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

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K. Jensen[1](https://orcid.org/0000-0003-0261-3831), S.F. Thormose[1], N.K. Noer[2](https://orcid.org/0000-0002-4430-0342), T.M. Schou[2](https://orcid.org/0000-0001-7545-7572), M. Kargo[3](https://orcid.org/0000-0003-1981-2722), A. Gligorescu[4], J.V. Nørgaard[1](https://orcid.org/0000-0002-0580-0184), L.S. Hansen[3,4](https://orcid.org/0000-0002-4270-6365), R.M. Zaalberg[3](https://orcid.org/0000-0002-2609-3458), H.M. Nielsen[3](https://orcid.org/0000-0002-8001-5629) and T.N. Kristensen[5](https://orcid.org/0000-0001-6204-8753)*

[1]Department of Animal and Veterinary Sciences, Aarhus University, 8830 Tjele, Denmark
[2]Enorm Biofactory A/S, 8762 Flemming, Denmark
[3]Center for Quantitative Genetics and Genomics, Aarhus University, 8000 Aarhus C, Denmark
[4]Department of Biology, Aarhus University, 8000 Aarhus C, Denmark
[5]Department of Chemistry and Bioscience, Aalborg University, 9220 Aalborg E, Denmark
*Correspondence: kj@anivet.au.dk

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 for the 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. This lekking behavior is described as crucial for establishing suitable conditions and achieving successful mating, and polygyny is therefore assumed to be rare. We show that when exposing a virgin male to a virgin female, they readily mate, demonstrating the ability to mate without prior lekking and that it is possible to mate selected pairs. Furthermore, we show that successful mating can be achieved between an individual male and at least four virgin females within a four-hour time span, and that nearly all matings result in live offspring. Our findings pave the way for moving *H. illucens* breeding programs beyond mass selection towards advanced selective breeding designs demanding controlled mating. Such designs enable selection for multiple traits simultaneously, are used to prevent 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 many insect species have been used as food and feed for centuries (Olivadese and Dindo, 2023). In the Western world, large-scale factories, daily producing tons of insect-derived products, have only been established recently (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. This knowledge should be considered when developing breeding programs aimed at improving traits of interest in commercial insect species using selective breeding.

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, sheep, and poultry, the effectiveness of breeding programs has relied heavily on the ability to master the reproduction of the animals, such as through artificial insemination. Another reason for the effectiveness of livestock selective breeding is the ability to keep track of pedigrees. Tracking pedigrees has been aided by efficient individual animal identification systems. Knowing the pedigree of individuals generations back in time contributes strongly to increase the precision by which genetically superior animals can be identified, and such knowledge can therefore boost 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 infancies (Jensen *et al.*, 2017). Yet, the potential for genetic improvement in insects is high given that 1) there is plenty of genetic variation within relevant species (Cai *et al.*, 2024; Eriksson and Picard, 2021; Hoffmann *et al.*, 2021), 2) they have short generation intervals spanning from weeks to a few months, and 3) 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.

The black soldier fly (*Hermetia illucens* L.; Diptera: Stratiomyidae) is reported to mate under lekking behavior (Giunti *et al.*, 2018; Julita *et al.*, 2020; Tomberlin and Sheppard, 2001), implying that many individuals of both sexes are exposed to each other. Under such circumstances, controlled matings where one specific male is mated to one specific female, or where one certain male is mated to several females and offspring are later collected, are impossible to perform. This constitutes a major challenge for developing effective selective breeding in *H. illucens* because it means that traditional and effective breeding designs such as the full-/half-sibling design (Falconer and Mackay, 1996) cannot be used. Such designs would be highly valuable for performing experiments aiming to estimate trait heritabilities, variance components, and genetic correlations between traits of interest, and when performing selective breeding in a population to improve its production traits. Recent studies attempting to genetically improve production traits in commercial insects have used mass

selection, also denoted phenotypic selection, where parents producing the next generation are mated in a mass reared environment and the pedigree structure therefore is unknown (Facchini *et al.*, 2022; Gligorescu *et al.*, 2023; Morales-Ramos *et al.*, 2019). Phenotypic selection can be very efficient and studies on *H. illucens* have shown that traits related to developmental time, feed conversion efficiency, and weight gain can be markedly changed through phenotypic selection (Facchini *et al.*, 2022). However, this type of selection does not allow for controlling inbreeding rate, 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).

