lekking species (Jones & Hamilton, 1998; Shelly *et al*., 2012; Jarrige *et al*., 2015; Mir & Mir, 2016).

Genetic diversity may also be influenced by the presence of selective mating in a population. In populations exhibiting random mating, genotypic frequencies remain stable over time. However, when sexual selection occurs, genotypic frequencies are disrupted, potentially affecting genetic diversity (Mayo, 2008). Negative assortative mating occurs when individuals mate with partners that are either genotypically or phenotypically dissimilar. This leads to a higher incidence of intermediate phenotypes, which increases heterozygosity (Baniel, 2018). Selection for mates of dissimilar colour in the white-throated sparrow is an example of negative assortative mating (Hedrick *et al*., 2018). When individuals mate with partners that are either genotypically or phenotypically similar, positive assortative mating occurs. This favours extreme phenotypes, thereby increasing homozygosity in the population (Baniel, 2018). Selection for larger mates in the American rubyspot damselfly is an example of positive assortative mating (Serrano-Meneses *et al*., 2018). Inbreeding is also a form of positive assortative mating, as it increases homozygosity in populations through the mating of genetically related individuals. When assortative mating occurs based on phenotypic selection, changes in heterozygosity are limited to genes relating to the phenotype (Hedrick *et al*., 2018). Inbreeding, however, increases homozygosity across the entire genome (Reed & Frankham, 2003).

Although no formal research has been done on the black soldier fly mating system, it has been hypothesised that this species is monogamous. Adult black soldier flies do not feed and are thus unable to replenish energy between mating events (Oliveira *et al*., 2016). This may leave their gamete stocks depleted after one mating, without energy available to produce more. In addition to this, no evidence for discriminate mating has been observed in black soldier fly populations. The aim of this study was thus to test the degree of genetic monogamy, as well as random mating, in the black soldier fly. This was done by genotyping one mother and one candidate father, as well as 25 offspring, from each of five families. Each family was tested for evidence of multiple paternity. The findings were subsequently compared to genetic diversity estimates to determine the relationship between the mating system and genetic diversity within the sample groups.

3.2 Methods and Materials

3.2.1 Sample Collection and DNA Preparation

Samples for the parentage analysis study were collected from the 48th generation of a mass-reared black soldier fly colony. Five mating pairs were captured *in copula* and placed in separate containers, each containing a block for the female to oviposit on. Upon oviposition, both the male and female were collected and stored in 90% ethanol. The female was considered the known mother of the offspring, while the male was considered a candidate father. Each egg clutch was incubated and reared separately. After hatching, neonate larvae were fed a standard artificial diet for six days before being collected and stored in 90% ethanol. Twenty-five larvae per clutch were selected at random, to test for the presence of multiple paternity. To test for assortative mating, thirty additional flies were randomly collected from the source population.

3.2.2 Genotyping

Genomic DNA was extracted using a modified CTAB method, as described in chapter 2 (Saghai-Maroof *et al*., 1984; Wang & Wang, 2012). Whole larvae were used for extraction, while head, thorax, and leg tissue were used for extraction from adult flies. A panel of seven microsatellite markers, previously developed by Rhode *et al*. (2020), was amplified in two multiplex PCR reactions (table 3.1; table A.1). Each reaction mixture contained KAPA2G™ Fast Multiplex PCR Mix, 20ng of genomic DNA and 0.8µM of each fluorescently labelled primer. The final reaction volume was 10µl. The initial denaturing step heated the reaction mixture to 95°C for 5 minutes. This was followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at the annealing temperature (T_a) for 30 seconds, and an extension step at 72°C for 90 seconds. Finally, an additional extension step was done at 72°C for 30 seconds. PCR products were visualised through 1.5% agarose gel electrophoresis. PCR amplicons were then separated via capillary electrophoresis at the Central Analytical Facilities at Stellenbosch University. The scoring of alleles was performed using GeneMapper v5.03 (Applied Biosystems).

Table 3.1: Panel of seven microsatellite markers previously developed by Rhode *et al.* (2020). *Hill_42* showed poor amplification in larvae and was excluded from further analyses.

Multiplex 1	**Multiplex 2**
Hill_41	Hill_25
Hill_23	Hill_33
Hill_14	
Hill_42	
Hill_6	

3.2.3 Genetic Data Analysis

Microsoft Excel (2016) and Microsatellite Toolkit v3.1 (Park, 2001) were used to create input files. The data was tested for null alleles, stuttering and allelic drop out in Microchecker v2.2.3 (1 000 randomisations, 95% confidence interval) (Van Oosterhout *et al*., 2004). Null allele frequencies were estimated using the Brookfield 1 method (Brookfield, 1996). Due to the small number of markers used, individuals with missing data at two or more loci were removed from the dataset. Genepop on the web v4.7 (Raymond & Rousset, 1995; Rousset, 2008) was used to perform exact tests, to test for the conformation of loci to Hardy-Weinberg equilibrium (HWE; 10 000 dememorization, 500 batches, 5 000 iterations per batch). The unbiased expected and observed heterozygosity (uH_e and H_o, respectively) and per locus F_{IS} were calculated for three samples: the candidate parents (F0), the offspring (F1) and the colony that the parents were sourced from (S). This was done in GenAlEx v6.503 (Peakall & Smouse, 2012). These estimates were then compared between the three cohorts by performing a Kruskal-Wallis test ($p<0.01$), to test for significant changes between the source population and offspring. The Queller and Goodnight method was then used to calculate the mean relatedness (r) in the source population and the candidate parent generation, as well as the individual pairwise relatedness estimates between each of the five parent pairs. (Queller & Goodnight, 1989). This was also performed in GenAlEx (1 000 permutations, 95% confidence interval).

Each of the five families was tested for multiple paternity separately using two different methods: genotypic exclusion and full-pedigree likelihood. Vitassign v8.2.1 (Vandeputte *et al*., 2006) was used for the genotypic exclusion method, while the full-pedigree likelihood method was performed in Colony v2.0.5.0 (Jones & Wang, 2010). To implement the full-pedigree method in Colony, allele frequencies were calculated within each family. All markers were given a genotypic rate of 0.1 and a polygamous mating system was assumed for both sexes. Assuming monogamy for males, as well as assuming a population with or without inbreeding, yielded similar results.

3.3 Results

3.3.1 Assortative Mating

Amplification of *Hill_42* was poor and *Hill_6* was monomorphic across all samples. These two markers were therefore excluded from further analyses. *Hill_14* deviated from Hardy-Weinberg equilibrium in both the source population and F1 ($p<0.01$; table B.1). Potential null alleles were also detected at this allele, due to an excess in homozygosity. No further evidence for null alleles, stuttering or allelic drop out was found. Of the five remaining markers, three deviated from HWE in F1 and the exact test could not be performed on a fourth, as it was monoallelic (table B.1). This locus, *Hill_41*, was also monoallelic in F0.

Diversity estimates for each of the three cohorts can be seen in figure 3.1. No significant differences in F_{IS} (p=0.997), uH_e (p=0.978) or H_o (p=0.954) were found between the three groups. The increase in relatedness between the source population and F0 was also not significant (figure 3.2), but pairwise relatedness between the candidate parents in four of the five families was found to be higher than in the generation as a whole (table 3.2). The parent pair in family 4 was an exception, with a pairwise relatedness of -0.430. However, the mother had a novel allele in *Hill_23* that had previously only been observed in the wild (chapter 2). If this marker were removed, the pairwise relatedness between these two individuals would have been similar to the other four parent pairs (r=0.583). Pairwise relatedness for all five parent pairs, as well as mean pairwise relatedness values for samples S and F0 with the exclusion of *Hill_23* can be found in table B.2.

