Apis mellifera (Hymenoptera: Apidae) drone sperm quality in relation to age, genetic line, and time of breeding

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Abstract—A honey bee (*Apis mellifera* Linnaeus; Hymenoptera: Apidae) queen's life expectancy is strongly dependent on the number of sperm she obtains by mating with drones during nuptial flights. Unexplained replacement of queens by the colony and young queens showing sperm depletions have been reported in North America, and reduced drone fertility has been a suspected cause. The aim of this study was to evaluate drone reproductive qualities during the queen-rearing season, from May to August. Drones from two different genetic lines were reared six times during the 2012 beekeeping season at our research centre in Québec (Canada). Semen volume as well as sperm number and viability were assessed at the ages of 14, 21, and 35 days. Results showed 1) a greater proportion of older drones with semen at the tip of the genitalia after eversion; 2) an influence of rearing date on semen production; and 3) no influence of drone genetic line, age or time of breeding on sperm viability. These results highlight the necessity of better understanding drone rearing and how it can be improved to ensure optimum honey bee queen mating.

Résumé—La durée de vie de la reine de l'abeille (*Apis mellifera* Linnaeus; Hymenoptera: Apidae) est dépendante du nombre de spermatozoïdes qu'elle acquiert durant les vols nuptiaux. Des remplacements de reines ainsi que de jeunes reines ayant épuisé leurs réserves de spermatozoïdes sont rapportés en Amérique du Nord et des problèmes de fertilité chez les faux-bourdons sont suspectés. Le but de cette étude était d'évaluer les qualités reproductives du faux-bourdon durant la saison de production des reines abeilles de mai à août. Des faux-bourdons de deux lignées différentes ont été élevés à six reprises au cours de la saison apicole 2012 au Centre de recherche en sciences animales de Deschambault, Québec (Canada). Le volume de sperme, le nombre de spermatozoïdes et la viabilité ont été évalués aux âges de 14, 21 et 35 jours de vie. Les résultats montrent que 1) le volume de sperme augmente avec l'âge des faux-bourdons testés; 2) le moment de l'élevage influence la production du sperme et 3) le nombre de spermatozoïdes et la viabilité des gamètes ne semblent pas influencés par la lignée génétique, l'âge ou le moment de l'élevage. Cette étude souligne la nécessité d'en connaître davantage sur l'élevage des faux-bourdons afin d'obtenir des reines abeilles adéquatement fécondées.

Introduction

The polyandrous mating system of the European honey bee, *Apis mellifera* Linnaeus (Hymenoptera: Apidae), is unique among domesticated animals (Koeniger 1990; Tarpy and Page 2000). Young queens make 1–3 nuptial flights to a drone congregation area (Schlüns *et al.* 2005; Tarpy and Page 2000). A study by Baudry *et al.* (1998), reported the presence of thousands of drones from 238 colonies in a single day at the congregation area. On average, 14 drones mate with the queen and then die (Estoup *et al.* 1995). Once she has mated, the queen returns to the hive with approximately 80–90 million spermatozoa in her lateral oviducts (Woyke 1962). An average of 4–7 million spermatozoa received over the course of all matings reaches the spermatheca (Laidlaw and Page 1984), where they are stored until used by the queen (Roberts and Mackensen 1951; Woyke 1962). In recent years, high numbers of deficient queens, *i.e.*, with incidence of early supersedure, unexplained death, premature drone egg laying, or interruption of egg laying, have been reported worldwide (Camazine *et al.* 1998; Rhodes 2008; vanEnglesdorp and Meisner 2010). Epidemiological surveys from the United States of America and Canada have identified poor queen quality as one of the main concerns for the honey-bee industry (vanEngelsdorp *et al.* 2010, 2011; vanEngelsdorp and Meisner 2010). According to Tarpy *et al*. (2012) the quality of commercially produced queens is linked to the production of large amounts of viable brood and the mating health of a queen can be assessed by investigating how well a queen was mated. These researchers found that queens had an average of 4.37 million stored sperm in their spermathecae with an average viability of 83.7%.

Their results also showed significant variations in viable sperm in the spermatheca of commercially produced queens in the United States of America, with some queens having less than 20% live sperm. In Australia, Rhodes *et al*. (2010) investigated sperm quality of honey-bee drones and found a low number of mature drones and a relatively low average number of sperm per drone. They suggested poor drone reproductive qualities may contribute to the low number of sperm in the spermatheca of commercially-produced queens.