Full-/half-sibling designs have been used successfully in other insect species, such as the house fly, *Musca domestica* (Boatta *et al.*, 2023; Hansen *et al.*, 2024). However, little is known about whether *H. illucens* males readily mate with several females (polygyny). It has recently been demonstrated that both male and female *H. illucens* can mate more than once in their lifetime (Chiabotto *et al.*, 2024; Jones and Tomberlin, 2021; Muraro *et al*., 2024). If controlled and polygynous mating is possible, this would enable the development of efficient pedigree-based breeding programs for this species. Furthermore, polygynous mating would create opportunities for advancing our selective breeding strategy, for example by selecting for multiple traits of economic value simultaneously (Zaalberg *et al.*, 2024).

Here, we set out to investigate 1) whether males and females will mate without prior lekking behavior, 2) whether males and females will mate when they are exposed to a restricted set of mating partners including little or no chance for mate choice, and 3) whether individually maintained males can consecutively mate multiple virgin females within a short mating session and produce full- and half-sibling offspring with these. We furthermore investigated the time frame required for a male to mate multiple females, and whether the number of offspring produced by a female is affected by the number of females the male has mated. We show that lekking is not a requirement for mating in *H. illucens* under captive rearing conditions, and that a male can mate successfully with an individual female or up to four females within two to three hours, producing viable offspring with each female. These novel 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, and 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 (27:17:6 cm L:W:H) each containing two kg of chicken feed (Paco Start, Danish Agricultural Grocery Company, Fredericia, Denmark) and 4 L of water. Roughly 1500 larvae (15 g) were added to each tray. Pupae were collected and maintained in individual plastic vials (115:23.5 mm height:diameter) secured with a foam stopper and checked daily for eclosion. Emerged adults were given 48-72h upon eclosion to sexually mature in the vial before being used in mating tests.

Experimental design

For the mating experiments, male and female flies were put together in transparent plastic containers (20:20:15 cm L:W:H) closed with a transparent lid with an 8 cm Ø hole for ventilation covered with a fine mesh. All flies were allocated to the containers by simultaneously removing all foam stoppers and shaking flies out of their rearing 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 location for mating, and a timer was started.

In a first round of experiments, we established 18 replicates of one male exposed to either one, two, three, four, or five females and given 48 h to mate under a fluorescent tube placed 50 cm above the containers and an 18:6 h light:dark cycle. In a second round of experiments, we established 30 replicates of one male exposed to one female and 25 replicates of one male exposed to 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. This light source covered the full spectrum, with a narrow peak intensity in the mid-blue range. Females were then placed in individual plastic vials held upside down with a coarse foam stopper in the bottom for egg laying, and a tray with water was placed 10 cm underneath to ensure that RH did not drop below the 70%.

An additional 247 adult virgin females were collected as pupae in individual plastic vials sealed with coarse foam stoppers and kept under the same conditions as females set up to lay eggs after mating. These females never had a chance to mate.

Trait recording

In the first round of experiments, the number of females that produced an egg clutch was recorded and egg clutches laid in the pre-weighed foam stopper were weighed. In the second round, we recorded the time until start and end of each mating, the number of females laying an egg clutch, the number of laid egg clutches where larvae hatched (evaluated by daily inspection), and the number of larvae that had hatched from each egg clutch. The larvae were counted after letting them develop on the chicken feed diet for eight days. The egg clutches were not weighed in the second round. Egg-laying was assessed daily from day one to six following mating. Cases where a female laid an egg clutch without having had a chance to mate were recorded by inspecting the vials with virgin females for egg clutches until death of the female.

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. 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 number of females laying an egg clutch and egg clutch mass across males given one to five virgin females for mating, when comparing time in between or before mating and mating duration across consecutive matings, and when testing for an effect of multiple mating on the number of larvae produced per female egg clutch. If the Kruskal-Wallis 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 (up to four) 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

In the first round of experiments, we found that the number of females from each container that laid an egg clutch increased with the number of females present in the container (Figure 1). Up to four females from the same container laid an egg clutch (Figure 1). The median mass of an egg clutch was 22.8 mg (10th percentile: 5.8, 25th: 16.7, 75th: 27.0, 90th: 30.7) and was not affected by the number of females in the container that laid eggs (Kruskal-Wallis: $\square^2_{3,85} = 1.81$, $P = 0.61$). The variation in egg clutch mass differed depending on the number of females in the container that produced an egg clutch (Levene: $F_{3,81} = 3.47$, $P = 0.020$). The variation in egg clutch mass was highest when four females produced eggs and lowest when only one female produced eggs, but with inconsistent pattern in between.

