



Does *Hermetia illucens* recognize sibling mates to avoid inbreeding depression?

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With 3 figures and 3 tables

Abstract: Management of genetic diversity in *Hermetia illucens* L. (Diptera: Stratiomyidae) commercial colonies is a key problem. Inbreeding depression can affect the maintenance and sustainability of insect farming, in particular small-scale mass-rearing, by reducing population fitness or causing colony collapse. In animals, kin recognition could play a role avoiding inbreeding and mating with sibling.

To determine inbreeding impact in a *H. illucens* laboratory strain, the courtship and mating behaviour occurring between unrelated or full-sibling mates were compared in controlled crosses. Furthermore, selected lifetime parameters, including egg laying and fertility, developmental time, total fecundity, and sex ratio, were compared. Inbreeding depression can be observed by second generation of full-sibling mating, with a severe reduction of produced progeny (emerged adults per female, No-Sib: 234.9 ± 38.0 vs. Sib2: 64.3 ± 16.7). No differences attributable to kin recognition were noted in the pre-copula and post-copula behavioural sequences. However, copulation was prolonged in inbred mates (No-Sib: 35.5 ± 2.8 min vs. Sib2: 52.2 ± 6.4 min), probably due to the physiological characteristics of the mates. Nevertheless, a valuable restoring impact of backcross on several life-time parameters was also observed, mitigating the impact of inbreeding depression.

This research provides valuable information about inbreeding and mate selection to avoid population depression in *H. illucens*. Further studies are needed to investigate the occurrence of other selection mechanisms in this species, as well as the possible impact of outbreeding on population fitness.

Keywords: black soldier fly; inbreeding avoidance; reproduction; fecundity; fitness

1 Introduction

Entomofarming has increased in the last years, due to the growing interest about alternative food and feed (Lange & Nakamura 2021). Ensuring food security and environmental sustainability, as well as reducing land and inputs requirements, are key issues of traditional livestock that can be addressed by insect farming (2020). The black soldier fly, *Hermetia illucens* L. (Diptera: Stratiomyidae) is a perfect candidate since it has already been domesticated for other purposes, mainly feed production and waste management (Alagappan et al. 2022; Lange & Nakamura 2021). Furthermore, in recent years, this insect has been mass-reared to produce metabolites and polymers, including proteins, lipids, and chitin, that can be used as food ingredients or for other industrial applications such as pharmaceuticals (Franco et al. 2021; Triunfo et al. 2022).

Several studies have investigated the sustainability of small-scale *H. illucens* mass-rearing for local supply (Gougbedji et al. 2021; Santos et al. 2022). Nevertheless, in small-scale conditions, inbreeding could be a key factor limiting production. Indeed, small populations are characterized by a limited number of breeders, that, generation after generation, can cause a genetic drift. Traits valuable under natural conditions can be lost, while random characteristics, including deleterious alleles, can be fixed altering the population fitness, causing inbreeding depression (Kaya et al. 2021). Furthermore, strict selection in small-scale conditions can trigger other challenges, such as the loss of resilience to changing environmental factors (Baek et al. 2014). Inbreeding depression can be countered by genetic purging, a natural process reducing the frequency of deleterious alleles and restoring fitness. Whereas in the wild this process can be effective in the short term, in mass-rearing the outcome

of gene purging can determine an additional loss of genetic diversity (Hedrick & Garcia-Dorado 2016).

Since inbreeding can result in reduced offspring fitness, the hypothesis that kinship can influence mate selection has been tested, although with different results, in several animal species (Pike et al. 2021). The chance of mating between siblings can be reduced using several mechanisms, such as sex-biased dispersion and multiple copulations with different partners (Li & Kokko 2019), and behavioural adaptation including kin recognition and avoidance either by males or females during courtship and mating (Penn & Frommen 2010). Female selection has been quite extensively investigated and confirmed in social and gregarious insects (Whitehorn et al. 2009), while little evidence is available about other insect groups (Fischer et al. 2015; Loyau et al. 2012; Metzger et al. 2010). Kin recognition in mate choice is an adaptive behaviour that can play a role when: *i*) the interactions between strictly related mates are frequent, and *ii*) inbreeding depression led to severe consequences (Pike et al. 2021). Indeed, if the chance of kin mating or the fitness decline are low or negligible, adaptive selection for mates is not likely to occur.

These conditions can be easily found in *H. illucens* mass-rearing and inbreeding depression has been proven in this species (Hull et al. 2024; Rhode et al. 2020); thus, mate selection could play a role to avoid excessive inbreeding and its consequences. The black soldier fly is a lekking species and males are supposed to be able to choose among the approaching mates. However, Giunti et al. (2018) demonstrated that males of this species are unable to recognize conspecific males from females, approaching both sexes and performing genital contact also toward same-sex individuals. In contrast, stratiomyid females have been generally considered unable to select their partners (Barbosa 2009). Nevertheless, *H. illucens* females proved to prefer mates performing characteristic courtship parameters, linked to male wing fanning's quality (Giunti et al. 2018). In this scenario, females could be able to select mates based also on their relatedness.

In this study, the occurrence of kin avoidance in a *H. illucens* laboratory population was evaluated by comparing the behavioural responses of females to full-sibling and non-related males during mate selection, as well as the courtship and mating sequences in controlled crosses. Furthermore, to confirm the existence of actual inbreeding depression and its consequences in the test colony, several life history traits of the produced progenies were also recorded and discussed.