Multiple factors have been found to affect mating health of queens: rearing conditions or queen age at mating can affect the number of sperm migrating to the spermatheca (Cobey, 2007). Moreover, the mating success of queens has been linked to male numbers and sperm quality at the drone congregation area (Cobey 2007; Nur *et al.* 2012). Several studies have shown that drone age, rearing date and genetic origin affect semen properties (Woyke and Jasinski 1978; Locke and Peng 1993; Zaitoun *et al.* 2009). Woyke and Jasinski (1978) showed that the number of spermatozoa entering the spermatheca of inseminated queens tends to decrease as drone age increases; 4097 million spermatozoa were found in queens' spermatheca inseminated with semen from two-week old drones, compared to 3175 million in queens inseminated with semen from nine-week old drones. Zaitoun *et al*. (2009) found that colonies produced drones from February to July in the semiarid conditions of Jordan. They also found that drones reared in May (swarming period) weighed more and had the highest sperm counts, higher fertility levels (defined as the presence of semen after manual eversion of drones) as well as less sperm abnormalities than drones sampled during the rest of the year. Drone

72 genetics seem to influence most aspects of sperm production and properties (Rhodes *et al.*
73 2010), including semen volume and number of sperm produced by each drone.

74 In northern climates such as that of eastern Canada, beekeeping is characterised by a
75 long overwintering period, followed by one of the shortest active seasons for the honey bee
76 (May to September) around the world. The races commonly bred have been selected for several
77 features essential for high survival rates in this climate. To our knowledge, no information on
78 drone reproductive qualities in northern climates exists, despite the particularities of these
79 environmental conditions.

80 During summer beekeeping season of 2013, we actively sampled drones from honey-
81 bee colonies. The aim of this study was to evaluate semen quality of drones with different
82 genetic origins, at different ages and at different seasonal breeding periods. Differences in
83 semen production and sperm qualities are expected in relation to these variables. Our prediction
84 was that sperm from young drones would be of highest quality during the swarming period.

Materials and methods

Drone rearing and sampling

This study was conducted at the Centre de Recherche en Sciences Animales de Deschambault (CRSAD, Deschambault, 46°40'26.85"N, 71°54'54.39"W), Québec, Canada. Mature drones were obtained from honey-bee colonies with open-mated queens belonging to two different lines: hybrid Italian stock *n* = 4 colonies (Rustique Apiculture, Saint-Camille, Québec, Canada) and Buckfast stock *n* = 4 colonies (Keld Branstrup, Ruds Vedby, Denmark). All colonies were fed sucrose syrup 1:1 and protein supplement patties (Global Patties Inc., Airdrie, Alberta, Canada standard 15% pollen patty) during April before the beginning of drone rearing.

Six successive batches of drones were bred during the 2012 beekeeping season (1 and 14 May, 12 and 20 June, 18 and 28 July). Drones were obtained by isolating each queen for 48 hours within a queen exclusion cage which allowed nurse bees to feed the queen freely. Each cage held a drone brood cell frame placed in the centre of the brood chamber. After this period, queens were freed from the excluder cage and drone broods were removed and replaced in the centre of the brood chamber. Upon emergence, 300 young drones were marked with a water-based Uni Poscapen (Mitsubishi Pencil Co. Ltd., Tokyo, Japan)on their upper thorax and returned to their respective colonies. Different colours were used to distinguish different drone cohorts within each colony. Marked drones were released in the honey super and prevented from leaving their colony by placing a queen excluder between the brood chamber and the honey

105 super, which allowed workers to move freely between the two, while confining drones to the
106 honey super.

107 Thirty marked drones were collected at 14, 21, and 35 days after emergence for semen
108 analysis. When captured, drones were kept alive in a flight cage with young nurse bees and a
109 small incandescent light until evaluation (within one hour). In this cage, drones were able to fly
110 and defecate, which helps initiate semen ejaculation (Collins 2004).

111

112 Semen volume

113 Semen collection was accomplished by manual eversion of sexual organs as described
114 by Woyke (2008). We selected this technique because it has been used in several studies
115 assessing properties of drone semen (Collins and Donoghue 1999; Rhodes *et al*. 2010; Gençer
116 et Kahya 2011; Nur *et al*. 2012) and is also commonly practiced for semen collection in
117 instrumental insemination of honey-bee queens (Harbo 1985; Mackensen and Tucker 1970).

