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Controlled and polygynous mating in the black soldier fly: advancing breeding programs utilizing quantitative genetic designs

Page header: CONTROLLED AND POLYGYNOUS MATING IN THE BLACK SOLDIER FLY

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Abstract

In animal breeding programs, utilizing quantitative genetic designs such as the full-/half-sibling design is fundamental. A full-/half-sibling design demands that mating can be controlled, and individuals can be tracked, enabling construction of a pedigree. In nature, black soldier fly (*Hermetia illucens* L.) males are reported to gather in lekking groups to engage in competitive displays and courtship rituals before mating, and this lekking behavior is described as crucial for mating in this species. We show that a randomly chosen male and a randomly chosen virgin female can readily mate under LED lights in a small container at 28 °C and 70% RH, demonstrating the ability to mate selected pairs. For males given access to four females, all mated and the time until first mating was considerably shorter than for males given only one female, where 80% of the males mated. Furthermore, when adding four virgin females to a single male, males successfully mated all four females in 52% of cases within our four-hour trial, with 96% of males mating at least twice. Number of matings observed for a male correlated with the number of females from the container that laid an egg clutch, and almost all egg clutches produced offspring. Observed mating thus lead to fertilization, and consecutive matings were with new females resulting in full- and half-sibling offspring. Males with four females that mated a second female did so at a median time of less than three minutes upon ending previous mating, showing that polygynous mating occurred rapidly in this setup. Our findings pave the way for moving *H. illucens* breeding programs beyond mass selection towards advanced selective breeding designs where controlled mating is typically required. Such designs enable selection for multiple traits simultaneously while controlling inbreeding and can drastically increase rates of selection responses compared to mass selection.

Keywords

consecutive polygyny, *Hermetia illucens*, individual males, offspring siring, pedigree tracking

Conflict of interest

The authors declare no conflict of interest.

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Introduction

Insects are currently being proposed as a sustainable future protein and lipid source (Dobermann *et al.*, 2017; Liceaga, 2021; van Huis, 2020), and some insect species have been used as food and feed for centuries (Olivadese and Dindo, 2023). In the Western world, large-scale factories producing insect-derived products have only recently been established (Montanari *et al.*, 2021). Especially species of flies, beetles, and crickets are target taxonomic groups gaining attention (Oonincx *et al.*, 2015; van Huis, 2021). These species differ from traditional livestock in many aspects of biology and management. Since the domestication of most insect species began recently and is still in progress, there is much to be learned about their biology, adaptation, and performance under artificial rearing conditions. One prerequisite for efficient selective breeding is the ability to perform controlled matings of selected pairs, but some insect species may have specific requirements to engage in mating (Burdfield-Steel and Harari, 2021). Knowing how to mate selected pairs is essential when developing breeding programs aimed at improving traits of interest in commercial insect species through selective breeding (Hansen *et al*. 2024b).

Selection has been instrumental in securing continued and cumulative genetic progress for production traits in livestock for decades (Hill and Kirkpatrick, 2010). Genetic changes along with technological advancements involving feeding optimization, use of reproductive technologies, and animal health management play key roles in securing sustainably produced future food and feed for a growing human population. In traditional livestock such as cattle, pigs, and poultry, the effectiveness of breeding programs has relied heavily on the ability to master the reproduction of the animals. This has resulted in more profitable animals that e.g. utilize feed for meat growth at a much higher efficiency (Emmerson, 1997; Lashley, 1978; Lush, 1937; Schultz *et al.* 2020). Another reason for the effectiveness of livestock selective breeding is the ability to keep track of pedigrees across generations, which greatly improves the precision by which genetically superior animals can be identified, which lead to boosting breeding responses (Clark *et al.*, 2012; Meuwissen, 1997). This does not only allow for fast genetic progress across generations but also for control of the rate of inbreeding in a population (Meuwissen, 1997), as well as for effective selection on multiple traits simultaneously (Cole *et al.*, 2021).