2 Materials and methods

2.1 Colony and mass-rearing

Hermetia illucens was reared at the laboratories of the Department of Agriculture of the University of Reggio Calabria, Italy since 2016, from an original colony

(1 kg of larvae, around 5000 prepupae) provided by Agripetgarden S.r.l. (Padova, Italy) [detail in Giunti et al. (2018)]. Since establishment, no population augmentation with wild flies occurred, while one augmentation event using larvae (1 kg) from Agripetgarden S.r.l. was provided in April 2020. Each generation (i.e., about 50 from the foundation) was separately reared, with no overlapping among cohorts. Population size was maintained between 1000–6000 specimens per generation. Adult flies were maintained inside rearing cages (BugDorm-6S610®, MegaView Science Co., Ltd., Taichung, Taiwan) and they were provided with oviposition devices (i.e., cardboards, 10 × 5 × 1 cm, replaced every 2 days) and water *ad libitum*. Egg masses were periodically collected and hatched larvae were reared on a standardized commercial diet (i.e., maize kernels and flour, soybean flour and oil, and sugar cane molasses, with a minimum protein and fat content of 16% and 3.5% respectively, by Pezzullo Mangimi®, Sun Company, Casalpusterlengo, Italy) until pupation.

2.2 Experimental design

2.2.1 Test insects and controlled crosses

To obtain the virgin black soldier flies used for the experimentations, 2 days old females were collected immediately after mating and then kept individually in separate plastic containers (50 mL) with ventilated caps and provided with water *ad libitum*. Older mated females were discarded to ensure that tested females mated only once with a single male partner, since multiple mating can occur later in females' life (Hoffmann et al. 2021). The selected females were checked daily to account for oviposition and survival. Single egg clutches were collected and placed inside a container with standard diet media *ad libitum* to allow hatching and larval development. Thus, the developed pupae were stored individually inside plastic containers (15 mL) until adult eclosion. Emerged flies were sexed by visual inspection of their genitalia (Julita et al. 2020). Using this procedure, *H. illucens* virgin adults were produced and their degree of relatedness was clearly determined, since flies originated from the same females were full siblings. The whole process was carried out inside climatic chambers illuminated by fluorescent tubes ($T = 26 \pm 1^\circ\text{C}$; $\text{RH} = 65 \pm 5\%$; Photoperiod = 12:12, light:dark; light intensity: approx. 8000 Lux, ca. $110 \mu\text{mol m}^{-2}\text{s}^{-1}$). Virgin flies (2–3 days old) were then sexed and used for the following controlled crosses to attain 4 different mating types (Supplementary Fig. S1):

1. **Not-sibling mates (No-Sib):** unrelated male and female;
2. **Sibling mates (Sib):** full sibling male and female;
3. **Second generation sibling mates (Sib2):** full sibling male and female, both originated from sibling mate;
4. **Not-sibling backcross mates (Backcross):** unrelated male and female, male from parental line and female from sibling mate.

For every controlled cross, test virgin flies originated from 6 different cohorts were used. Furthermore, tested females in cross 1 (No-Sib) and 2 (Sib) derived from the same cohorts, as well as the ones used in cross 3 (Sib2) and 4 (Backcross), to account for cohort effect. This phenomenon led to cohorts within a population differing from each other in some characteristics, such as fecundity, and may increase variability, although it can be uninfluential when the resources are not limited (Lindström & Kokko 2002). For every mating type, 40 virgin females and 400 virgin males were observed (i.e., 40 replicates); all flies were replaced with new ones after every replicate.

2.2.2 Courtship, mating and mate recognition

Observations were accomplished during approximately 3 months (from May to July 2022), during the optimal mating season for this species (Park et al. 2010). Trials were performed at constant temperature ($26 \pm 1^\circ\text{C}$) and relative humidity ($65 \pm 5\%$). Courtship and mating behaviour were observed as described by Giunti et al. (2018), using a cubic observation chamber (1m^3) with led illumination on the top. Insects were gently released in a testing cage (clear Perspex, cubic 60cm), placed inside the observation chamber. Observations were performed between 10:00–14:00 h and were carried out over several days to account for daily variability.

Courtship and mating behaviour for the 4 different controlled crosses were observed by releasing ten virgin males and one virgin female (2–3 days old) in the testing cage. Previous studies reported that *H. illucens* males performed lekking behaviour, consisting of male aggregations to attract females, in which males fight to defend their territory (Tomberlin & Sheppard 2001). For this reason, a single male is generally unable to attain successful courtship. Virgin males were acclimated inside the testing cage for 10 minutes before the release of a virgin female. Behavioural trials began ten minutes after the female release, and insect behaviour was directly observed for 60 minutes (or until the end of post-copulatory behaviours). Characteristic courtship (preliminary genital contact, wing fanning), copula (success, duration) and post-copulatory (grooming) behaviours, previously described by Giunti et al. (2018), were compared to determine behavioural differences attributable to the degree of relatedness between the mates.

2.2.3 Reproductive performances

Mated *H. illucens* females were retrieved immediately after post-copulatory activity from the testing cage used for the behavioural observation. Mated females from the controlled crosses were individually collected and released inside cylindrical plastic containers (50 mL) with ventilated caps and water *ad libitum* under controlled conditions ($T = 26 \pm 1^\circ\text{C}$; $\text{RH} = 65 \pm 5\%$; Photoperiod = 12:12, light:dark). The females were checked daily for oviposition and survival, until their death. Oviposition occurrence, number of egg clutches, time

intercurrent from mating to oviposition and longevity of mated females were recorded.

Each egg clutch was collected, and egg fertility was accounted by recording hatching. Hatched larvae were reared on the standard larval diet used for mass-rearing and food was provided *ad libitum* until pupation. When around 75% of the larvae concluded their development (i.e., their colour became darker indicating prepupal and pupal stages), pupae and larvae were manually removed from the substrates, counted, and weighted. Developmental time (i.e., time from egg to pupa) was also calculated.