[FIGURE 1: Number of females laying an egg clutch depending on number of females present]

In the second round where either one or four virgin females were exposed to a single male, the first mating occurred considerably sooner when males were together with four females compared to when they were together with only one female (Figure 2). Across the 25 males accompanied by four virgin females the median of first mating was 7:17 minutes (10th: 3:46, 25th: 5:04, 75th: 10:46, 90th: 21:38), and 100% had initiated mating within 35 minutes (Figure 2). At this point, only 37% of males exposed to a single virgin female had initiated mating (Figure 2), and these initiated their first mating at a median of 53:58 minutes (10th: 7:40, 25th: 13:29, 75th: 01:04:24, 90th: 02:50:00). 72% of males accompanied by four females had initiated mating 10 minutes into the trial, while only 10% of males accompanied by a single female had initiated mating at this time point (Figure 2). After 15 minutes, 88% of males accompanied by four females had initiated mating, while 23% accompanied by a single female had initiated mating (Figure 2). Over the entire four-hour period, 80% of males accompanied by only a single female mated, which was a significantly lower proportion than for males accompanied by four females (Pearson: $\square^2_{1,55} = 5.61$, $P = 0.018$; Figure 2).

[FIGURE 2: Time until males initiated mating depending on number of females present]

Most males exposed to four virgin females also mated a second (96%) and a third time (88%), and just above half of the males (52%) mated four times. Two males mated five and one also six times, implying remating with a previously mated female. Males that mated a second time did so at a median of 2:43 minutes (10th: 0:48, 25th: 1:28, 75th: 8:11, 90th: 10:44), all within 30 minutes after terminating the first mating, and one within less than 30 seconds (Figure 3A). After the second mating, the median time in between matings increased significantly (Figure 3A). The variation in time until next or first mating varied significantly in a similar pattern as the time in between or until mating (Levene: $F_{3,76} = 16.85$, $P < 0.0001$; Figure 3A). 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 3B). The variation in mating duration showed a similar, significant pattern (Levene: $F_{3,76} = 10.43$, $P < 0.0001$; Figure 3B).

[FIGURE 3: Time 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: 16, 25th: 79, 75th: 210, 90th: 310) were produced from each egg clutch. For the 25 males with access to four females, only one female produced an egg clutch for two of the males (8%), two females produced an egg clutch for 11 of the males (44%), three females produced an egg clutch for nine of the males (36%), and all four females produced an egg clutch for three of the males (12%). We found a significant correlation between the number of times a male mated and the number of females producing an egg clutch (Pearson: $\chi^2_{9,25} = 18.32$, $P = 0.032$). Within females mated with males with access to four females, 58 (92%) out of the total 63 egg clutches laid by individual females produced larvae. There was no significant effect of the number of females mated by the male on clutch hatching (Pearson: $\chi^2_{3,63} = 4.73$, $P = 0.19$). The egg clutches each produced a median of 138 larvae (10th: 33, 25th: 69, 75th: 219, 90th: 269), again with no effect of the number of females mated by the male and ovipositing (Kruskal-Wallis: $\chi^2_{3,58} = 3.41$, $P = 0.33$), and with no significant difference in variance (Levene: $F_{3,54} = 1.07$, $P = 0.37$). Similarly, there was 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: $\chi^2_{1,81} = 0.007$, $P = 0.93$). There was no significant variation in the number of larvae per egg clutch depending on whether one or four females were available to a male (Levene: $F_{1,79} = 0.0002$, $P = 0.99$).

Out of the 247 virgin female flies that were maintained in their vials without a chance to mate, a total of 10 (4%) oviposited before they died after more than seven days as adults. These egg clutches were generally very small, and no larvae hatched from them.

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 three main questions of central importance for the ability to execute efficient selective breeding programs in *H. illucens*. The overall aims were to investigate: 1) whether *H. illucens* males and females will mate without prior lekking behavior, 2) whether *H. illucens* males and females will mate when they are exposed to a restricted set of mating partners including little or no chance for mate choice, and 3) 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. The answers to all three questions are affirmative; 1) *H. illucens* males and females do mate readily without prior aggregation of males in competitive displays and lekking rituals, 2) controlled mating between single mating pairs of selected individuals is possible, and 3) successful matings do occur between one male and several females, showing that *H. illucens* is polygynous. These findings are of central importance for developing pedigree-based breeding programs for *H. illucens*.