Genetic improvement of insect species for food and feed is still in its infancy (Gowda *et al.*, 2025; Hansen *et al.*, 2024b; Jensen *et al.*, 2017). Yet, the potential for fast genetic improvement in insects is high given that there is plenty of genetic variation within relevant species (Cai *et al.*, 2024; Eriksson and Picard, 2021; Hoffmann *et al.*, 2021), most insects have short generation intervals spanning from weeks to a few months, and the small size of individuals means that high census population sizes can be maintained on relatively little space. However, to utilize this potential in selective breeding we need to be able to control who mates with who, as we want to facilitate mating between individuals that we have selected to be parents of the next generation, thus excluding any chance for polyandry or sexual selection.

Recent studies attempting to genetically improve production traits in commercially produced insects have done so employing phenotypic mass selection (Facchini *et al.*, 2022; Gligorescu *et al.*, 2023; Morales-Ramos *et al.*, 2019). However, under mass rearing conditions no information is available about which flies mate and contribute genetically to the next generation, and the pedigree structure is therefore unknown. Pedigree information can facilitate the estimation of trait heritabilities, variance components, and genetic correlations between traits of interest (Falconer and Mackay, 1996). One commonly used method for estimating such genetic parameters is through a full-/half-sibling design, where phenotypes of

siblings and half-siblings are compared. Many insect species will readily mate when placed together as virgins in pairs, especially amongst insects typically maintained in laboratory cultures, including crickets (Hawkes *et al.*, 2022; Rapkin *et al*., 2017), cockroaches (Bunning *et al.*, 2015; Jensen and Silverman, 2018), silkworms (Abdelmegeed, 2015; Sarkar *et al*., 2009), mealworms (Sellem *et al.*, 2024; Worden and Parker, 2001), fruit flies (Jensen *et al.*, 2015; Perez-Staples *et al.*, 2007), and house flies (Hansen *et al*. 2024a; Laursen *et al*., 2024). Full-/half-sibling designs have been successfully applied in the house fly (Boatta *et al.*, 2023; Hansen *et al.*, 2024a), and half-sibling designs have been applied in mealworm (Sellem *et al.*, 2024). However, many insect species require specific conditions to induce mating (Burdfield-Steel and Harari, 2021; Lloyd, 1979), and mating selected individuals is therefore not easily attainable for all species.

The black soldier fly (*Hermetia illucens* L.; Diptera: Stratiomyidae) has become a main species of interest in the insect production industry (Tomberlin and van Huis, 2020; van Huis *et al.*, 2020), and there is great interest in genetically improving this species for production through breeding (Athanassiou *et al.*, 2024). Studies on *H. illucens* have shown that traits related to developmental time, feed conversion efficiency, and weight gain can be improved under mass selection (Facchini *et al.*, 2022, Gligorescu *et al.* 2023). However, this type of selection does not easily allow for controlling rates of inbreeding, for selecting on multiple traits simultaneously, and long-term responses to selection are expected to be lower than what can be obtained when pedigree information is available (Falconer and Mackay, 1996). In nature, *H. illucens* have so-called lekking behavior (Barrett *et al.*, 2023; Chiabotto *et al.*, 2024; Giunti *et al.*, 2018; Jones and Tomberlin, 2021; Julita *et al*., 2020; Kortsmit *et al.*, 2023; Lemke *et al.*, 2023; Meneguz *et al.*, 2023; Muraro *et al*. 2024; Tomberlin and Sheppard, 2001). Lekking occurs when several males perform sexual display together to attract females while each male defends a territorial patch (Alcock, 1990; Fiske et al. 1998; Tomberlin and Sheppard, 2001), implying that several males occur together simultaneously. It is thus believed that a single male cannot mate as he is unable to attain successful courtship (Laudani *et al.*, 2024). If this is the case, controlled mating where a specific male is mated to a specific female is difficult to perform. Using marked flies, keeping track of mated pairs is possible (Chiabotto *et al.*, 2024; Jones and Tomberlin, 2021; Muraro *et al*., 2024), but clearly tedious. If the presence of multiple males is necessary to induce mating in *H. illucens*, as reported to this point (Barrett *et al.*, 2023; Chiabotto *et al.*, 2024; Giunti *et al.*, 2018; Jones and Tomberlin, 2021; Julita *et al.*, 2020; Kortsmit *et al.*, 2023; Laudani *et al.*, 2024; Lemke *et al*., 2023; Manas *et al*., 2024; Meneguz *et al.*, 2023; Muraro *et al*. 2024; Tomberlin and Sheppard, 2001), this is a major challenge for developing effective selective breeding programs involving pedigree tracking and full-/half-sibling designs for this species.