Lastly, every pupa was placed inside a plastic container (15 mL) and checked daily for adult eclosion, to assess the total fecundity (i.e., number of emerged adults, percentage of eclosion) and the sex ratio of the produced progeny.

2.3 Data analyses

Statistical analyses were carried out using JMP 11 software (SAS Institute). Categorical data, such as mating outcomes, oviposition occurrence, and egg hatching, were compared using Chi-square test with Yate's correction. Nominal logistic regressions (LR) were used to assess the possible relationships between binary responses (e.g., copula success) and continuous variables (e.g., wing-fanning duration).

Differences among continuous data were analysed using specific Generalized Linear Mixed Models (GLMMs), described by the general following formula: $y = X\beta + Zu + \varepsilon$ where y is the vector of the observations (e.g., copula duration), X is the fixed effect matrix, β is the vector of fixed effects (i.e., cross-type), Z is the random effect matrix, u is the vector of random effects (i.e., individual, cohort) and ε is the vector of the residual effects. Tukey HSD post-hoc test was performed on every continuous parameter analysed for both fixed and random effects.

3 Results

3.1 Courtship, mating and mate recognition

The behavioural sequence observed for all the tested mating types was consistent with those reported by Giunti et al. (2018). After an initial latency phase, responsive females started the flying activity which stimulated males' courtship responses and inflight approaches. Courting males performed peculiar wing-fanning behaviour and attempted genital contact. In this phase, females can dismount males refusing copulation or can accept male mounting, assuming the acceptance position and proceeding with copula. At the end of the copula, both males and females performed typical grooming behaviours before leaving the mating site.

Considering responses to courtship, females were divided into "unresponsive" (i.e., females displaying no flight activity), "unsuccess" (i.e., responsive females rejecting male courtships) and "success" (i.e., responsive females accepting mating). Females' responses varied according to the different

cross-type ($\chi^2_3 = 12.99, P = 0.005$) (Fig. 1), with the lowest success rate in Sib2 cross (37.5%), while the highest was recorded in No-Sib mates (57.5%).

Wing fanning did not change depending on mate type ($F_{3,122} = 0.08, P = 0.97$), underling that males did not change their courtship according to mate kinship. Similarly, no differences were recorded in post-copulatory behaviours both in females ($F_{3,77} = 0.02, P = 0.99$) and males ($F_{3,77} = 0.05, P = 0.97$). However, successful courtship approaches were characterized by shorter wing fanning in all the crosses ($[LR]\chi^2_{1,122} = 50.91, P < 0.0001$). Furthermore, copula duration was significantly influenced by the kinship of mates ($F_{3,77} = 4.27, P = 0.013$), with longer copula in Sib2 crosses compared to the other treatments (Table 1). Random variance explained by “cohort” random effect was low for all the tested parameters ($< 8.1\%$) and no differences were highlighted among cohorts by Tukey HSD post-hoc test.

3.2 Reproductive performances

Mated females were divided according to oviposition occurrence in “absent”, when it did not occur and “present”, when females laid eggs. Furthermore, the number of oviposition events was also recorded, separating *i*) “single oviposition”, i.e., females laid eggs during a single oviposition event in their lifetime, and *ii*) “multiple oviposition”, females laid eggs in two distinct events (i.e., two different days). Oviposition occurrence varied depending on the mating type ($\chi^2_3 = 7.86, P = 0.049$) (Fig. 2), with the highest proportion of non-ovipositing females in Sib2 crosses (26.7%). In contrast, no differences were accountable for multiple or single oviposition events among the various cross-types ($\chi^2_3 = 6.16, P = 0.104$).

Egg laying was not influenced by copula duration ($[LR]\chi^2_{1,77} = 0.21, P = 0.65$). In contrast, longer lifespan was positively associated to oviposition occurrence

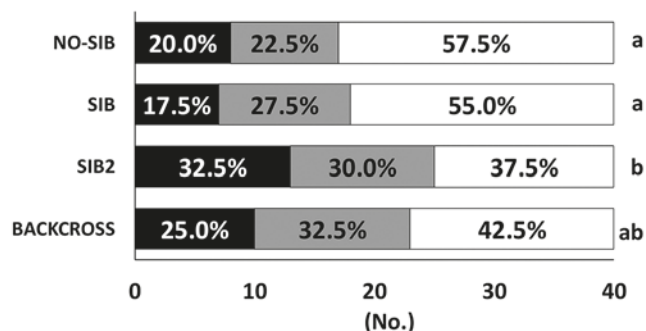


Fig. 1. Responses of females to males' courtship in different controlled crosses in *H. illucens*: number unresponsive females = black bars; number unsuccessful courtship = grey bars; number successful courtship = white bars. Different letters indicate statistical differences among cross-types (Chi square test, $P < 0.05$). No-Sib = Not-sibling mates; Sib = Full-sibling mates; Sib2 = Second generation full-sibling mates; Backcross = Not-sibling backcross mates. No. = number of flies.