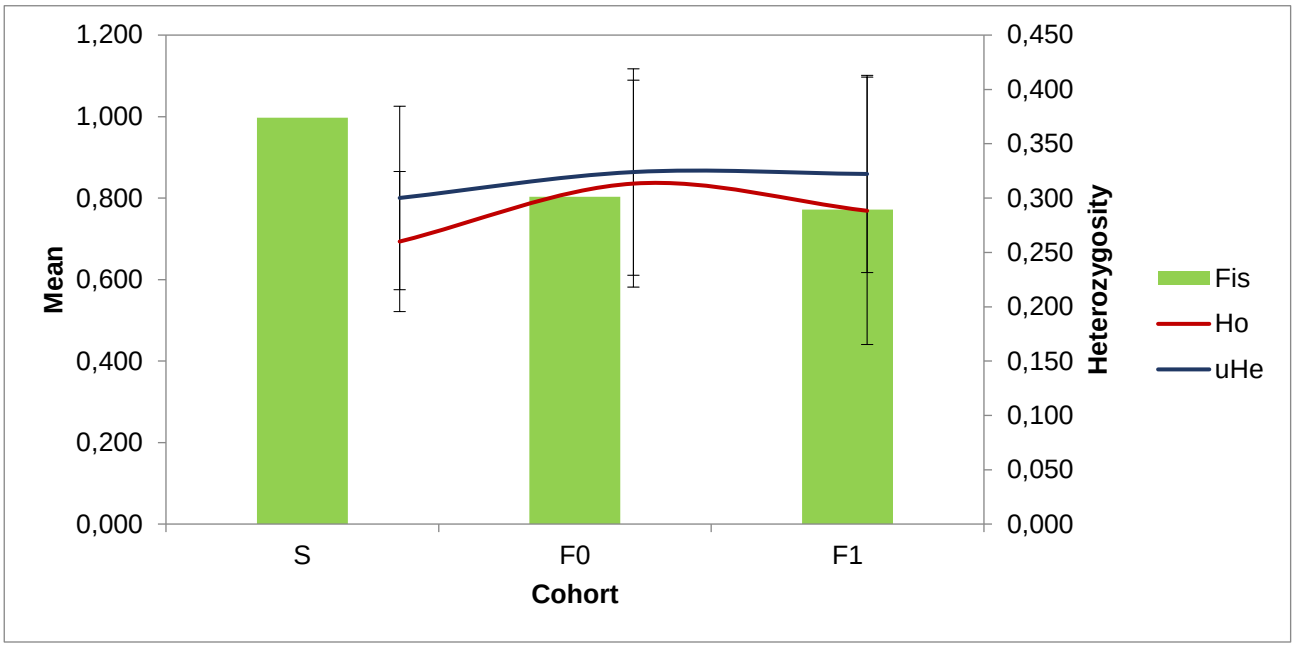

Figure 3.1: Genetic diversity estimates for the three cohorts: the source population (S), the candidate parents (F0) and the offspring (F1). The mean per locus F_{IS} (Fis), observed heterozygosity (H_o) and unbiased expected heterozygosity (uH_e) for each group are displayed in this figure. The error bars represent standard error.

Table 3.2: The pairwise relatedness between the mother and candidate father of each family.

Parent Pair	Pairwise Relatedness
Family 1	0.455
Family 2	0.455
Family 3	1.000
Family 4	-0.430
Family 5	0.700

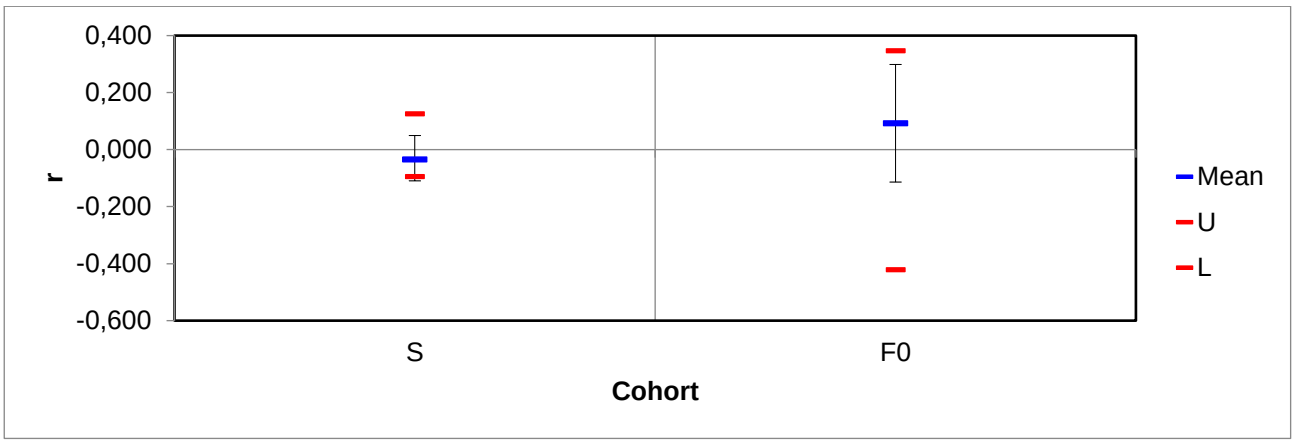

Figure 3.2: The mean pairwise relatedness (r) of the source population (S) and the candidate parents (F0). Relatedness is indicated by the blue bars, with error bars representing standard error. The limits of the 95% confidence interval for the null hypothesis of no difference are indicated by red bars.

3.3.2 Parentage Analysis

Two of the five families, families 3 and 4, were found to have more than one contributing father. The estimated number of fathers, as well as their respective contributions, differed based on the method used. The genotypic exclusion method found evidence for two potential fathers in both families, with the candidate father contributing to 33% of offspring in family 3 and 63% of offspring in family 4 (figure 3.3). The full-pedigree likelihood method, however, identified three potential fathers in each of the two families. The candidate father was found to contribute only 11% and 37% of offspring in families 3 and 4, respectively (figure 3.4).