Optimal conditions for mating to occur in *H. illucens* includes moderately high temperatures between 26 to 29 °C and a high relative humidity of 65 to 85 % (Giunti *et al.*, 2018; Julita *et al.*, 2020). Bright light with high intensity in the blue-green spectrum is critical for encouraging mating (Barrett *et al*., 2022; Heussler *et al*., 2018; Liu *et al*., 2020; Oonincx *et al*., 2016; Schneider, 2020; Tomberlin and Sheppard, 2002), and LED lights have proven to be the best artificial light source in comparative tests (Heussler *et al*., 2018, Hoc *et al*., 2019; Julita *et al.*, 2020, Liu *et al.*, 2020, Macavei *et al.*, 2020; Oonincx *et al*., 2016). Although it has been demonstrated that both male and female *H. illucens* can mate more than once (Chiabotto *et al.*, 2024; Dickerson *et al.*, 2024; Hoffmann *et al.*, 2021; Jones and Tomberlin, 2021; Muraro *et al*., 2024), little is known about how quickly *H. illucens* males will mate polygynously when multiple females are present. If controlled polygynous mating of individual males is possible, this would enable the development of efficient pedigree-based breeding programs for this species (Slagboom *et al*., 2024). Furthermore, controlled

polygynous mating would create opportunities to advance selective breeding strategies, for example by simultaneously selecting for multiple traits of economic value (Zaalberg *et al.*, 2024). Our primary goals were to investigate whether males and females will mate in individual pairs within small containers with no chance for mate choice, and whether and how quickly individually maintained males can mate multiple virgin females consecutively. We then investigated whether mating led to egg clutches and offspring, and whether the number of offspring produced by a female was affected by the number of females the male had mated. Our findings provide a foundation for implementing controlled mating in *H. illucens* breeding programs, contributing to the advancement of pedigree-based selective breeding in commercial insect production.

Materials and methods

Fly population and rearing

We used a laboratory culture of *H. illucens* established in 2017 at Enorm Biofactory A/S (Hedelundvej 15, 8762 Flemming, Denmark) from a batch of >100,000 juveniles. The culture was maintained in a large population of >5,000 adults per generation prior to this experiment. Rearing of flies and all experiments were performed in climate-controlled rooms kept at 28 ± 0.5 °C and 70 ± 10% relative humidity (RH). Larvae were collected from Enorm at 5-6 days of age and reared in open trays (57:37:8 cm L:W:H - internal measures) each containing two kg of chicken feed (Paco Start, Danish Agricultural Grocery Company, Fredericia, Denmark) and 4 L of water (66.67% substrate humidity by mass). Roughly 1500 larvae (15 g) were added to each tray. To control fly age since eclosion and ensure virginity (Permana *et al*. 2020), pupae were collected at their final stage and maintained in individual drosophila vials (95:25 mm height:diameter; VWR) secured with a foam stopper, and checked daily for emergence. Emerged adults were then given another 48-72 h in their individual vials to sexually mature. They were then sexed in the vial by visual inspection of the genitalia (Julita *et al.* 2020) and used in mating trials. All flies in mating trials thus were of a similar age (48-72 h), ensuring virility and sexual maturity (Dickerson *et al.*, 2024; Tomberlin and Sheppard 2002). To test for occurrence of unfertilized oviposition, a parallel set of 247 female flies were maintained in their individual vials since collection as pupae and monitored for the appearance of egg clutches until their death.