($[LR]\chi^2_{1,77} = 4.81, P = 0.028$), although no differences were recorded in the female longevity among treatments ($F_{3,77} = 0.23, P = 0.87$). However, mate kinship influenced the time elapsed between the copula and the first egg laying ($F_{3,69} = 3.59, P = 0.018$), with the longest time in Sib2 females (Table 2). Furthermore, hatching was influenced by mating type ($\chi^2_3 = 9.8, P = 0.02$). Fertility ratio in Sib2 females was significantly lower compared to those from other controlled crosses (Fig. 3). Developmental time was also significantly different among the cross-types ($F_{3,58} = 6.39, P = 0.002$), again highlighting the longest extent in Sib2 progeny. The average weight of the single pupa was not significantly different among the various controlled crosses ($F_{3,58} = 1.66, P = 0.204$), while statistical differences could be acknowledged when considering the total number of pupae produced by every mated female ($F_{3,58} = 3.45, P = 0.030$) and their whole weight ($F_{3,58} = 3.40, P = 0.033$) (Table 3).

Similar to other lifetime parameters, the eclosion ratio ($F_{3,58} = 5.65, P = 0.002$) and the number of emerged adults ($F_{3,58} = 2.87, P = 0.046$) were significantly lower in Sib2 compared to No-Sib (Table 3). Interestingly, also the sex ratio was affected by the kinship of the mates, revealing that No-Sib progeny was almost equally distributed between males and females, whereas for all the other crosses the progeny was significantly more male-biased ($F_{3,58} = 4.96, P = 0.004$).

None of the above discussed continuous data highlighted significant differences among the tested cohorts according to Tukey HSD test, with an explained variance attributable to “cohort” random effect always lower than 16.1%.

4 Discussion

Overall, direct kin recognition and avoidance were not evidenced in the present *H. illucens* population. Tested

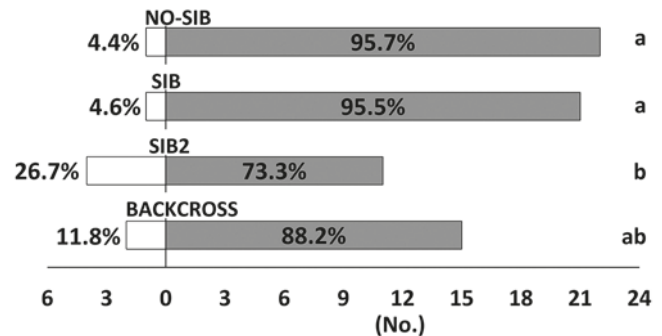


Fig. 2. Oviposition outcomes by mated females in different controlled crosses in *H. illucens*: number of females not ovipositing = white bars; number of females ovipositing = grey bars. Different letters indicate statistical differences among cross-types (Chi square test, $P < 0.05$). No-Sib = Not-sibling mates; Sib = Full-sibling mates; Sib2 = Second generation full-sibling mates; Backcross = Not-sibling backcross mates. No. = number of flies.

Table 1. Behavioural parameters (mean ± standard error) observed in courtship and mating in different controlled crosses in *H. illucens*. Different letters indicate significant differences among the cross-types (GLMM followed by Tukey HSD post-hoc test, $P < 0.05$). No-Sib = Not-sibling mates; Sib = Full-sibling mates; Sib2 = Second generation full-sibling mates; Backcross = Not-sibling backcross mates. WS: wing fanning. n = sample size.

Cross	Unsuccessful WF (s)	Successful WF (s)	Copula (min)	Male grooming (s)	Female grooming (s)
No-Sib	38.11 ± 7.7 (n = 9)	16.4 ± 1.7 (n = 23)	35.5 ± 2.8a	71.0 ± 17.3	126.8 ± 18.5
Sib	39.3 ± 6.7 (n = 11)	15.9 ± 2.3 (n = 22)	34.0 ± 3.1a	71.9 ± 15.9	124.4 ± 18.9
Sib2	34.9 ± 3.9 (n = 12)	15.7 ± 2.6 (n = 15)	52.2 ± 6.4b	73.4 ± 17.7	129.5 ± 27.0
Backcross	37.0 ± 4.7 (n = 13)	16.4 ± 2.2 (n = 17)	33.3 ± 3.2a	77.5 ± 12.6	134.4 ± 12.6

Table 2. Longevity of mated females and oviposition parameters (mean ± standard error) recorded for different controlled crosses in *H. illucens*. Different letters indicate significant differences among the cross-types (GLMM followed by Tukey HSD post-hoc test, $P < 0.05$). No-Sib = Not-sibling mates; Sib = Full-sibling mates; Sib2 = Second generation full-sibling mates; Backcross = Not-sibling backcross mates. n = sample size.

Cross	Mated ♀♀ longevity (d)	Oviposition event (%)		Time from mating (d)	
		Single	Multiple	1 st oviposition	2 nd oviposition
No-Sib	8.1 ± 0.5 (n = 23)	81.82% (n = 18)	18.18% (n = 4)	2.3 ± 0.2 a	5.3 ± 1.1
Sib	8.4 ± 0.6 (n = 22)	90.48% (n = 19)	9.52% (n = 2)	2.3 ± 0.1 a	6.0 ± 1.7
Sib2	8.8 ± 0.8 (n = 15)	90.91% (n = 10)	9.09% (n = 1)	3.5 ± 0.6 b	7.0 ± 0.0
Backcross	8.4 ± 0.6 (n = 17)	93.33% (n = 14)	6.67% (n = 1)	2.7 ± 0.2 ab	7.0 ± 0.0

Table 3. Developmental time (DT), progeny production, and sex ratio (mean ± standard error) recorded in different controlled crosses in *H. illucens*. Different letters indicate significant differences among the cross-types (GLMM followed by Tukey HSD post-hoc test, $P < 0.05$). No-Sib = Not-sibling mates; Sib = Full-sibling mates; Sib2 = Second generation full-sibling mates; Backcross = Not-sibling backcross mates. n = sample size.