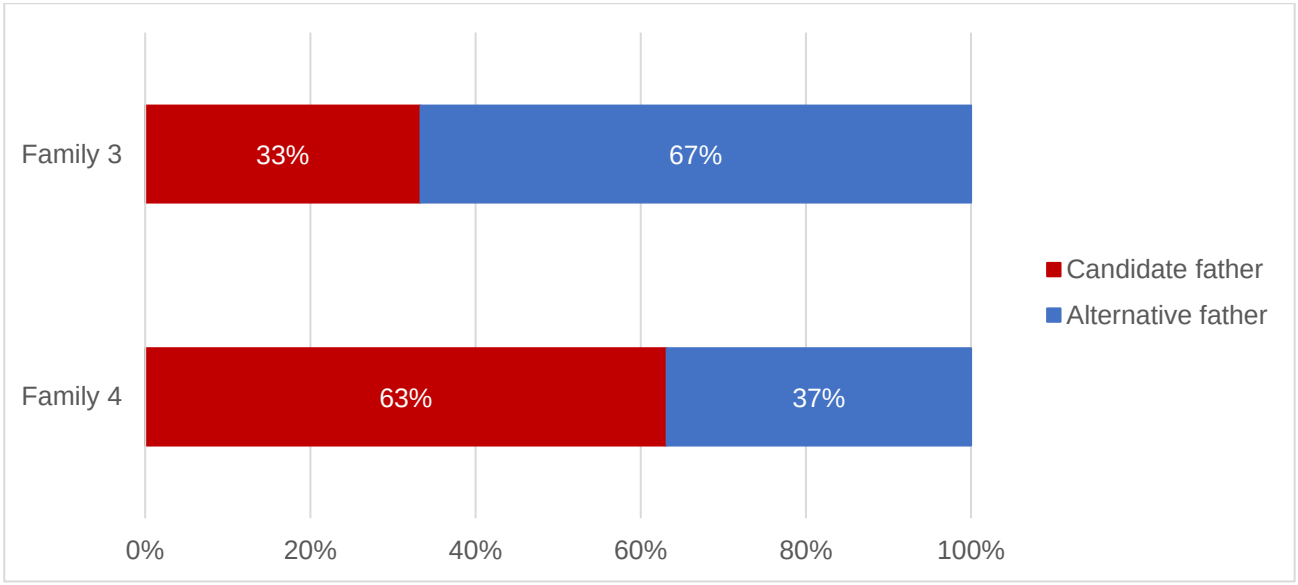

Figure 3.3: The percentage of offspring within each family that are descended from the candidate father (in red), as well as a potential alternative father (in blue), based on the genotypic exclusion method.

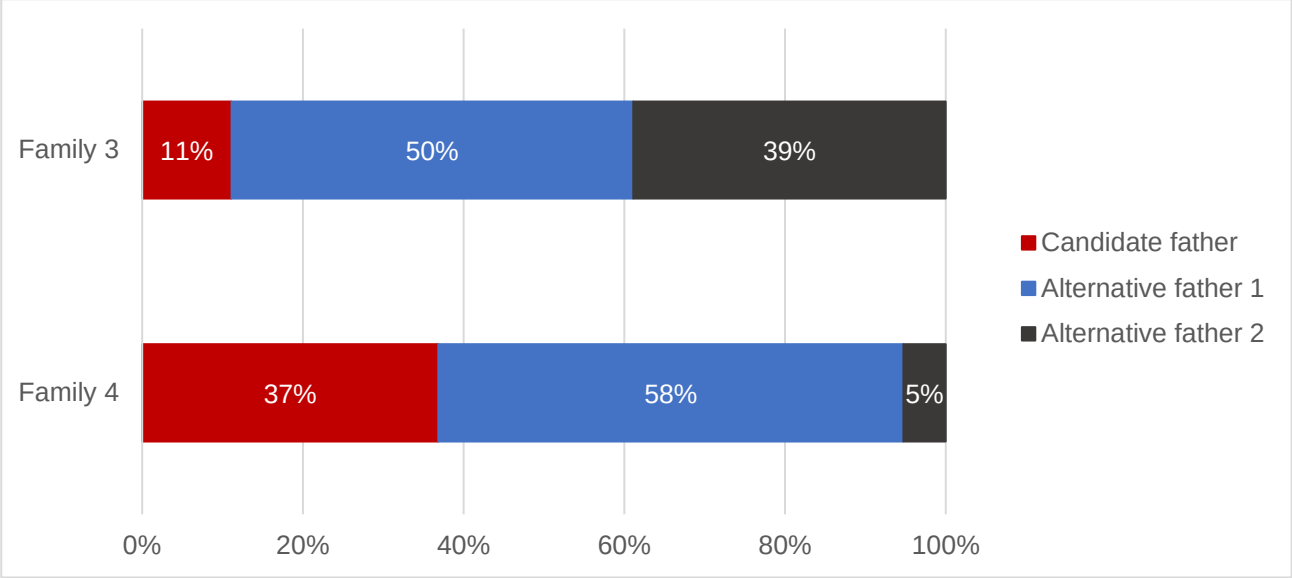

Figure 3.4: The contribution of the candidate father (red) and two potential alternative fathers (blue and black, respectively) to the offspring of families 3 and 4, based on the full-pedigree likelihood method.

3.4 Discussion

3.4.1 Assortative Mating

In a panmictic population, genotypic frequencies would be expected to remain stable over time (Mayo, 2008). No significant differences in heterozygosity would therefore be expected between the source population and offspring descended from it. Based on the mean per locus F_{IS}, expected- and observed heterozygosity in F1 (figure 3.1), random mating would thus be expected in the factory population. However, three of the five markers used in this study deviated significantly from Hardy-Weinberg equilibrium in F1. A fourth was found to be monoallelic, indicating that diversity estimates may have been misleading. Additionally, while no significant change in mean relatedness was found between the source population and F0, individual parent pairs within this group displayed high levels of relatedness. Increased levels of relatedness and inbreeding are associated with positive assortative mating (Baniel, 2018). A study on high-altitude *Drosophila* colonies by Lack *et al*. (2016) found that directional selection for traits such as large wing size led to inbreeding and increased wing abnormalities in these populations. It is therefore likely that positive assortative mating occurred between members of F0, rather than the expected indiscriminate mating.

A common driver of positive assortative mating in insects is size. Larger bodies aid males in the defence of their territories, which could prove advantageous to lekking insects like the black soldier fly. Larger body size has also previously been associated with increased female fecundity in tephritid fruit flies (Serrano-Meneses *et al*., 2018; Shelly, 2018a). Natural selection for the largest mate may therefore have contributed to positive assortative mating in this colony. Additionally, larger larval size is often actively selected for as a production trait in commercial insect species (Jensen *et al*., 2017). Rearing practices within the black soldier fly factory may have influenced the available choice of

mates, encouraging mating between genetically similar individuals. The mating of closely related individuals is also a common sign of inbreeding in captive populations (Briscoe *et al*., 1992; Dixon *et al*., 2008). As discussed in chapter 2, the population that F0 was sourced from has seen a significant increase in relatedness over time, coupled with a decline in diversity estimates. Additionally, the increased relatedness between parent pairs was detected using loci from random genomic regions, as opposed to genes associated with known phenotypes. This would suggest that positive assortative mating in F0 could be attributed to the effects of inbreeding, rather than active mate selection for specific traits (Reed & Frankham, 2003; Hedrick *et al*., 2018).

3.4.2 Parentage Analysis

Two of the five families genotyped in this study showed evidence of multiple paternity. While the genotypic exclusion method could only identify two candidate fathers per family, the full-pedigree likelihood method found that both families could have up to three candidate fathers. These findings suggest the presence of polyandry in black soldier flies. Polyandry has been observed in many other lekking species, including the lesser wax moth. Females of this species showed a 19% remating rate under experimental conditions (Engqvist *et al*., 2014). Various species of lekking fruit flies also exhibit polyandrous behaviour, with female remating frequencies of as low as 3% and as high as 76%, depending on environmental conditions (Shelly, 2018b). The absence of parental care in flies allows a larger allocation of energy for mating in females, increasing the likelihood of polyandrous matings (Kvarnemo, 2018).