118 An initial vertical pressure on the head of the drone with the thumb and index finger
119 produced partial eversion of the endophallus. Subsequent horizontal pressure from the anterior
120 to the posterior of the abdomen resulted in full eversion of the genitals. In mature drones, semen
121 is cream-coloured and found at the tip of the genitalia on a bed of white mucus. Using a Harbo
122 Large Capacity Syringe (GS 1100, Fisher Scientific, Ottawa, Ontario, Canada), semen was
123 collected from five drones in each colony for each combination of drone breeding date and age.
124 Semen was collected only from drones producing at least 0.2 μL (the minimum amount required

for the syringe) and volume was recorded to the nearest 0.1 μL. Care was taken to avoid mucus collection. Semen from each drone was stored in a separate glass capillary tube until sperm quality analysis could be performed later on the same day; *i.e.*, within 12 hours of collection.

Sperm count

Sperm count was conducted using a Neubauer Improved Haemacytometer, BS.748 (Hawksley Technology, Lancing, United Kingdom, depth 0.1 mm, 1/400 mm^2). A semen volume of 0.2 μL was diluted in 1.5 mL Tris buffer in a sterile Eppendorf and gently mixed by inversion (dilution factor = 7500). Sperm were counted in five squares (0.1 mm^3 = 0.1 μL) at the four corners and centre of each end of the haemocytometer, and counts were repeated three times (with new slide preparations) under a light microscope at 400x magnification for a total number of sperm in 15 squares for each drone. To obtain sperm numbers per drone, the following formula was applied:

Sperm cells per drone = (*n* sperm cells in 15 squares x dilution factor 7500 x semen volume) \ 1.5

Sperm viability

Sperm viability was assessed the day of semen collection using a Live/Dead Sperm Viability Kit (L-7011, Life Technology Inc., Burlington, Ontario, Canada and a modified version of the method used by Collins and Donoghue (1999). For each drone pool, we used 0.2 μL of

144 semen for the sperm count; the rest of the semen was diluted for the viability test in an individual
145 Eppendorf containing 40 μL Tris buffer and mixed gently. After allowing SYBR-14 and
146 propidium iodide to thaw and come to room temperature, 1.5 μL of SYBR-14 was added to the
147 semen dilution, mixed and allowed to stain 10 minutes. An additional 1.5 μL was added, mixed
148 and allowed to stain 10 more minutes. In a separate analysis of each individual semen sample,
149 1.5 μL of stained semen solution was diluted in 1.5 μL Tris buffer and mixed gently. A drop of
150 this solution was placed on a slide preparation and sperm viability was assessed using a Zeiss
151 Observer Z1 microscope equipped with fluorescence filters. For each slide, five different fields
152 of view were observed (200x), photographed and saved. Five slides of coloured semen were
153 prepared per drone. Each spermatozoid was scored as either alive (green), or, if sperm had lost
154 membrane integrity, dead (red). For each drone, the mean viability percentage was obtained
155 from the five slides.

156

157 Statistical analysis

158 The probabilities of semen presence after manual eversion were compared across
159 drone genetic lines, breeding dates and drone ages using a mixed logit model with repeated
160 measures. Measurements were taken from drones across all breeding dates and drone ages
161 in each colony, the latter being the experimental units for drone genetic lines. We integrated
162 this variability between colonies by using data on individual drones instead of means data for
163 each colony, and added a 4^{th} error term in the model (four error terms: line, rearing date, age

and sampling error, which integrate the variability of pseudo repetitions). Values of semen volume, sperm count, and sperm viability were analysed using a mixed Ancova model with the same repeated measurements. For each response variable, the best transformation was chosen among the Box-Cox family to meet the assumptions of the model. In the particular case of sperm viability, angular transformation was chosen, the recommended transformation for percentage variables (Sokal and Rohlf 1995).

When a significant effect was found in any analysis, multiple comparisons were performed using the protected Fisher's least significant difference (LSD) method. The normality assumption was tested using the Shapiro-Wilk's statistic, while the homogeneity of variances was verified using traditional residuals plots. All analyses were performed at the 0.05 level of significance, and models were adjusted to data using SAS software (release 9.4; SAS Institute Inc., Cary, North Carolina, United States of America,) via the Glimmix and Mixed procedures.