Experimental design

For the mating experiments, male and female flies were maintained together in transparent 3.0 L (19.5:19.5:11.3 cm L:W:H) plastic containers (TPS192, Dansk Transport Emballage A/S, Vojens, Denmark). An eight cm diameter hole was cut in the lid for ventilation and covered with a fine mesh. All flies were allocated to the containers by simultaneously removing all foam stoppers and shaking flies out of their vials into the containers through an opened corner of the lid. This was done in a room at 20 °C, 50% RH, and lighting from fluorescent tubes in the roof, which stimulated very limited activity and no mating display. The containers with flies were then moved to the climate-controlled room for mating trials.

We established 30 replicates of one male with one female, and 25 replicates of one male with four females. Here, the flies were given four hours to mate under two LED lights (112.5 cm 50W Philips LED*life* VerticalGrow120 full spectrum) placed 8 cm above the container lids. All replicates involving one male with one female were performed on one day, and the

replicates involving one male with four females were performed over the following two days with ten replicates on the first day and 15 on the second. Number of trials on a day fitted with the observation capacity of a single observer. We recorded the time until start and end of each mating by manual observation and recording using a timer and notebook. The timer was started when all flies were placed in the climate-controlled room underneath the LED lights, and the LED lights were turned on. No mating related activity occurred before this point. After the four hours with the male in the container, females were caught and placed in individual drosophila vials held upside down with coarse polyurethane foam stoppers (SuperFish aquarium filter, one-two mm spaces) clogging the bottoms for ovipositing. To enhance egg survival, a tray (57:37:8 cm L:W:H) with water was placed 10 cm underneath the vials, ensuring that RH did not drop below 70%, and vials were checked daily for egg clutches over the following six days. All egg clutches were placed in individual drosophila vials by collecting the foam stopper with the eggs and plugging it deep into the vial so the eggs were about one cm from the surface of a two cm layer substrate of 5 g chicken feed with 10 mL water. After seven days, the vials with larvae were recorded and larvae with remaining substrate were transferred to an eight cm diameter plastic container with 30 g chicken feed and 60 mL water and given seven more days to grow. The container was covered with a lid with a six cm diameter hole with a fine mesh for ventilation. The larvae were then collected from their media and counted.

Statistical analysis

Homogeneity of variances were compared between groups with a Levene's test, and Shapiro-Wilk tests were used to test for normal distribution of residuals. Variances were in most cases unequal (Levene tests, $P < 0.05$), and data were generally not normally distributed (Shapiro–Wilk tests, $P < 0.05$). Therefore, nonparametric tests were used. A Kruskal-Wallis test was used when comparing time until each consecutive mating since start or since the end of the previous mating, as well as when comparing mating duration across consecutive matings. It was also used when testing for an effect of the number of times a male mated on the number of larvae produced per egg clutch by females from the same container. If the Kruskal-Wallis test indicated significant differences, it was followed by a Dunn's test for multiple comparisons. A Wilcoxon test was used to compare the time until first mating and the number of larvae produced per female egg clutch by males paired with one *versus* four females. A Pearson's chi-squared test was used to test for a difference in the proportion of males that mated within the four-hour mating trial depending on whether they were exposed to one or four females. This test was also used to test for a correlation between the number of times a male was observed mating and the number of females that laid an egg clutch, and to test for an effect of multiple mating on the proportion of egg clutches that resulted in larvae within males exposed to four females. Statistical analyses were performed in JMP 15.0.0 (SAS Institute Inc., Cary, NC, USA).

Results

All males exposed to four females mated, while 80% of males provided access to only a single female mated, which was a significantly lower proportion (Pearson: $\chi^2_{1,55} = 5.61$, $P = 0.018$; Figure 1). Most males exposed to four females also mated a second (96%) and a third time (88%), and just above half of the males (52%) mated four times. The time to first mating was considerably shorter when males were together with four females than when they were

together with only one female (Figure 1). Across the 25 males provided access to four females, the median time until first mating was 07:17 (mm:ss) minutes (10th percentile: 03:46, 25th: 05:04, 75th: 10:46, 90th: 21:38), while males exposed to only one female initiated mating at a median of 53:58 minutes (10th percentile: 07:40, 25th: 13:29, 75th: 01:04:24 (hh:mm:ss), 90th: 02:50:00). All males exposed to four females had initiated mating within 35 minutes (Figure 1).