Cross	DT (d)	Pupa weight (g)	Pupae per ♀♀		Progeny per ♀♀		Sex ratio
			Nb.	Weight (g)	% Eclosion	Nb adults	
No-Sib (n = 21)	37.6 ± 0.9a	0.193 ± 0.007	262.4 ± 39.8a	50.66 ± 8.24a	90.4 ± 2.1a	234.9 ± 38.0a	1.03 ± 0.02a
Sib (n = 18)	39.4 ± 1.1a	0.172 ± 0.011	277.6 ± 61.3a	47.83 ± 11.85a	85.9 ± 3.0ab	231.4 ± 58.5a	1.21 ± 0.07b
Sib2 (n = 7)	46.1 ± 3.2b	0.160 ± 0.015	100.0 ± 25.6b	16.04 ± 4.97b	62.3 ± 5.6b	64.3 ± 16.7b	1.35 ± 0.16b
Backcross (n = 12)	37.3 ± 1.0a	0.171 ± 0.014	194.9 ± 46.0ab	33.40 ± 8.22ab	82.1 ± 3.3ab	157.6 ± 34.8ab	1.27 ± 0.09b

specimens showed courtship and mating sequences consistent with those reported by previous research (Giunti et al. 2018). Notably, no differences were reported among the courtship sequence among the tested mating types, with uniform pre- and post-copulatory behaviours among the various controlled crosses. In all the tested crosses, females preferentially mated with males performing short-wing fanning, highlighting that inbred adults behave similarly to non-inbred ones and that females can actively choose mates based on courtship quality.

Similarly to other insect species (de Boer et al. 2021; Reece et al. 2004; Ruf et al. 2010), *H. illucens* females seemed to be unable to recognize kin from unrelated mates. Indeed, no significant differences in success rate resulted in females approaching different mates (i.e., No-Sib vs. Sib and Sib2 vs. Backcross). Nevertheless, based on produced fecundity and progeny, mate selection seemed important. This species has been considered monogamous for long time

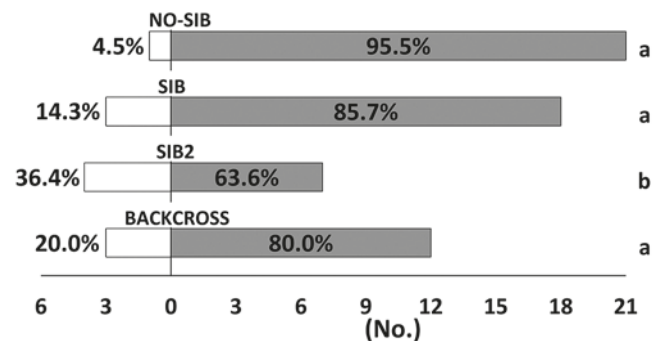


Fig. 3. Fertility of eggs laid by mated females in different controlled crosses in *H. illucens*: number of females ovipositing unfertile eggs = white bar; number of females ovipositing fertile eggs = grey bars. Different letters indicate statistical differences among cross-types (Chi square test, $P < 0.05$). No-Sib = Not-sibling mates; Sib = Full-sibling mates; Sib2 = Second generation full-sibling mates; Backcross = Not-sibling backcross mates. No. = number of flies.

(Giunti et al. 2018), but recent research demonstrated that females can mate multiple times (Muraro et al. 2024). In this scenario, polyandry can play a role to avoid inbreeding depression (Michalczyk et al. 2011). Indeed, from an anatomical point of view, *H. illucens* females present three spermathecae (Munsch-Masset et al. 2023), which can be used to control paternity of the progeny by *H. illucens* females, suggesting that cryptic female choice (CFC) by post-mating selection may occur (Bretman et al. 2009).

Overall, significant differences were noted in the mating performances among the controlled crosses. In Sib2, a higher ratio of unresponsive females and a lower success rate compared to No-Sib and Sib females were accounted. The decline in the reproductive performance could be related to a generally lower quality of Sib2 mates, although backcross of inbred females with unrelated males reduced female unresponsiveness and increased the success rate. Thus, the inferior performances in Sib2 crosses could be mainly linked to a lower courtship quality by inbred males, and just marginally to the lower propensity to mate of inbred females.

Besides, copulation was significantly longer in Sib2 controlled crosses. Variations in copula duration have been explained by several external and internal factors; usually, longer copula increases sperm transfer and/or prevents female from remating by decreasing their receptivity (Simmons & Wedell 2020). Prolonged copula can be also linked to adaptive cryptic male choice (CMC), expecting males to selectively spend more resources (i.e., time, ejaculate, energy) when mating with high-quality and more fecund females (Bonduriansky 2001). This adaptive behaviour has been reported in another stratiomyid fly, *Merosargus cingulatus* Schiner (Barbosa 2011), although it is not consistent with our results in which longer copula took place in less fecund females. Instead, females might also control copula duration by acting adaptive cryptic female choice (CFC) (Barbosa 2009), by preferring males performing longer copula. Yet, based on our results, this seems not the case of *H. illucens*. Therefore, copula in Sib2 took the longest time, although this could be dangerous for both mates by increasing the risk of predation and decreasing their foraging chances. This result can indicate that *H. illucens* inbred males produce low-quality and quantity of ejaculates (Baeshen et al. 2014), which can determine longest copula, as reported in other insect species (Bloch Qazi 2003).