Results

Drone rearing

Drones were bred in six batches for each colony between 1 May 1 and 28 July 2012. Since drones from the 28 July cohort reached the age of 14 days in September, when colonies expulse drones in preparation for overwintering, too few were available for evaluation, and the cohort was excluded from statistical analyses. Also, a colony in the hybrid Italian stock (Québec line) died at the beginning of the experiment. Initial analysis showed no significant difference between genetic lines for all variables, thus colonies from both lines were pooled for added statistical strength. While the experiment had a total of 105 treatment combinations (7 colonies x 5 breeding dates x 3 drone ages), observations were only available for 72 of them, mainly due to poor rearing success in the first rearing cohort of both genetic lines. Several variance components of the model that were estimated to 0 were removed from the model. Because of the missing data pattern and problems with estimation of some variance components, degrees of freedom were estimated using the Kenward-Roger method (Kenward and Roger 1997).

Manual eversion

We sampled and manually everted 472 marked drones from May to August 2012. Of these drones, 55.3% produced a sufficient amount of semen to be collected with the Gilmont syringe and were used for semen evaluation (Table 1). Statistical analysis showed that the proportion of drones releasing semen after manual eversion was dependent on drone age ($F_{2,450}$

197 =3.94; P = 0.020; Fig. 1). The interaction between drone age effect and breeding date effect
198 was non-significant ($F_{8,450}$= 1.93; P = 0.054). Multiple comparisons made to evaluate the
199 significant effect of drone age showed that fewer 14-day old drones produced at least 0.2 μL of
200 semen (63.5 ± 8.5%) than 35-day old drones (87.8 ± 6.2%).

201

202 Semen volume

203 For drones that released semen after manual eversion (n = 261 or 55.3% of total drones
204 sampled), the mean semen volume was 1.01 ± 0.03 μL, ranging from 0.4–2.4 μL. Results
205 showed that semen volume was influenced by the combined effect of drone age and breeding
206 date ($F_{8,15.7}$ = 2.97; P = 0.031; Fig. 2). At 14 and 35 days old, there was no difference in semen
207 volume throughout the drone rearing season ($F_{4,21}$ = 0.44; P = 0.776 and $F_{4,15.1}$ = 2.25; P = 0.112
208 respectively).

209

210 Sperm count

211 Of the total number of drones with semen after eversion (n = 261), 177 produced at
212 least 0.2 μL of semen. These drones had an average total sperm count of 1.80 ± 1.65 million
213 (range 0.008–7.77) spermatozoa. There was no significant effect of any source of variation on
214 sperm count (Table 2.)

215

216 Sperm viability

217 The mean percentage of sperm viability was 64.2 ± 1.07 % (range 36.79–86.66). There
218 was no observable effect of drone line ($F_{1,128}$ = 0.49; P = 0.485), breeding date ($F_{4,128}$ = 0.43;
219 P = 0.788) or drone age ($F_{2,128}$ = 0.31; P = 0.736) on sperm viability (Table 2).

220

Discussion

The purpose of this study was to assess the drone semen quality of different genetic lines, at different ages and reared at several periods during the beekeeping season in Québec, Canada. Our data showed that age, and rearing date have an impact on semen volume but not on sperm count or viability. One key finding of this study is that a large proportion of mature drones failed to release semen by manual eversion, as previously observed by Rhodes *et al.* 2010.

We did not find differences in measured variables between the two genetic lines. Rhodes *et al*. (2010) examined the influence of genetics on semen production in the honey bee and found differences in semen volume between lines. They were even able to identify a line of drones producing higher volumes of semen. This observation is interesting for honey-bee breeding because adequate queen fecundation is dependent on the volume of semen produced by each drone, and migration of sperm into the spermatheca is dose dependent (Cobey 2007). Furthermore, a drone's number of descendants is maximised by semen production (Schlüns *et al*. 2003). The four major Québec queen breeders maintain between four and six different maternal lines and there is an unknown number of lines coming from queen imports from Hawaii, New Zealand, Chile, and California. We selected only two honey-bee lines available from among those in our breeding program (10–14), and we recommend that more research be conducted on a wider range of honey-bee lines in order to verify whether honey bees could be bred for higher drone fertility.