It should, however, be noted that artificial conditions within the black soldier fly mass-rearing facility may be more conducive to polyandrous mating than natural environments. External conditions in the factory setting are controlled to maintain the optimal temperature and light intensity for mating (Dortmans *et al*., 2017). Additionally, fly cages are stocked to maintain a high density of flies, ensuring that a maximal amount of mating takes place. Wild populations are often subjected to more varied conditions, leaving individuals with fewer opportunities to mate (Tomberlin & Sheppard, 2002). A study on Mediterranean fruit flies showed that only 3-12% of females remated in lower density populations, while 15-76% remated in crowded conditions (Mossinson & Yuval, 2003). For this reason, polyandry may be more common in a factory setting than in the wild. Furthermore, higher levels of relatedness amongst captive flies may increase the reproductive life span of females, giving them more opportunities to mate. In a study by Le Page *et al*., (2017) male *Drosophila* flies that were related and reared together were less competitive, causing less harm to females in the process of mating. This in turn increased female reproductive life span, as well as mating success. Increased competition between unrelated males may therefore decrease the incidence of female remating in wild black soldier fly populations. To gain a better understanding of the natural mating patterns of the black soldier fly, this experiment would need to be repeated with wild mating pairs caught *in copula*.

Regardless of its occurrence in nature, polyandry in an artificial environment has positive implications for genetic management. A polyandrous mating system would be more favourable than monogamy in both the maintenance of genetic diversity and the reintroduction of genetic diversity into homogenous populations. This is due to the increased heterozygosity that is observed in offspring when females mate multiply. Effective population size is also larger when more males contribute to the next generation (Kvarnemo & Simmons, 2013; Taylor *et al.*, 2014). However, positive assortative mating in domesticated populations may counteract these positive effects of polyandry. The mechanism behind assortative mating in this species would need to be investigated to determine its long-term effects on domesticated BSF colonies.

The evidence for polyandry further demonstrates that adult flies may have enough energy to participate in multiple matings, despite not being able to replenish energy through feeding. This creates the potential for polygyny, as observed in other lekking insects (Shelly, 2018b). In the lesser wax moth, adult males emerge with a fixed number of fertilising sperm. Sperm is then divided amongst partners, until the sperm stock is depleted. This reduces the energy cost of mating, as no new sperm is produced between matings (Jarrige *et al*., 2015). Ejaculate size also often decreases with each successive mating in lekking insects, as both sperm and energy reserves deplete over time (Engqvist *et al*., 2014). Black soldier flies could therefore have the potential for polygyny, even if they do not produce new sperm as adults. However, more extensive research would be required to determine whether male black soldier flies mate multiply.

3.5 Conclusion

Based on increased relatedness within parent pairs in F0, evidence for positive assortative mating was found in a mass-reared black soldier fly population. This may be due to selection for beneficial traits such as larger mate size or the genetic effects of inbreeding. Multiple paternity was found in two out of five families tested, providing evidence for polyandry in this lekking species. Polyandry has positive implications for genetic management, as polyandrous populations show increased heterozygosity in offspring and larger effective population sizes, when compared to monogamous populations. However, the extent of positive assortative mating in this species, as well as its long-term impact on genetic health, is unknown. Additionally, as the artificial environment is optimised to ensure as much mating as possible, polyandry may be more common in the factory environment than in nature. A similar study would therefore need to be performed on wild individuals to determine the true degree of polyandry in a natural setting. Further, as multiple mating has now been deemed possible, the potential for polygyny in this species needs to be investigated. This study was limited by a small sample size and a small marker panel. Future studies would benefit from both larger sample sizes and larger, more informative marker panels.

3.6 References

Alcock, J. 1990. A large male competitive advantage in a lekking fly, *Hermetia comstockiwilliston* (Diptera: Stratiomyidae). *Psyche*, 97(3–4): 267–279.

Arnqvist, R.A.N. & Nilsson, T. 2000. The evolution of polyandry: multiple mating and female fitness in insects. *Animal Behaviour*, 60: 145–164.

Baniel, A. 2018. Assortative mating. In J. Vonk & T. Shackelford, eds. *Encyclopedia of Animal Cognition and Behavior*. Springer International Publishing.

Baruch, O., Mendel, Z., Scharf, I. & Harari, A.R. 2017. Mating system, mate choice and parental care in a bark beetle. *Bulletin of Entomological Research*, 107(5): 611–619.

Booy, G., Hendriks, R.J.J., Smulders, M.J.M., Van Groenendael, J.M. & Vosman, B. 2000. Genetic diversity and the survival of populations. *Plant Biology*, 2(4): 379–395.

Boulay, R., Arnan, X., Cerdá, X. & Retana, J. 2014. The ecological benefits of larger colony size may promote polygyny in ants. *Journal of Evolutionary Biology*, 27(12): 2856–2863.

Briscoe, D.A., Malpica, J.M., Robertson, A., Smith, G.J., Frankham, R., Banks, G. & Barker, J.S.F. 1992. Rapid loss of genetic variation in large captive populations of *Drosophila* flies : Implications for the genetic management of captive populations. *Conservation Biology*, 6(3): 416–425.

Brookfield, J.F.Y. 1996. A simple new method for estimating null allele frequency from heterozygote deficiency. *Molecular Ecology Notes*, 5: 453–455.

Cotton, A.J., Cotton, S., Small, J. & Pomiankowski, A. 2015. Male mate preference for female eyespan and fecundity in the stalk-eyed fly, *Teleopsis dalmanni*. *Behavioral Ecology*, 26(2): 376–385.

Dixon, T.J., Coman, G.J., Arnold, S.J., Sellars, M.J., Lyons, R.E., Dierens, L., Preston, N.P. & Li, Y. 2008. Shifts in genetic diversity during domestication of Black Tiger shrimp, *Penaeus monodon*, monitored using two multiplexed microsatellite systems. *Aquaculture*, 283(1–4): 1–6.

Dortmans, B.M.A., Diener, S., Verstappen, B.. & Zurbrügg, C. 2017. *Black Soldier Fly Biowaste Processing - A Step-by-Step Guide*. P. Donahue, ed. Dübendorf, Switzerland: Eawag: Swiss Federal Institute of Aquatic Science and Technology.

Engqvist, L., Cordes, N., Schwenniger, J., Bakhtina, S. & Schmoll, T. 2014. Female remating behavior in a lekking moth. *Ethology*, 120(7): 662–671.

Ficetola, G.F., Padoa-Schioppa, E., Wang, J. & Garner, T.W.J. 2010. Polygyny, census and effective population size in the threatened frog, *Rana latastei*. *Animal Conservation*, 13(Supplementary

1): 82–89.

Flintham, E.O., Yoshida, T., Smith, S., Pavlou, H.J., Goodwin, S.F., Carazo, P. & Wigby, S. 2018. Interactions between the sexual identity of the nervous system and the social environment mediate lifespan in *Drosophila melanogaster*. *Proceedings of the Royal Society B: Biological Sciences*, 285(1892).

Gerofotis, C.D., Yuval, B., Ioannou, C.S., Nakas, C.T. & Papadopoulos, N.T. 2015. Polygyny in the olive fly — effects on male and female fitness. *Behavioral Ecology and Sociobiology*, 69: 1323–1332.

Hedrick, P.W., Tuttle, E.M. & Gonser, R.A. 2018. Negative-assortative mating in the white-throated sparrow. *Journal of Heredity*, 109(3): 223–231.