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 polygyny and polyandry in mass breeding of *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*. This breeding design was recently used in *M. domestica* (Boatta *et al.*, 2023; Hansen *et al.*, 2024), 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*.

We observed that mating is initiated faster when more females were presented to a male, indicating that the presence of more females speeds up the process of initiating mating. This may be induced by females disturbing and activating each other, facilitating flight and availability to the male. It could also be that presenting only one male to multiple females increases the chance that a present female finds the male acceptable for mating. Mating is highly dependent on female choice including a threshold for male quality (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).

Our experiments showed no evidence that male fertility declines following one or multiple matings. 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). It should be stated that we did not follow the offspring, and potential effects on offspring performance can therefore not be concluded upon.

The low egg clutch production by unmated females (4%) indicates that far the most of egg clutches laid in our first round of experiments were fertilized. It also matches the high proportion of egg clutches that produced larvae in our second round of experiments. We therefore conclude that egg clutch production is a reliable, although not perfect indicator of mating and fertilization, at least under our experimental conditions.

In conclusion, our demonstration that a selected male *H. illucens* can be mated individually, without prior lekking, with one or multiple virgin females and produce viable offspring with these showed that the reproductive biology of *H. illucens* allows for quantitative genetic designs in breeding programs. These involve full-/half-sibling designs, enabling identification of superior families under selective breeding and for estimating genetic and environmental variance components, heritabilities, and phenotypic and genetic correlations between traits. The knowledge generated from such designs, i.e. heritabilities, genetic correlations and variance component estimates for traits of interest (Falconer and Mackay, 1996), are important for designing sustainable and efficient breeding programs and for active breeding because it allows for pedigree-based selection programs that include control of inbreeding rates, facilitate selection on multiple traits simultaneously, and sustain cumulative responses to selection across generations.