241 Sexual maturation of drones is attained at the age at which sperm has completed
242 migration from the testes to the seminal vesicles and when mucus glands are fully developed
243 (Rhodes 2008). In our study, the higher proportion of 35-day old drones with semen after manual
244 eversion compared to 14-day-old drones (respectively 87% and 64%) shows that not all 14-day-
245 old drones can expulse semen after manual eversion. According to several authors who used
246 this technique, drones aged 10–21 days are mature, and semen properties are most suitable
247 for queen insemination (Woyke and Jasinsky 1978; Harbo and Williams 1987). Nevertheless,
248 our findings indicate that a large proportion of young drones do not release semen through
249 manual eversion. The efficiency of the technique in the case of young drones requires further
250 investigation, particularly in commercial queen breeding open-mating areas, where there is a
251 greater natural variety of drones.

252 In-hive drone containment and its impact on semen expulsion also require further
253 investigation. In our study, only a small proportion of marked drones survived 35 days in
254 confined hives (from 0 to 2.82%), as observed by Rhodes *et al.* (2010). Under field conditions,
255 the estimated life span of *Apis mellifera* drones is 20–40 days (Page and Peng 2001). The
256 confinement of drones during the rearing period could have reduced drone survival through
257 flight deprivation and absence of defecation during their development as suggested by Laidlaw
258 (1979). In our study, drones were able to fly and defecate only when removed from rearing
259 colonies and placed within the flight cage. Due to the cumulative effect of these disturbances,
260 only a few of the 35-day-old drones were available for semen analysis.

We found that only a low proportion of drones, 53%, produced the 0.2 µL of semen required for collection and analysis. Rhodes *et al.* (2010) and others (Collins and Pettis 2001; Andersen 2004) found a similarly low proportion of drones (40.6%) aged 14–35 days with sufficient semen production for analysis, and expressed doubts about the capability of these drones to mate with a queen in nature. Woyke (2008) studied manual eversion and suggested that the pressure inside the partly everted endophallus is probably too weak to provoke further eversion and release of semen for drones arrested at the partly everted stage but did not find drones showing a total eversion without releasing semen. This phenomenon requires further investigation to determine whether drones not releasing semen after manual eversion are able to mate with the queen, and if so, whether semen is transferred into the oviducts. The effect of age on proportion of drones with semen may also be due to a bias in sampling. Only a few drones survived to 35 days, and we could expect that these were the fittest individuals. The higher proportion found with semen may be due to the fact that the strong individuals who survived longer may also be those with the highest proportion having semen.

The mean sperm count we measured (1.80 ± 1.65 million) was highly variable and within the range obtained in several previous studies (Andersen 2004; Koeniger *et al.* 2005; Rhodes *et al.* 2010; Gençer and Kahya 2011; Nur *et al.* 2012). Because of this variance, a higher number of sampled drones and a second year of data could have provided us with more accurate information on the influence of drone age on sperm number.

Based on our findings, the proportion of drones with semen after manual eversion seems to fluctuate during the beekeeping season in eastern Canada. This variability has also

282 been observed in Australia (Rhodes *et al.* 2004) and in Jordan (Zaitoun *et al.* 2009). We found
283 lower proportions of drones with semen in early May and mid-June (respectively 43% and 72%).
284 Interestingly, semen volume was also lowest at these two breeding dates. We speculate that
285 changes in the active season of semen production might be explained by the same factors
286 affecting drone production. A honey-bee colony regulates male production throughout the
287 growing season according to environmental factors such as colony size and food availability
288 (Lee and Winston 1987; McNally and Schneider 1994; Boes 2010). Because drones are more
289 costly for the colony to produce than workers (Seeley 2002; Hrassnigg and Crailsheim 2005),
290 colonies reduce drone production during a period in which limited resources are available in
291 their environment (Rowland and McLellan 1987). Honey bees rarely face a total dearth of pollen
292 in their environment, but are rather confronted with variability of pollen resources, abundance,
293 type and diversity across both time and space (Di Pascale *et al*. 2013). In early May in Québec,
294 honey bees can benefit from several blooming shrubs and trees, as well as a few indigenous
295 wild plant sources of pollen and nectar, including the ubiquitous dandelion (*Taraxacum* Wiggers,
296 Asteraceae). However, the pollen and nectar of the majority of melliferous plants are available
297 only in June and July in Québec (Chabot 1948), and only 32 of the 143 main melliferous species
298 flower during May, compared to 64 in June and 88 in July. None of the crops considered
299 important melliferous plants for the honey bee, such as alfalfa (*Medicago sativa* Linnaeus,
300 Fabaceae), white clover (*Trifolium repens* Linnaeus, Fabaceae) or buckwheat (Fagopyrum
301 Miller, Polygonaceae), flower in May. Resource availability for honey-bee colonies could have
302 influenced drone's production of semen during the season, and further research on dietary
303 effects on drones is needed. In this regard, it is important to note that we encountered great