Hughes, A.R., Inouye, B.D., Johnson, M.T.J., Underwood, N. & Vellend, M. 2008. Ecological consequences of genetic diversity. *Ecology Letters*, 11(6): 609–623.

Jaffé, R. 2014. An updated guide to study polyandry in social insects. *Sociobiology*, 61(1): 1–8.

Jaffé, R., Pioker-Hara, F.C., Dos Santos, C.F., Santiago, L.R., Alves, D.A., Kleinert, A. de M.P., Francoy, T.M., Arias, M.C. & Imperatriz-Fonseca, V.L. 2014. Monogamy in large bee societies: A stingless paradox. *Naturwissenschaften*, 101(3): 261–264.

Jarrige, A., Riemann, D., Goubault, M. & Schmoll, T. 2015. Strategic sperm allocation in response to perceived sperm competition risk in a lekking insect. *Animal Behaviour*, 109(October 2017): 81–87.

Jensen, K., Kristensen, T.N., Heckmann, L.L. & Sørensen, J.G. 2017. Breeding and maintaining high-quality insects. In A. Van Huis & J. K. Tomberlin, eds. *Insects as food and feed: from production to consumption*. Wageningen: Wageningen Academic Publishers: 174–198.

Jones, O.R. & Wang, J. 2010. Colony: a program for parentage and sibship inference from multilocus genotype data. *Molecular Ecology Resources*, 10(3): 551–555.

Jones, T.M. & Hamilton, J.G.C. 1998. A role for pheromones in mate choice in a lekking sandfly. *Animal Behaviour*, 56(4): 891–898.

Kvarnemo, C. 2018. Why do some animals mate with one partner rather than many? A review of causes and consequences of monogamy. *Biological Reviews*, 93(4): 1795–1812.

Kvarnemo, C. & Simmons, L.W. 2013. Polyandry as a mediator of sexual selection before and after mating. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 368(1613).

Lack, J.B., Monette, M.J., Johanning, E.J., Sprengelmeyer, Q.D. & Pool, J.E. 2016. Decanalization of wing development accompanied the evolution of large wings in high-altitude *Drosophila*.

Proceedings of the National Academy of Sciences of the United States of America, 113(4): 1014–1019.

Mattila, H.R. & Seeley, T.D. 2007. Genetic diversity in honey bee colonies enhances productivity and fitness. *Science*, 317(5836): 362–364.

Mayo, O. 2008. A century of Hardy-Weinberg equilibrium. *Twin Research and Human Genetics*, 11(3): 249–256.

Mir, S.H. & Mir, G.M. 2016. Lekking behaviour and male-male rivalry in the melon fly *Bactrocera cucurbitae* (Coquillett) (Diptera: Tephritidae). *Journal of Insect Behavior*, 29(4): 379–384.

Mossinson, S. & Yuval, B. 2003. Regulation of sexual receptivity of female Mediterranean fruit flies: Old hypotheses revisited and a new synthesis proposed. *Journal of Insect Physiology*, 49(6): 561–567.

Oliveira, F., Doelle, K. & Smith, R. 2016. External morphology of *Hermetia illucens* Stratiomyidae: Diptera (L.1758) based on electron microscopy. *Annual Research & Review in Biology*, 9(5): 1–10.

Van Oosterhout, C., Hutchinson, W.F., Wills, D.P.M. & Shipley, P. 2004. Micro-Checker: Software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, 4(3): 535–538.

Le Page, S., Sepil, I., Flintham, E., Pizzari, T., Carazo, P. & Wigby, S. 2017. Male relatedness and familiarity are required to modulate male-induced harm to females in *Drosophila*. *Proceedings of the Royal Society B: Biological Sciences*, 284(1860): 11–14.

Park, S.D.E. 2001. Microsatellite Toolkit. http://animalgenomics.ucd.ie/sdepark/ms-toolkit.

Peakall, R. & Smouse, P.E. 2012. GenALEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics*, 28(19): 2537–2539.

Pörschmann, U., Trillmich, F., Mueller, B. & Wolf, J.B.W. 2010. Male reproductive success and its behavioural correlates in a polygynous mammal, the Galápagos sea lion (*Zalophus wollebaeki*). *Molecular Ecology*, 19(12): 2574–2586.

Queller, D.C. & Goodnight, K.F. 1989. Estimating relatedness using genetic markers. *Evolution*, 43(2): 258–275.

Raymond, M. & Rousset, F. 1995. Genepop (version 1.2): Population genetics software for exact tests and ecumenicism. *Journal of Heredity*, 86: 248–249.

Reed, D.H. & Frankham, R. 2003. Correlation between fitness and genetic diversity. *Conservation Biology*, 17(1): 230–237.

Rhode, C., Badenhorst, R., Hull, K.L., Greenwood, M.P., Bester-van der Merwe, A.E., Andere, A.A., Picard, C.J. & Richards, C. 2020. Genetic and phenotypic consequences of early domestication in black soldier flies (*Hermetia illucens*). *Animal Genetics*, 51(5): 752–762.

Rousset, F. 2008. Genepop'007: A complete re-implementation of the Genepop software for Windows and Linux. *Molecular Ecology Resources*, 8(1): 103–106.

Saghai-Maroof, M.A., Soliman, K.M., Jorgensen, R.A. & Allard, R.W. 1984. Ribosomal DNA spacer-length polymorphisms in barley: mendelian inheritance, chromosomal location, and population dynamics. *Proceedings of the National Academy of Sciences*, 81(24): 8014–8018.

Serrano-Meneses, M.A., López-García, K. & Carrillo-Muñoz, A.I. 2018. Assortative mating by size in the American rubyspot damselfly (*Hetaerina americana*). *Journal of Insect Behavior*, 31(6): 585–598.

Shelly, T., Edu, J. & Pahio, E. 2012. Mate choice by lekking males: Evidence from the Mediterranean fruit fly from field cage trials (Diptera: Tephritidae). *Annals of the Entomological Society of America*, 105(2): 368–376.

Shelly, T.E. 2018a. Larval host plant influences male body size and mating success in a tephritid fruit fly. *Entomologia Experimentalis et Applicata*, 166(1): 41–52.

Shelly, T.E. 2018b. Sexual selection on leks: A fruit fly primer. *Journal of Insect Science*, 18(3): 1–16.

Taylor, M.L., Price, T.A.R. & Wedell, N. 2014. Polyandry in nature: A global analysis. *Trends in Ecology and Evolution*, 29(7): 376–383.

Tomberlin, J.K. & Sheppard, D.C. 2002. Factors influencing mating and oviposition of black soldier flies (Diptera: Stratiomyidae) in a colony. *Journal of Entomological Science*, 37(4): 345–352.

Tomberlin, J.K. & Sheppard, D.C. 2001. Lekking behavior of the black soldier fly (Diptera: Stratiomyidae). *The Florida Entomologist*, 84(4): 729–730.

Vandeputte, M., Mauger, S. & Dupont-Nivet, M. 2006. An evaluation of allowing for mismatches as a way to manage genotyping errors in parentage assignment by exclusion. *Molecular Ecology Notes*, 6(1): 265–267.

Vargo, E.L. 2019. Diversity of termite breeding systems. *Insects*, 10(2): 1–12.

Wang, Q. & Wang, X. 2012. Comparison of methods for DNA extraction from a single chironomid for PCR analysis. *Pakistan Journal of Zoology*, 44(2): 421–426.