Reproductive performances were greatly impacted by inbreeding depression in *H. illucens* after two generations of strict inbreeding. Egg hatchability, as well as pupation and eclosion showed a significant decline. In other fly populations, while egg fertility was influenced by inbreeding, no impact was observed for pupation and eclosion (Rhode et al. 2020). Rhode et al. (2020) have reported colony collapse after 5 generations in a small experimental *H. illucens* colony, suggesting that the collapse should be attributable to the fixation of deleterious alleles. However, in our population, inbreeding affected oviposition more than hatchability and

pupation, proving that the decline of population is primarily linked to mating occurrence and subsequently to mate quality. Moreover, Munsch-Masset et al. (2023) reported that *H. illucens* females produced oocytes synchronously and can lay eggs just one time in their life. Ovarian synchronicity was also observed in a *H. illucens* laboratory strain by Tomberlin et al. (2002), while this trait was not retrieved in our laboratory strain, which proved multiple egg-laying events in a small fraction of our population, as also demonstrated in a recent research (Muraro et al. 2024).

Developmental time was prolonged in inbred *H. illucens*, whereas pre-imaginal stage duration could decrease after backcross. In fly species with short adult lifespan and almost monogamous females mating and ovipositing early in their life, delayed pupation and eclosion could greatly affect offspring fitness (Edwards 2000). Besides, considering *H. illucens* rearing for entomofarming, the yield of mature pupae, in terms of numerosity and weight produced *per* female, was affected by inbreeding, prompting colony collapse in small mass-rearing.

Usually shortened lifespans are expected for insects after several generations of inbreeding (Bilde et al. 2009), since most of the energy is allocated to reproduction rather than survival. This aspect should have been particularly important for this species, which may not feed at the adult stage and uses the resources accumulated during the larval stages for adult survival and performances (Lupi et al. 2019). No effect was associated to inbreeding on *H. illucens* female adult longevity, as reported for other insect species (Harano 2011). Moreover, while the original population was composed of equal proportions of male and female adults, inbreds were more male-biased. This feature of our laboratory strain was dissimilar to those of other *H. illucens* laboratory populations, which are female-biased (Tomberlin et al. 2002).

In conclusion, this study poses a first step to investigate the mating behavioural ecology of this species, in terms of mate choice and kin avoidance, and demonstrates inbreeding depression in *H. illucens*. Moreover, flies were not able to discriminate siblings from unrelated mates, with potential negative consequences on colony maintenance and health. However, the existence of cryptic mate behaviour, as well as the impact of inbreeding on insect physiological features need further consideration. In contrast, a valuable restoring impact of backcross was noted, suggesting that this mechanism can help to maintain optimal population levels and avoid colony collapse. Overall, it is acknowledged that mass-reared insect colonies benefit from the re-introduction of genetic diversity from other colonies (Whiteley et al. 2015). Nevertheless, the introduction of wild insects in a domesticated or early-domesticated colony can trigger an additional decline in the offspring fitness; this process, known as outbreeding depression, can be linked to the introduction of complete different behavioural or genetic characteristics, that worse adapt to artificial conditions (Whitlock et al. 2013). Therefore, research about inbreeding and outbreeding dynamics is still

pivotal to understand and estimate the long-term sustainability of *H. illucens* mass-rearing and to maintain and manage genetic diversity in commercial colonies.