304 difficulty rearing drones in early spring and late summer. Apparently, even if drone production
305 is forced by caging a queen in a drone frame, workers will not rear drones when there is a
306 shortage of resources, especially pollen, which can lead workers to cannibalise the brood
307 (Schmickl and Crailsheim 2001), a phenomenon we observed with drones from the first
308 breeding date (1 May).

309 Results obtained for sperm viability are of concern due to the generally low rates found
310 (mean 64.2 ± 1.07%) throughout the active season. Previous studies on drone sperm viability
311 have shown means ranging between 70 and 90% (Locke and Peng 1993; Rhodes 2008; Nur *et*
312 *al.* 2012). According to Collins (2000), queens inseminated with semen with 42.5% sperm
313 viability resulted in reduced numbers of worker brood. Many factors could have contributed to
314 reduced sperm viability: drone semen is sensitive to bacterial infection (Andere et al. 2011), and
315 contamination can significantly reduce its viability (Locke and Peng 1993). Collins (2004) also
316 found that the act of collecting semen in a syringe can injure sperm and reduce its viability.
317 Sturüp *et al*. (2013) found that drones exposed to a slight temperature increase from 35–39 °C
318 resulted in reduced sperm viability. Even semen samples evaluated shortly after collection may
319 have been damaged or contaminated, thereby reducing viability. The possible impact of the
320 generalized poor sperm viability observed here on queen fertility problems identified by the
321 industry also requires closer investigation.

322 In conclusion, this study demonstrated that semen volume of drone honey bees in
323 eastern Canada fluctuates during the short beekeeping season, and that drones reared in spring
324 (May) show the lowest fertility levels. We recommend that queen breeders test drones for

325 semen quality, to ensure mating with the highest possible reproduction levels at commercial
326 queen bee mating apiaries.

327

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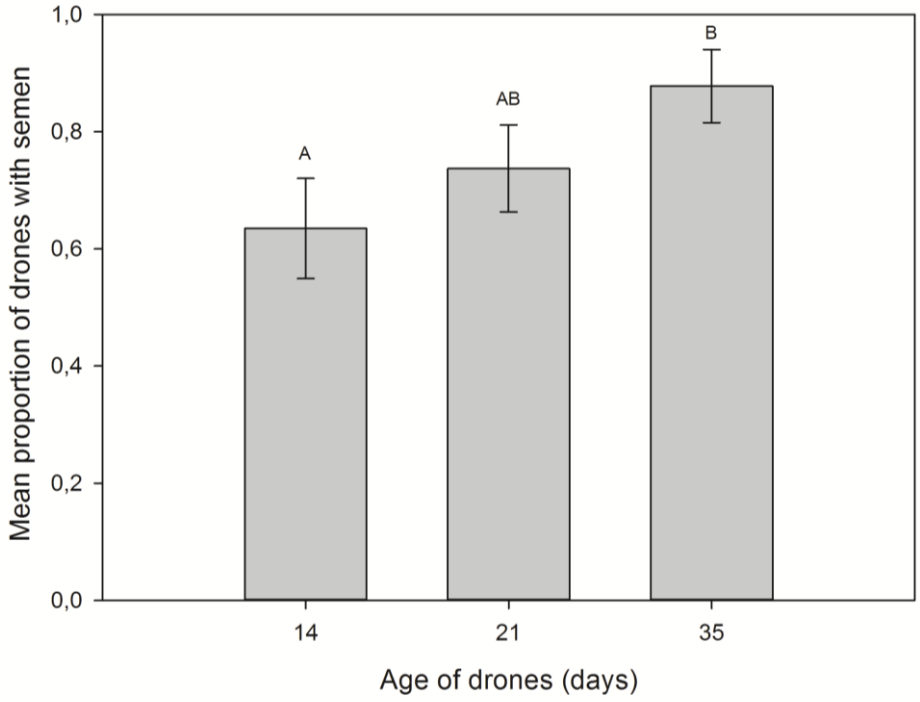