[FIGURE 1: Time until males initiated mating depending on number of females present]

Males given four females that mated a second time initiated their second mating at a median of 02:43 minutes (10th percentile: 00:48, 25th: 01:28, 75th: 08:11, 90th: 10:44) after the first mating had ended, and all had initiated their second mating within 30 minutes after terminating the first (Figure 2A). After the second mating, the median time in between matings increased significantly (Figure 2A). Down to 10 seconds was recorded for two males to initiate the third mating after finishing the second. Mating duration generally increased for each mating from around 20 minutes for the first mating to around 40 minutes for the third, with an intermediate 30 minutes of mating for the fourth mating (Figure 2B).

[FIGURE 2: Time until and between matings and mating durations of subsequent matings]

Out of the 30 females set up individually with a male, 23 subsequently produced an egg clutch, and all egg clutches produced larvae. A median of 149 larvae (10th percentile: 16, 25th: 79, 75th: 210, 90th: 310) were produced from these egg clutches. For the 25 males with access to four females, in 8% (2) of the cases only one female produced an egg clutch, in 44% (11) of the cases two females produced an egg clutch, in 36% (9) of the cases three females produced an egg clutch, and in 12% (3) of the cases all four females produced an egg clutch. We found a significant correlation between the number of times a male was observed mating and the number of females from the container that produced an egg clutch (Pearson: $\square^2_{9,25}$ = 18.32, P = 0.032). Within these 63 egg clutches, 92% (58) produced larvae. This demonstrates that most matings lead to fertilization, and that matings were predominantly with different, virgin females. In the 247 female flies maintained in their individual vials since collection as pupae, 4% (10) laid an egg clutch within their lifetime, confirming that little oviposition occurred without prior mating and fertilization.

There was no significant effect of the total number of times a male was observed mating on the proportion of laid clutches that produced offspring sired by the male (Pearson: $\square^2_{3,63}$ = 4.73, P = 0.19). The egg clutches each produced a median of 138 larvae (10th percentile: 33, 25th: 69, 75th: 219, 90th: 269), with no effect of the number of females from the container that oviposited (Kruskal-Wallis: $\square^2_{3,58}$ = 3.41, P = 0.33), and there was similarly no significant difference in the number of larvae produced per egg clutch depending on whether one or four females were available to a male (Wilcoxon: $\square^2_{1,81}$ = 0.007, P = 0.93). These findings indicate that no sperm depletion occurred.

Discussion

The importance of adult behavior is increasingly recognized in the breeding and maintenance of *H. illucens* populations (Lemke *et al.*, 2023; Meneguz *et al.*, 2023). With this study, we aimed to answer questions of central importance for the ability to execute efficient selective breeding programs in *H. illucens*. The main aims were to investigate whether *H. illucens* males and females can mate in individual pairs under controlled conditions with no chance for mate choice, and whether individually maintained *H. illucens* males can consecutively

mate multiple virgin females within a short mating session and produce full- and half-sibling offspring with these. Our results show that *H. illucens* males and females do mate readily in controlled mating pairs of selected individuals without prior lekking within our setup. In effect, we have heavily relaxed sexual selection, allowing for predominantly artificial selection which is optimal in selective breeding. We furthermore show that multiple successful matings occur quickly between one male and several females within our setup, showing that *H. illucens* can be highly polygynous and can readily produce full- and half-sibling offspring. These findings are of central importance for developing pedigree-based breeding programs for *H. illucens*. Although *H. illucens* females are known to lay unfertilized egg clutches (Dickerson *et al.*, 2024; Harjoko *et al.*, 2023; Tomberlin and Sheppard, 2002), it occurred at a low level even in the absence of males, which together with the high proportion of egg clutches that produced larvae shows that oviposition was a good but not perfect indicator of mating in our experiment.