References

- Alagappan, S., Rowland, D., Barwell, R., Mantilla, S. M. O., Mikkelsen, D., James, P., ... Hoffman, L. C. (2022). Legislative landscape of black soldier fly (*Hermetia illucens*) as feed. *Journal of Insects as Food and Feed*, 8(4), 343–356. <https://doi.org/10.3920/JIFF2021.0111>
- Baek, M. J., Yoon, T. J., Kang, H. J., & Bae, Y. J. (2014). Biological and genetic characteristics of *Glyptotendipes tokunagai* (Diptera: Chironomidae) on the basis of successive rearing of forty-two generations over seven years under laboratory conditions. *Environmental Entomology*, 43(5), 1406–1418. <https://doi.org/10.1603/EN14052>
- Baeshen, R., Ekechukwu, N. E., Toure, M., Paton, D., Coulibaly, M., Traoré, S. F., & Tripet, F. (2014). Differential effects of inbreeding and selection on male reproductive phenotype associated with the colonization and laboratory maintenance of *Anopheles gambiae*. *Malaria Journal*, 13(1), 19. <https://doi.org/10.1186/1475-2875-13-19>
- Barbosa, F. (2009). Cryptic female choice by female control of oviposition timing in a soldier fly. *Behavioral Ecology*, 20(5), 957–960. <https://doi.org/10.1093/beheco/arp083>
- Barbosa, F. (2011). Copulation duration in the soldier fly: The roles of cryptic male choice and sperm competition risk. *Behavioral Ecology*, 22(6), 1332–1336. <https://doi.org/10.1093/beheco/arr137>
- Bilde, T., Maklakov, A. A., Meisner, K., La Guardia, L., & Friberg, U. (2009). Sex differences in the genetic architecture of lifespan in a seed beetle: Extreme inbreeding extends male lifespan. *BMC Evolutionary Biology*, 9(1), 33. <https://doi.org/10.1186/1471-2148-9-33>
- Bloch Qazi, M. C. (2003). A potential mechanism for cryptic female choice in a flour beetle. *Journal of Evolutionary Biology*, 16(1), 170–176. <https://doi.org/10.1046/j.1420-9101.2003.00501.x>
- Bonduriansky, R. (2001). The evolution of male mate choice in insects: A synthesis of ideas and evidence. *Biological Reviews of the Cambridge Philosophical Society*, 76(3), 305–339. <https://doi.org/10.1017/S1464793101005693>
- Bretman, A., Newcombe, D., & Tregenza, T. (2009). Promiscuous females avoid inbreeding by controlling sperm storage. *Molecular Ecology*, 18(16), 3340–3345. <https://doi.org/10.1111/j.1365-294X.2009.04301.x>
- de Boer, R. A., Vega-Trejo, R., Kotrschal, A., & Fitzpatrick, J. L. (2021). Meta-analytic evidence that animals rarely avoid inbreeding. *Nature Ecology & Evolution*, 5(7), 949–964. <https://doi.org/10.1038/s41559-021-01453-9>
- Edwards, A. W. F. (2000). The genetical theory of natural selection. *Genetics*, 154(4), 1419–1426. <https://doi.org/10.1093/genetics/154.4.1419>
- Fischer, K., Karl, I., Heuskin, S., Janowitz, S., & Dötterl, S. (2015). Kin recognition and inbreeding avoidance in a butterfly. *Ethology*, 121(10), 977–984. <https://doi.org/10.1111/eth.12410>
- Franco, A., Scieuzo, C., Salvia, R., Petrone, A. M., Tafi, E., Moretta, A., ... Falabella, P. (2021). Lipids from *Hermetia illucens*, an innovative and sustainable source. *Sustainability (Basel)*, 13(18), 10198. <https://doi.org/10.3390/su131810198>
- Giunti, G., Campolo, O., Laudani, F., & Palmeri, V. (2018). Male courtship behaviour and potential for female mate choice in the black soldier fly *Hermetia illucens* L. (Diptera: Stratiomyidae). *Entomologia Generalis*, 38(1), 29–46. <https://doi.org/10.1127/entomologia/2018/0657>
- Gougbedji, A., Agbohessou, P., Lalèyè, P. A., Francis, F., & Caparros Megido, R. (2021). Technical basis for the small-scale production of black soldier fly, *Hermetia illucens* (L. 1758), meal as fish feed in Benin. *Journal of Agriculture and Food Research*, 4, 100153. <https://doi.org/10.1016/j.jafr.2021.100153>
- Harano, T. (2011). Inbreeding depression in development, survival, and reproduction in the adzuki bean beetle (*Callosobruchus chinensis*). *Ecological Research*, 26(2), 327–332. <https://doi.org/10.1007/s11284-010-0787-y>
- Hedrick, P. W., & Garcia-Dorado, A. (2016). Understanding inbreeding depression, purging, and genetic rescue. *Trends in Ecology & Evolution*, 31(12), 940–952. <https://doi.org/10.1016/j.tree.2016.09.005>
- Hoffmann, L., Hull, K. L., Bierman, A., Badendorst, R., Bester-van der Merwe, A. E., & Rhode, C. (2021). Patterns of genetic diversity and mating systems in a mass-reared black soldier fly colony. *Insects*, 12(6), 480. <https://doi.org/10.3390/insects12060480>
- Hull, K. L., Greenwood, M. P., Lloyd, M., Brink-Hull, M., Bester-van der Merwe, A. E., & Rhode, C. (2024). Drivers of genomic diversity and phenotypic development in early phases of domestication in *Hermetia illucens*. *Insect Molecular Biology*, imb.12940. <https://doi.org/10.1111/imb.12940>
- Julita, U., Fitri, L. L., Putra, R. E., & Permana, A. D. (2020). Mating success and reproductive behavior of black soldier fly *Hermetia illucens* L. (Diptera, Stratiomyidae) in tropics. *Journal of Entomology*, 17(3), 117–127. <https://doi.org/10.3923/je.2020.117.127>
- Kaya, C., Generalovic, T. N., Ståhls, G., Hauser, M., Samayoa, A. C., Nunes-Silva, C. G., & Sandrock, C. (2021). Global population genetic structure and demographic trajectories of the black soldier fly, *Hermetia illucens*. *BMC Biology*, 19(1), 94. <https://doi.org/10.1186/s12915-021-01029-w>
- Lange, K. W., & Nakamura, Y. (2021). Edible insects as future food: Chances and challenges. *Journal of Future Foods*, 1(1), 38–46. <https://doi.org/10.1016/j.jfutfo.2021.10.001>
- Li, X. Y., & Kokko, H. (2019). Sex-biased dispersal: A review of the theory. *Biological Reviews of the Cambridge Philosophical Society*, 94(2), 721–736. <https://doi.org/10.1111/brv.12475>
- Lindström, J., & Kokko, H. (2002). Cohort effects and population dynamics. *Ecology Letters*, 5(3), 338–344. <https://doi.org/10.1046/j.1461-0248.2002.00317.x>
- Loyau, A., Cornuau, J. H., Clobert, J., & Danchin, E. (2012). Incestuous sisters: Mate preference for brothers over unrelated males in *Drosophila melanogaster*. *PLoS One*, 7(12), e51293. <https://doi.org/10.1371/journal.pone.0051293>
- Lupi, D., Savoldelli, S., Leonardi, M. G., & Jucker, C. (2019). Feeding in the adult of *Hermetia illucens* (Diptera Stratiomyidae): Reality or fiction? *Journal of Entomological and Acarological Research*, 51(1). <https://doi.org/10.4081/jea.2019.8046>
- Metzger, M., Bernstein, C., Hoffmeister, T. S., & Desouhant, E. (2010). Does kin recognition and sib-mating avoidance limit the risk of genetic incompatibility in a parasitic wasp? *PLoS One*, 5(10), e13505. <https://doi.org/10.1371/journal.pone.0013505>