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Figures

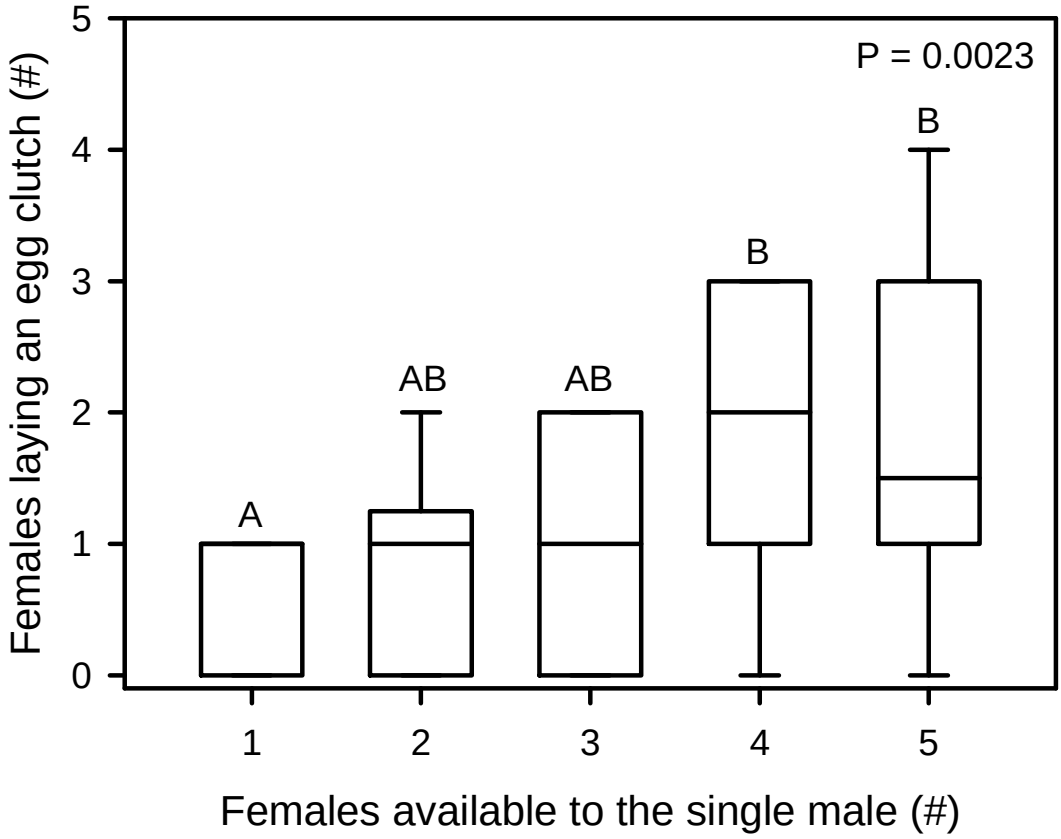

FIGURE 1. Number of females that laid an egg clutch after 48h of exposure to a single male, depending on the number of females (1 to 5) that were available to the male in the container. Boxes show median and 10th, 25th, 75th, and 90th percentiles. No outliers were present. The *P*-value is from a Kruskal-Wallis test across the number of females available. Different letters indicate significant differences (Dunn test, $\alpha = 0.05$).

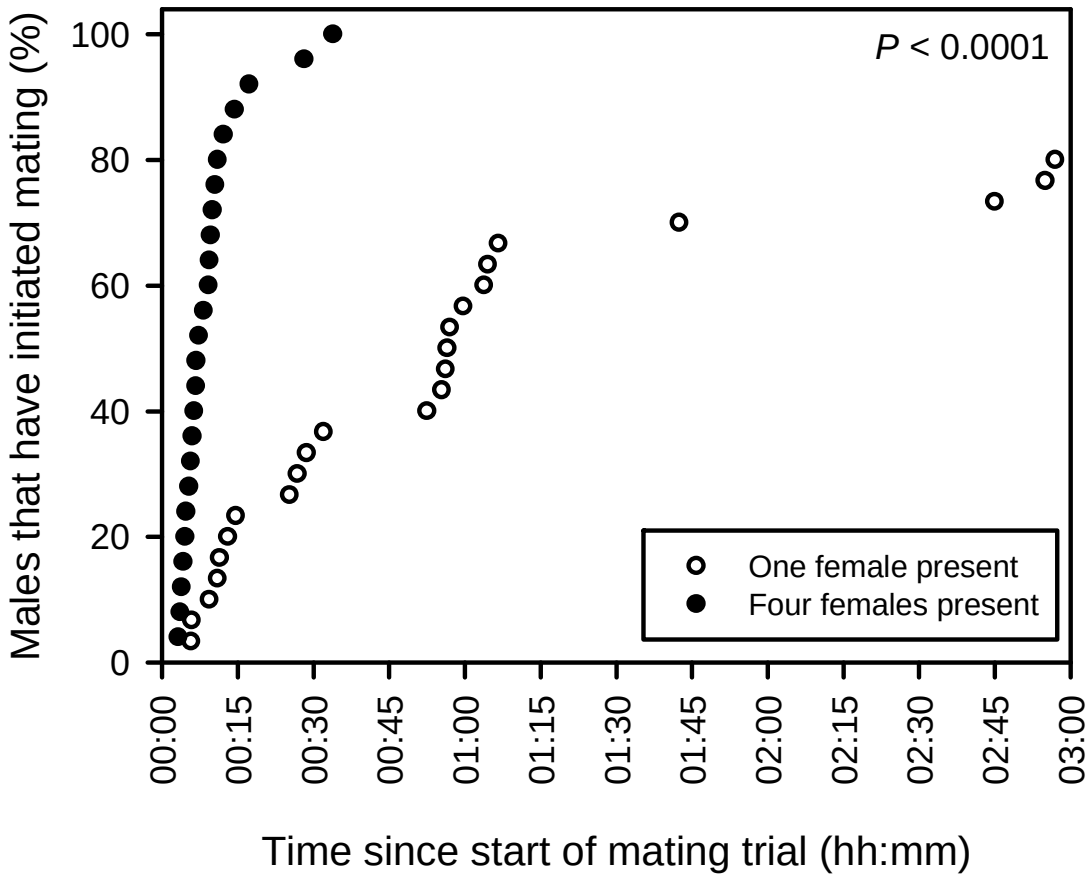

FIGURE 2. Percentage of males that had initiated their first mating over time for each individual male within the first three hours of exposure to one (white) or four (black) females in the container. The *P*-value is from a Wilcoxon test on time until mating depending on the number of females present, including only males that mated.