Earlier studies have suggested that monogamy and polyandry are the prevailing mating systems in *H. illucens* (Giunti *et al.*, 2018; Kotzé *et al.*, 2019; Tomberlin and Sheppard, 2002). Such a mating system would diminish the use of standard quantitative genetic designs, i.e. full-/half-sibling designs, because they require that males can successfully mate multiple females. We showed that polygyny occurs in laboratory reared *H. illucens*, supporting recent evidence of both polygyny and polyandry in mass breeding *H. illucens* populations (Chiabotto *et al.*, 2024; Hoffmann *et al.*, 2021; Jones and Tomberlin, 2021; Muraro *et al.*, 2024). Our demonstration that polygyny can occur readily in a small container with only one single male facilitates the planning and development of full-/half-sibling designs in breeding programs for *H. illucens*. Full-/half-sibling breeding designs were recently used in house flies (Boatta *et al.*, 2023; Hansen *et al.*, 2024a), supporting our suggestion that advanced breeding programs are applicable in insects produced for food and feed. The findings of the current study pave the way for efficient breeding programs aiming at genetic improvement of traits of interest in *H. illucens*.

The shorter time until first mating when four females were present to a male instead of only one may have been caused by females disturbing each other, inducing movement and flight, and thereby increasing availability to the male. In theory, presenting multiple females to a single male also increases the chance that one of the females finds the male acceptable for mating due to complementary male genes or lower threshold for male quality (Hunt and Sakaluk, 2014; Moore and Moore, 1988). In *H. illucens*, male iridescent coloring and flight display performance including wing fanning are among traits assessed by females (Giunti *et al.* 2018; Julita *et al.* 2020). Mating is often highly dependent on female choice (Hunt and Sakaluk, 2014; Thornhill and Alcock, 1983), and female choice also plays a role in *H. illucens* when deciding whether to accept or reject a mating partner (Julita *et al.*, 2020). Adding multiple females to a male will theoretically select for females that are more willing to mate under the given artificial conditions. This selection can also occur when only one female is present, as the male may not achieve mating the female. Our results showed no evidence that male fertility declines following one or multiple matings, and thus we saw no sign of sperm depletion. At the third and fourth consecutive mating, a presumed lower sperm cell concentration due to sperm depletion might have been counterbalanced by the generally longer time until next mating (Figure 3A) as well as the generally longer mating duration (Figure 3B), which might have aided to respectively produce and allocate more sperm (Manas *et al.*, 2024). Very limited polyandry was observed in the current study, evident through the high correlation between number of matings observed for a male and number of females laying egg clutches producing offspring sired by the same male. Although polyandry is demonstrated in *H. illucens* (Chiabotto *et al.*, 2024; Muraro *et al.*, 2024), this occurred to a

very limited extent in our study as similarly reported in Giunti *et al*. (2018). In our setup, females did not have access to multiple males which could have stimulated the motivation for polygyny by broadening the sperm gene pool (Hunt and Sakaluk, 2014). It should be stated that we did not follow the offspring, and potential effects on offspring performance can therefore not be concluded upon.

In our study, LED lights were highly sufficient at inducing mating, supporting earlier studies (Julita *et al.*, 2020, Oonincx *et al.*, 2016, Liu *et al*., 2020, Macavei *et al*., 2020, Heussler *et al*., 2018, Hoc *et al*., 2019). Adaptation to artificial lighting instead of sunlight could potentially have occurred during domestication (Barrett *et al*., 2022). This is also the case for the lack of requirement for lekking, as defending lekking sites in a cage is likely not advantageous as these do not represent good sources of substrate for egg laying and larval development compared to situations in nature (Tomberlin and Sheppard, 2001). Since our *H. illucens* culture had undergone several generations under commercial rearing, adaptation by natural selection to the production environment may have already occurred, and the investigated population might therefore be more willing to mate under our artificial conditions than recently wild-caught flies. Since the populations kept by companies are largely highly commercialized and the population that we used originates from within the European commercialized clutch (Kaya *et al*., 2021), our findings will be relevant to most companies and institutions interested in breeding and genetically improving their *H. illucens* populations.