Winternitz, J.C., Promerova, M., Polakova, R., Vinkler, M., Schnitzer, J., Munclinger, P., Babik, W., Radwan, J., Bryja, J. & Albrecht, T. 2015. Effects of heterozygosity and MHC diversity on

patterns of extra-pair paternity in the socially monogamous scarlet rosefinch. *Behavioral Ecology and Sociobiology*, 69(3): 459–469.

Chapter 4: Study Conclusions

4.1 Research Overview

The use of *Hermetia illucens* as an alternative source of animal protein has led to a recent surge in the mass production of this species. As the exploitation of black soldier flies is a relatively new field, research has mainly focused on the fly's basic life history and optimisation of the mass-rearing environment and production protocols. However, the continued growth of the commercial black soldier fly industry has raised new questions on the long-term effects of domestication on the genetic health of mass-reared colonies. Domesticated populations are often at risk of fitness loss due to the effects of inbreeding. The short generation time observed in insects may accelerate the process of inbreeding, thereby leaving mass-reared insect colonies at a greater risk of inbreeding depression than traditional livestock species. This study therefore aimed to determine the potential effects of domestication on the genetic diversity of a mass-reared black soldier fly colony. The mating behaviour of this colony and its potential impact on genetic diversity was also studied.

In chapter 2, the changes in genetic diversity in a mass-reared black soldier colony were tracked over the course of 52 generations. This was an expansion on the work of Rhode *et al*. (2020), who studied the effects of domestication on an experimental colony founded from 2 000 individuals. The degree of genetic monogamy in the mass-reared colony was then investigated through parentage analysis (chapter 3). Previous observations have suggested a monogamous mating system, as adult black soldier flies are unable to replenish energy for gamete production through feeding. Finally, estimates of relatedness and inbreeding were used to test for the presence of assortative mating in the population.

4.2 Domestication and Genetic Diversity

All genetic diversity estimates were found to have decreased significantly throughout 52 generations in the mass-reared population (chapter 2). Mean pairwise relatedness also increased to 0.430, close to a full-sib level of relatedness. This confirms the hypothesis that mass-reared black soldier fly colonies are at risk of inbreeding. Similar results have previously been observed in domesticated *Drosophila melanogaster* colonies (Briscoe *et al*., 1992). The presence of inbreeding in the mass-reared BSF colony is also supported by evidence of positive assortative mating in this population (chapter 3). The mean pairwise relatedness in the population was found to be less than the pairwise relatedness within randomly selected mating pairs. This increase in relatedness was observed in markers from random non-functional regions of the genome, as opposed to loci from genes of known function. It is thus likely indicative of inbreeding in the population, rather than positive assortative mating for desirable traits such as larger mate size (Reed & Frankham, 2003; Hedrick *et al*., 2018).

Inbreeding may have negative implications for domesticated populations, including reduced productivity, reduced response to selection and loss of fitness (James, 1971; Hughes *et al*., 2008; Hedrick & Garcia-dorado, 2016). All sample groups also showed low effective population sizes of between 22 and 59 (chapter 2). With estimates for the minimum sustainable effective population size ranging from 50 to 500, the mass-reared colony may be at great risk for both a loss of fitness and extinction (Toro *et al*., 2011; Frankham *et al*., 2014).

Genetic rescue, the augmentation of inbred populations with immigrants from donor populations, could be a potential solution to this problem. This method of reintroducing genetic diversity has been successful in various animal species in the past, including *Drosophila* (Heber *et al*., 2012; Whiteley *et al*., 2015). A wild black soldier fly population in Durban could be considered as a donor population for the mass-reared colony, as genetic diversity in the wild sample was found to be high (chapter 2). However, a combination of temporal population structure in the wild population and genetic drift in both colonies has led to increased differentiation between the wild and mass-reared colonies over time. To lower the risk of outbreeding depression, the compatibility of wild flies with the artificial environment would need to be established (Heber *et al*., 2012; Barmentlo *et al*., 2018). This could be done by simulating genetic rescue through the introduction of wild immigrants into a small experimental colony sourced from the mass-reared population.

4.3 Mating System

Lekking species are often associated with polygynous mating systems, while only some species exhibit polyandry (Jones & Hamilton, 1998; Shelly *et al*., 2012; Jarrige *et al.*, 2015; Mir & Mir, 2016). However, adult black soldier flies cannot feed, leaving them unable to replenish energy for the production of new gametes between matings (Oliveira *et al*., 2016). A monogamous mating system has therefore been hypothesized in this species, previously based on observational data. In chapter 3, evidence for multiple paternity was found in two out of five families. This suggests the presence of polyandry in the mass-reared population, despite the inability of adults to feed. As polyandrous mating systems tend to increase heterozygosity in populations, this could have positive implications for the future genetic management of domesticated black soldier fly colonies (Kvarnemo & Simmons, 2013; Taylor *et al*., 2014). These findings, however, are limited to mass-reared populations, as a captive environment may be more conducive to multiple mating than the wild.

4.4 Shortcomings and Future Undertakings

The first limitation of this study was its sample sizes. The wild and mass-reared populations, consisting of thousands of flies, were only represented by thirty individuals per generation. Each sample could therefore only represent a small fraction of the potential variation in the two populations. Although heterozygosity has been estimated accurately using sample sizes as low as twenty in the past, this was done in species with smaller census population sizes. Estimates of allelic

richness have further been estimated to require between 50 and 80 individuals, depending on marker polymorphism (Sánchez-Montes *et al*., 2017). However, marker polymorphism is negatively correlated to the required sample size. Diversity estimates in the mass-reared colony would therefore have been affected more by low sample sizes than diversity estimates in the wild colony. Additionally, while five families were sufficient to provide evidence for polyandry, the analysis of a larger number of families would improve estimates on the rate of female remating in the mass-reared population. While the marker panel used in this study was able to provide answers on the effects of domestication on genetic diversity, as well as black soldier fly mating behaviour, larger marker panels would also improve the accuracy of results.

Evidence for positive assortative mating in the mass-reared colony was hypothesized to be an indicator of inbreeding in the population, based on increased homozygosity in random genomic regions, as opposed to genes of known function (Reed & Frankham, 2003; Hedrick *et al*., 2018). However, inbreeding increases linkage disequilibrium across the genome, as the number of potential allelic combinations decreases (Slatkin, 2008). High levels of genome-wide linkage disequilibrium may lead to the detection of positive assortative mating for desirable traits in regions that are not directly related to the trait of interest. As the animals that were tested for assortative mating were sourced from an inbred population, the observed increased relatedness within mating pairs could therefore have been caused by either inbreeding or phenotypic mate selection. To test the full extent of positive assortative mating, mating pairs would need to be assessed based on both phenotypic performance and genotypic information.