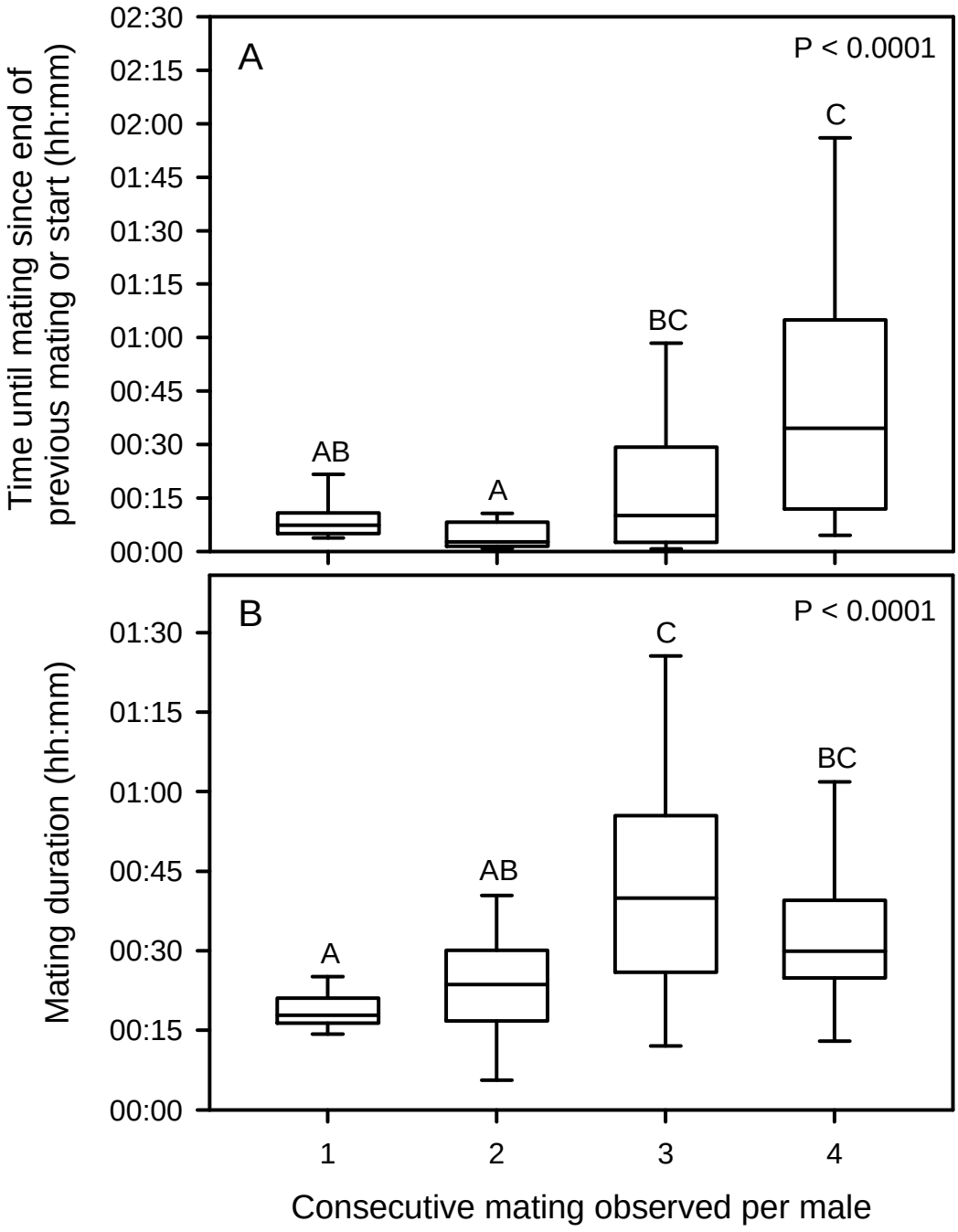

FIGURE 3. A) Time until first mating and in between subsequent matings and B) duration of each consecutive mating in males exposed to four virgin females for four hours. Boxes show median and 10th, 25th, 75th, and 90th percentiles. Outliers are not shown. The *P*-values are from Kruskal-Wallis tests across the consecutive matings. Different letters indicate significant differences (Dunn test, $\alpha = 0.05$).