In conclusion, our demonstration that a selected male *H. illucens* can be mated individually with one or multiple virgin females and produce viable offspring with these shows that the reproductive biology of *H. illucens* allows for using quantitative genetic designs in breeding programs such as full-/half-sibling designs. The knowledge generated from such designs, i.e. heritabilities, genetic correlations and variance component estimates for traits of interest, are important for designing sustainable and efficient breeding programs. Using pedigree-based selection programs it is possible to control inbreeding rates, facilitate selection on multiple traits simultaneously, and sustain cumulative responses to selection across generations. However, relaxing sexual selection which is a consequence of the proposed mating scheme might have fitness consequences (Promislow *et al.*, 1998), and potential risks related to this should be investigated in future studies.

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Figures

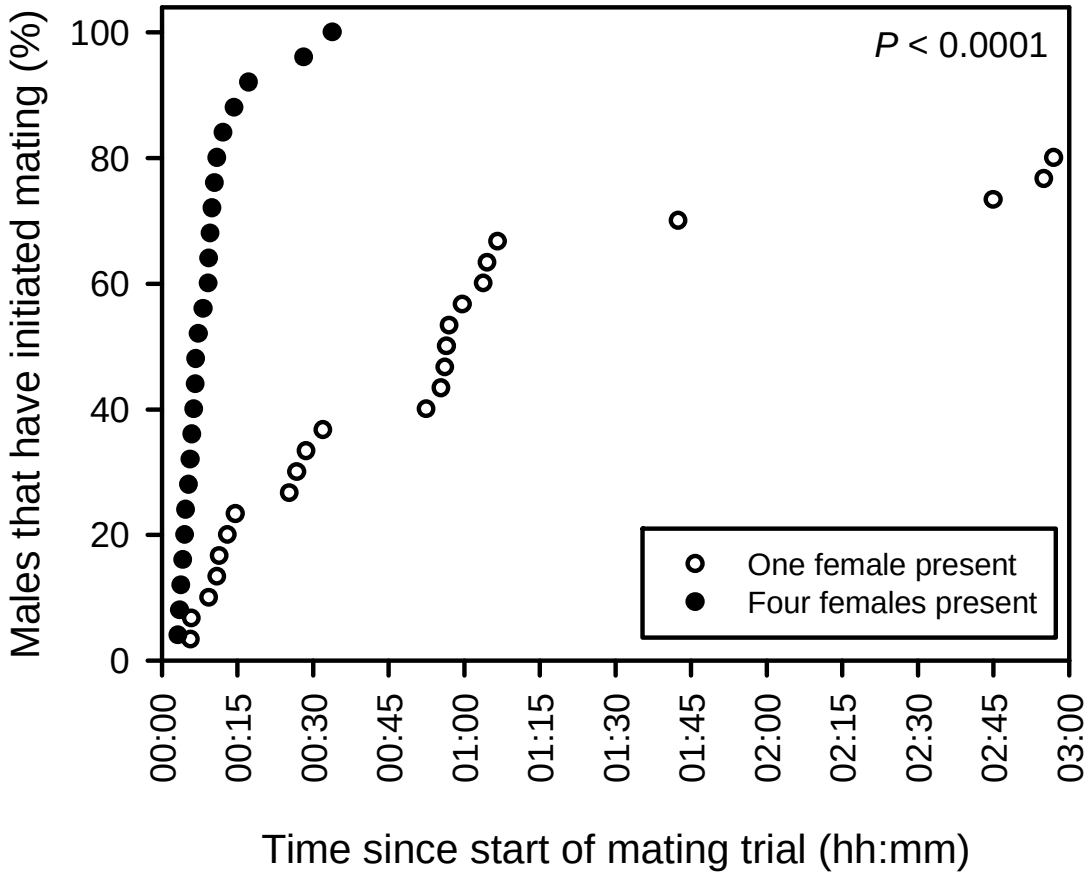

FIGURE 1. Timepoints and cumulative percentages of males initiating their first mating when given access to one (white) or four (black) females. The *P*-value is from a Wilcoxon test on time until mating depending on the number of females present, including only males that mated.