Further, this study focused on multiple mating in females. However, the discovery of polyandrous mating confirms that multiple mating is possible in adult flies, even though they cannot replenish energy between matings. This opens the possibility for polygynous mating, which is common in lekking species (Shelly *et al*., 2012; Jarrige *et al*., 2015; Mir & Mir, 2016). In the lekking lesser wax moth, males emerge with a limited number of sperm that is then allocated to various female partners until the sperm stock is depleted (Jarrige *et al*., 2015). A similar mechanism could allow male black soldier flies to mate multiply. Future studies could test for polygyny by placing an equal number of males and females in a cage and allowing them to mate. All adult flies and a sample of offspring from each clutch could then be subjected to parentage analysis to determine the contribution of each parent to the offspring. This method of parentage analysis would also give all females an equal opportunity to remate, thus giving an estimation of female remating rates. In the present study, mating pairs were sampled *in copula*, which deprived females of potential future matings. In addition to more comprehensive parentage analysis, future research could also be directed at investigating mating patterns of wild black soldier flies. To ensure the maximum production output, mass-reared black soldier fly populations are often reared at high population densities (Sheppard *et al.*, 2002). This increased density may lead to higher rates of female polygamy in captive populations than in the wild, as less energy is required to find potential mates. Increased rates of female polygamy have

previously been observed in captive populations of other lekking flies (Shelly, 2018). To better understand black soldier fly mating behaviour in a natural setting, the degree of genetic monogamy in wild populations would therefore need to be determined independently.

4.5 Concluding Remarks

This study investigated the true extent of diversity loss as a result of domestication in a mass-reared black soldier fly colony. It provided evidence for severe inbreeding in the colony, highlighting the importance of genetic management in domesticated populations. Positive assortative mating was also found between mating pairs, potentially providing further evidence for inbreeding in the mass-reared population. To counteract the effects of inbreeding, immigrant flies from a wild population in Durban could potentially be used to augment the mass-reared population. However, the performance of wild flies in the artificial environment would need to be assessed first, to lower the risk of outbreeding depression. Polyandry was observed for the first time in this species, which was previously thought to be monogamous. As the occurrence of multiple mating in adults has now been confirmed, the next step would be to determine the extent of polygynous mating in the black soldier fly.

4.6 References

Barmentlo, S.H., Meirmans, P.G., Luijten, S.H., Triest, L. & Oostermeijer, J.G.B. 2018. Outbreeding depression and breeding system evolution in small, remnant populations of *Primula vulgaris*: consequences for genetic rescue. *Conservation Genetics*, 19(3): 545–554.

Briscoe, D.A., Malpica, J.M., Robertson, A., Smith, G.J., Frankham, R., Banks, R.G. & Barker, J.S.F. 1992. Rapid loss of genetic variation in large captive populations of *Drosophila* flies: Implications for the genetic management of captive populations. *Conservation Biology*, 6(3): 416–425.

Frankham, R., Bradshaw, C.J.A. & Brook, B.W. 2014. Genetics in conservation management: Revised recommendations for the 50/500 rules, Red List criteria and population viability analyses. *Biological Conservation*, 170: 56–63.

Heber, S., Briskie, J. V. & Apiolaza, L.A. 2012. A test of the 'genetic rescue' technique using bottlenecked donor populations of *Drosophila melanogaster*. *PLoS ONE*, 7(8): e43113.

Hedrick, P.W. & Garcia-dorado, A. 2016. Understanding inbreeding depression , purging , and genetic rescue. *Trends in Ecology & Evolution*, 31(12): 940–952.

Hedrick, P.W., Tuttle, E.M. & Gonser, R.A. 2018. Negative-assortative mating in the white-throated sparrow. *Journal of Heredity*, 109(3): 223–231.

Hughes, A.R., Inouye, B.D., Johnson, M.T.J., Underwood, N. & Vellend, M. 2008. Ecological

consequences of genetic diversity. *Ecology Letters*, 11(6): 609–623.

James, J.W. 1971. The founder effect and response to artificial selection. *Genetical Research*, 16(3): 241–250.

Jarrige, A., Riemann, D., Goubault, M. & Schmoll, T. 2015. Strategic sperm allocation in response to perceived sperm competition risk in a lekking insect. *Animal Behaviour*, 109(October 2017): 81–87.

Jones, T.M. & Hamilton, J.G.C. 1998. A role for pheromones in mate choice in a lekking sandfly. *Animal Behaviour*, 56(4): 891–898.

Kvarnemo, C. & Simmons, L.W. 2013. Polyandry as a mediator of sexual selection before and after mating. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 368(1613).

Mir, S.H. & Mir, G.M. 2016. Lekking behaviour and male-male rivalry in the melon fly *Bactrocera cucurbitae* (Coquillett) (Diptera: Tephritidae). *Journal of Insect Behavior*, 29(4): 379–384.

Oliveira, F., Doelle, K. & Smith, R. 2016. External morphology of *Hermetia illucens* Stratiomyidae: Diptera (L.1758) based on electron microscopy. *Annual Research & Review in Biology*, 9(5): 1–10.

Reed, D.H. & Frankham, R. 2003. Correlation between fitness and genetic diversity. *Conservation Biology*, 17(1): 230–237.

Rhode, C., Badenhorst, R., Hull, K.L., Greenwood, M.P., Bester-van der Merwe, A.E., Andere, A.A., Picard, C.J. & Richards, C. 2020. Genetic and phenotypic consequences of early domestication in black soldier flies (*Hermetia illucens*). *Animal Genetics*, 51(5): 752–762.

Sánchez-Montes, G., Ariño, A.H., Vizmanos, J.L., Wang, J. & Martínez-Solano, Í. 2017. Effects of sample size and full sibs on genetic diversity characterization: A case study of three syntopic Iberian pond-breeding amphibians. *Journal of Heredity*, 108(5): 535–543.

Shelly, T., Edu, J. & Pahio, E. 2012. Mate choice by lekking males: Evidence from the Mediterranean fruit fly from field cage trials (Diptera: Tephritidae). *Annals of the Entomological Society of America*, 105(2): 368–376.

Shelly, T.E. 2018. Sexual selection on leks: A fruit fly primer. *Journal of Insect Science*, 18(3): 1–16.

Sheppard, D.C., Tomberlin, J.K., Joyce, J.A., Kiser, B.C. & Sumner, S.M. 2002. Rearing methods for the black soldier fly (Diptera: Stratiomyidae). *Journal of Medical Entomology*, 39(4): 695–698.

Slatkin, M. 2008. Linkage disequilibrium - Understanding the evolutionary past and mapping the medical future. *Nature Reviews Genetics*, 9(6): 477–485.

Taylor, M.L., Price, T.A.R. & Wedell, N. 2014. Polyandry in nature: A global analysis. *Trends in Ecology and Evolution*, 29(7): 376–383.

Toro, M.A., Meuwissen, T.H.E., Fernández, J., Shaat, I. & Mäki-Tanila, A. 2011. Assessing the genetic diversity in small farm animal populations. *Animal*, 5(11): 1669–1683.

Whiteley, A.R., Fitzpatrick, S.W., Funk, W.C. & Tallmon, D.A. 2015. Genetic rescue to the rescue. *Trends in Ecology and Evolution*, 30(1): 42–49.

Appendix A

Table A.1: The name, repeat motif, dye, size range and annealing temperature of each of the ten microsatellite markers used for genotyping. Markers were developed by Rhode *et al*. (2020).