- Michalczyk, L., Millard, A. L., Martin, O. Y., Lumley, A. J., Emerson, B. C., Chapman, T., & Gage, M. J. G. (2011). Inbreeding promotes female promiscuity. *Science*, 333(6050), 1739–1742. <https://doi.org/10.1126/science.1207314>
- Munsch-Masset, P., Labrousse, C., Beaugeard, L., & Bressac, C. (2023). The reproductive tract of the black soldier fly (*Hermetia illucens*) is highly differentiated and suggests adaptations to sexual selection. *Entomologia Experimentalis et Applicata*, 171(11), 857–866. <https://doi.org/10.1111/eea.13358>
- Muraro, T., Lalanne, L., Pelozuelo, L., & Calas-List, D. (2024). Mating and oviposition of a breeding strain of black soldier fly *Hermetia illucens* (Diptera: Stratiomyidae): polygynandry and multiple egg-laying. *Journal of Insects as Food and Feed*, 1(8), 1423. <https://doi.org/10.1163/23524588-20220175>
- Park, K., Kim, W., Lee, S., Choi, Y., & Nho, S. (2010). Seasonal pupation, adult emergence and mating of black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae) in artificial rearing system. *International Journal of Industrial Entomology*, 21(2), 189–191.
- Penn, D. J., & Frommen, J. G. (2010). Kin recognition: An overview of conceptual issues, mechanisms and evolutionary theory. In P. Kappeler (Ed.), *Animal Behaviour: Evolution and Mechanisms* (pp. 55–85). Berlin, Heidelberg: Springer. https://doi.org/10.1007/978-3-642-02624-9_3
- Pike, V. L., Cornwallis, C. K., & Griffin, A. S. (2021). Why don't all animals avoid inbreeding? *Proceedings of the Royal Society B*, 288(1956), 20211045. <https://doi.org/10.1098/rspb.2021.1045>
- Reece, S. E., Shuker, D. M., Pen, I., Duncan, A. B., Choudhary, A., Batchelor, C. M., & West, S. A. (2004). Kin discrimination and sex ratios in a parasitoid wasp. *Journal of Evolutionary Biology*, 17(1), 208–216. <https://doi.org/10.1046/j.1420-9101.2003.00640.x>
- Rhode, C., Badenhorst, R., Hull, K. L., Greenwood, M. P., Bester-van der Merwe, A. E., Andere, A. A., ... Richards, C. (2020). Genetic and phenotypic consequences of early domestication in black soldier flies (*Hermetia illucens*). *Animal Genetics*, 51(5), 752–762. <https://doi.org/10.1111/age.12961>
- Ruf, D., Mazzi, D., & Dorn, S. (2010). No kin discrimination in female mate choice of a parasitoid with complementary sex determination. *Behavioral Ecology*, 21(6), 1301–1307. <https://doi.org/10.1093/beheco/arq148>
- Santos, S. M., Sequenzia, P. L., Rodrigues, E. B., Martins, I. P., Pena, A. N. L., Costa, D. V., & Nogueira, W. C. L. (2022). System development for production of black soldier fly larvae (*Hermetia illucens*) in small scale, using wild flies. *Research. Social Development*, 11(1), e20311124747. <https://doi.org/10.33448/rsd-v11i1.24747>
- Simmons, L. W., & Wedell, N. (2020). Fifty years of sperm competition: the structure of a scientific revolution. *Philosophical Transactions of the Royal Society B*, 375(1813), 20200060. <https://doi.org/10.1098/rstb.2020.0060>
- Tomberlin, J. K., & Sheppard, D. C. (2001). Lekking behavior of the black soldier fly (Diptera: Stratiomyidae). *The Florida Entomologist*, 84(4), 729–730. <https://doi.org/10.2307/3496413>
- Tomberlin, J. K., Sheppard, D. C., & Joyce, J. A. (2002). Selected life-history traits of black soldier flies (Diptera: Stratiomyidae) reared on three artificial diets. *Annals of the Entomological Society of America*, 95(3), 379–386. [https://doi.org/10.1603/0013-8746\(2002\)095\[0379:slhtob\]2.0.co;2](https://doi.org/10.1603/0013-8746(2002)095[0379:slhtob]2.0.co;2)
- Triunfo, M., Tafi, E., Guarnieri, A., Salvia, R., Scieuzo, C., Hahn, T., ... Falabella, P. (2022). Characterization of chitin and chitosan derived from *Hermetia illucens*, a further step in a circular economy process. *Scientific Reports*, 12(1), 6613. <https://doi.org/10.1038/s41598-022-10423-5>
- van Huis, A. (2020). Insects as food and feed, a new emerging agricultural sector: A review. *Journal of Insects as Food and Feed*, 6(1), 27–44. <https://doi.org/10.3920/JIFF2019.0017>
- Whitehorn, P. R., Tinsley, M. C., & Goulson, D. (2009). Kin recognition and inbreeding reluctance in bumblebees. *Apidologie*, 40(6), 627–633. <https://doi.org/10.1051/apido/2009050>
- Whiteley, A. R., Fitzpatrick, S. W., Funk, W. C., & Tallmon, D. A. (2015). Genetic rescue to the rescue. *Trends in Ecology & Evolution*, 30(1), 42–49. <https://doi.org/10.1016/j.tree.2014.10.009>
- Whitlock, R., Stewart, G. B., Goodman, S. J., Piertney, S. B., Butlin, R. K., Pullin, A. S., & Burke, T. (2013). A systematic review of phenotypic responses to between-population outbreeding. *Environmental Evidence*, 2(1), 13. <https://doi.org/10.1186/2047-2382-2-13>

Manuscript received: May 23, 2024

Revisions requested: August 7, 2024

Revised version received: August 22, 2024

Manuscript accepted: August 30, 2024

The pdf version (Adobe JavaScript must be enabled) of this paper includes an electronic supplement:

Figure S1