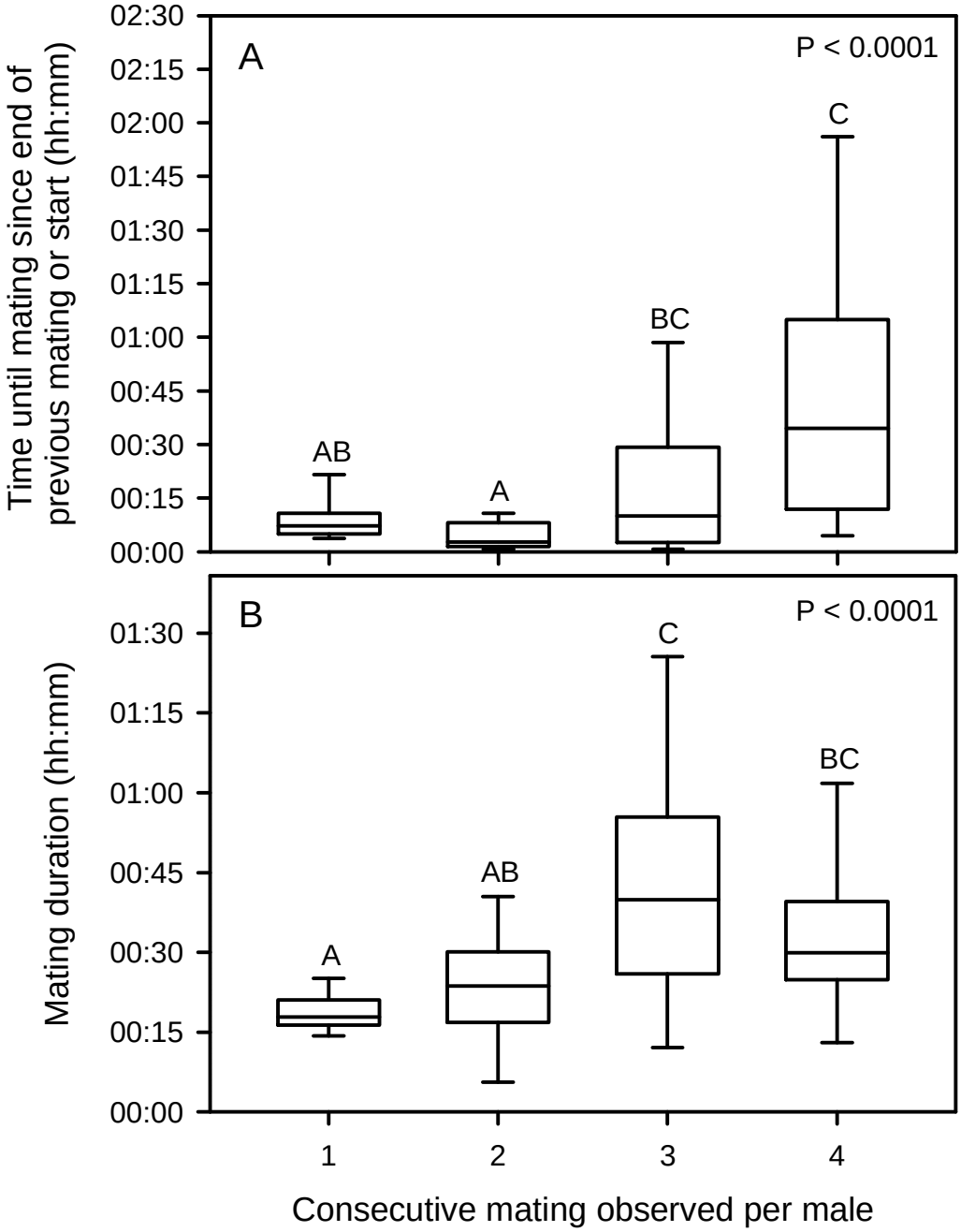

FIGURE 2. A) Time until first mating and in between subsequent matings and B) duration of each consecutive mating in males exposed to four virgin females for four hours. Boxes show median and 10th, 25th, 75th, and 90th percentiles. Outliers are not shown. The *P*-values are from Kruskal-Wallis tests across the consecutive matings. Different letters indicate significant differences (Dunn test, $\alpha = 0.05$).