Multiplex	Locus name	Repeat motif	Dye	Size range (bp)	T_a (°C)
1	Hill_17	(CCGA)n	NED	316-432	60
	Hill_29	(ACTT)n	VIC	303-583	61
	Hill_30	(ACAG)n	PET	208-428	60
2	Hill_41	(ATCC)n	FAM	205-221	58
	Hill_23	(ACGG)n	NED	405-417	58
	Hill_14	(ACAG)n	VIC	210-298	58
	Hill_42	(ACCT)n	FAM	450-502	58
	Hill_6	(AGAT)n	PET	230-302	59
3	Hill_25	(CCGT)n	PET	359-439	54
	Hill_33	(GGAT)n	VIC	153-173	54

Table A.2: Genetic diversity estimates at each individual locus, for each of the five groups. Sample size (N), number of alleles (A_n), allelic richness (A_r), private allelic richness (PA_r), Shannon's information index (I), observed heterozygosity (H_o), unbiased expected heterozygosity (uH_e), F_{IS}-estimates and Hardy-Weinberg (HW) p-values are included in the table.

Marker	Cohort	N	A_n	A_r	PA_r	I	H_o	uH_e	F_{IS}	HW P-Value
Hill_17	$Wild_{2015}$	30	9	8.116	3.630	1.520	0.267	0.688	0.606	0.000**
	$Wild_{2018}$	30	6	5.669	1.971	1.352	0.600	0.692	0.118	0.235
	F_{28}	29	9	7.954	4.321	1.518	0.241	0.705	0.652	0.000**
	F_{48}	30	5	4.589	1.755	0.903	0.300	0.474	0.356	0.006**
	F_{52}	26	3	2.966	0.290	0.750	0.115	0.471	0.750	0.000**
Hill_14	$Wild_{2015}$	29	7	6.705	2.746	1.725	0.448	0.815	0.441	0.000**
	$Wild_{2018}$	29	8	7.574	2.236	1.698	0.483	0.785	0.374	0.000**
	F_{28}	30	6	5.607	2.179	1.344	0.133	0.690	0.803	0.000**
	F_{48}	30	3	2.700	0.000	0.763	0.267	0.521	0.480	0.005**
	F_{52}	25	2	2.000	0.000	0.680	0.040	0.497	0.918	0.918

Marker	Cohort	N	A_n	A_r	PA_r	I	H_o	uH_e	F_{IS}	HW P-Value
	$Wild_{2015}$	30	4	3.994	0.000	1.118	0.533	0.615	0.118	0.278
	$Wild_{2018}$	30	4	3.700	1.000	1.148	0.467	0.677	0.299	0.025*
Hill_23	F_{28}	30	4	3.400	0.706	0.768	0.533	0.473	-0.147	0.848
	F_{48}	30	3	2.700	0.000	0.690	0.433	0.450	0.021	1.000
	F_{52}	30	2	2.000	0.000	0.476	0.233	0.305	0.221	0.225
	$Wild_{2015}$	30	4	3.970	0.293	1.006	0.267	0.572	0.526	0.000**
	$Wild_{2018}$	30	4	3.676	0.700	0.745	0.233	0.408	0.418	0.015*
Hill_41	F_{28}	29	3	2.724	0.000	0.418	0.069	0.220	0.680	0.001**
	F_{48}	30	3	2.614	0.017	0.230	0.100	0.098	-0.040	1.000
	F_{52}	27	1	1.000	0.000	0.000	0.000	0.000	-	-[a]
	$Wild_{2015}$	29	8	7.643	1.652	1.778	0.448	0.811	0.437	0.000**
	$Wild_{2018}$	27	8	7.462	0.778	1.570	0.519	0.739	0.285	0.002**
Hill_42	F_{28}	22	8	7.995	0.999	1.668	0.091	0.762	0.878	0.000**
	F_{48}	30	5	4.826	0.000	0.912	0.267	0.447	0.393	0.009**
	F_{52}	22	6	5.998	0.000	1.389	0.227	0.697	0.666	0.000**
	$Wild_{2015}$	30	9	8.098	1.760	1.756	0.433	0.780	0.435	0.000**
	$Wild_{2018}$	21	8	8.000	2.002	1.655	0.476	0.772	0.368	0.001**
Hill_6	F_{28}	30	4	3.741	0.274	0.435	0.067	0.190	0.643	0.001**
	F_{48}	30	2	1.914	0.000	0.146	0.000	0.066	1.000	0.017*
	F_{52}	28	3	2.691	0.750	0.243	0.071	0.105	0.304	0.055
	$Wild_{2015}$	30	11	9.481	4.017	1.800	0.467	0.775	0.387	0.000**
	$Wild_{2018}$	24	8	7.960	1.999	1.833	0.667	0.824	0.173	0.015*
Hill_25	F_{28}	30	4	3.613	1.124	0.550	0.233	0.271	0.123	0.051
	F_{48}	30	2	2.000	0.000	0.476	0.367	0.305	-0.224	0.551
	F_{52}	30	3	3.000	0.013	0.763	0.533	0.433	-0.252	0.572
	$Wild_{2015}$	30	6	5.290	2.546	0.912	0.300	0.443	0.311	0.043*
	$Wild_{2018}$	25	3	2.840	0.073	0.665	0.240	0.422	0.420	0.025*
Hill_33	F_{28}	30	3	3.000	0.024	0.817	0.533	0.472	-0.148	0.493
	F_{48}	30	2	1.994	0.000	0.245	0.133	0.127	-0.071	1.000
	F_{52}	30	2	2.000	0.000	0.325	0.133	0.183	0.259	0.241

* Statistical significance at the 5% nominal level

** Statistical significance at the 1% nominal level

[a] Exact test could not be performed, as locus was homozygous for one allele throughout all individuals.

Appendix B

Table B.1: Per locus estimates of F_{IS}, unbiased expected heterozygosity (uH_e) and observed heterozygosity (H_o), as well as the Hardy-Weinberg (HW) p-values for each locus.

Marker	Cohort	uH_e	H_o	F_{IS}	HW P-Value
Hill_14	Source (S)	0.521	0.267	0.480	0.005**
	Parents (F0)	0.521	0.300	0.394	0.246
	Offspring (F1)	0.503	0.090	0.820	0.000**
Hill_23	Source (S)	0.450	0.433	0.021	1.000
	Parents (F0)	0.511	0.600	-0.237	0.431
	Offspring (F1)	0.482	0.677	-0.414	0.000**
Hill_41	Source (S)	0.098	0.100	-0.040	1.000
	Parents (F0)	0.000	0.000	-	-[a]
	Offspring (F1)	0.000	0.000	-	-[a]
Hill_25	Source (S)	0.305	0.367	-0.224	0.551
	Parents (F0)	0.294	0.333	-0.200	1.000
	Offspring (F1)	0.349	0.448	-0.289	0.003**
Hill_33	Source (S)	0.127	0.133	-0.071	1.000
	Parents (F0)	0.294	0.333	-0.200	1.000
	Offspring (F1)	0.277	0.227	0.177	0.128

** Statistical significance at the 1% nominal level

[a] Exact test could not be performed, as locus was homozygous for one allele throughout all individuals.

Table B.2: Mean pairwise relatedness of the source population and candidate parents, as well as pairwise relatedness estimates for each of the five parent pairs, with the exclusion of the locus *Hill_23*. Standard errors for the two sample groups are indicated in brackets.

Population	Mean Pairwise Relatedness
Source population (S)	-0.034 (-0.152 - 0.075)
Candidate parents (F0)	0.307 (0.087 - 0.489)
Parent Pair	**Pairwise Relatedness**
Family 1	0.583
Family 2	0.583
Family 3	1.000
Family 4	0.583
Family 5	1.000