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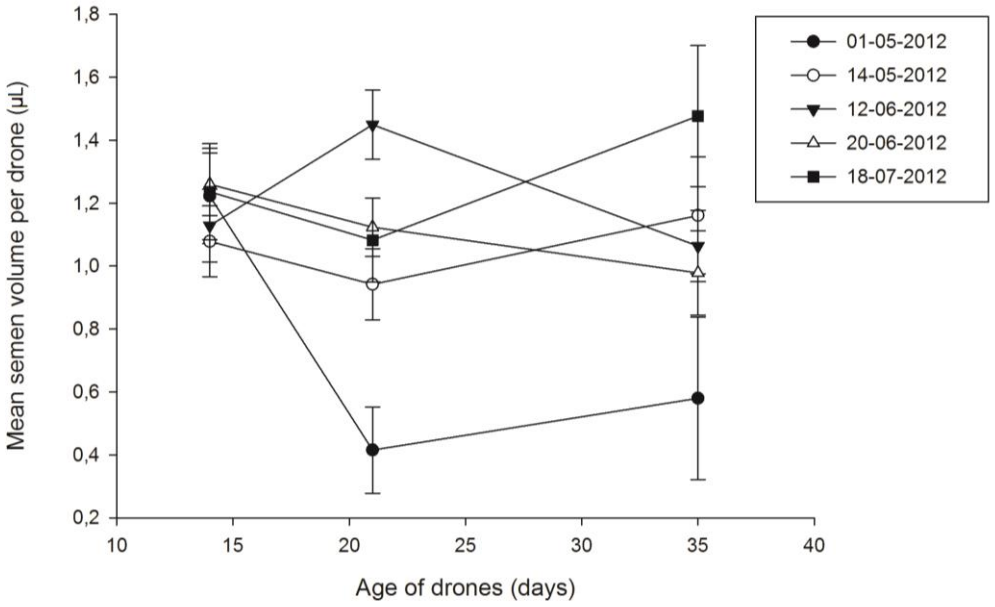
475 Fig. 1. Mean proportion of drones with semen at the tip of the endophallus after manual

476 eversion for the three ages (in days) (± se). Different letters indicate significant difference (*F*

477 2,450 = 3.94; *P* = 0.020).



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479 Fig. 2. Mean semen volume per drone (µL) for the three different ages (in days) and five

480 rearing dates (± se) (F 8,15.7) = 2.97; P = 0.031).

Table 1. Number of drones marked at emergence and proportion of drones with semen after manual eversion at various ages.

Rearing date	Number of drones marked at emergence	Proportion of drones with semen after manual eversion			Number of marked drones surviving to 35 days
		14	21	35	
1 May	830	21/85 (24.7%)	25/84 (29.8%)	5/9 (55.6%)	9/830 (1.08%)
14 May	597	21/23 (91.2%)	19/29 (65.5%)	13/14 (92.9%)	14/597 (2.35%)
12 June	900	22/38 (57.9%)	17/27 (63.0%)	19/24 (79.2%)	24/900 (2.67%)
20 June	972	26/51 (51.0%)	33/38 (86.8%)	10/12 (83.3%)	12/972 (1.23%)
18 July	355	10/15 (66.7%)	11/13 (84.6%)	9/10 (90.0%)	10/355 (2.82%)
Total of drones with semen after manual eversion: 261/472 (55.3%)					

Table 2. Results of the single factor ANOVA with repeated measurements for proportion of drones with semen, volume of semen, sperm count per drone, and sperm viability.

Effect	Proportion of drones with semen			Volume of semen			Number of sperm			Sperm viability		
	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Drone Line	1, 9.4	0.10	0.7595	1, 23.5	0.13	0.7197	1, 5.8	0.30	0.6060	1, 128	0.49	0.4850
Rearing date	4, 8.4	1.71	0.2359	4, 18.1	3.80	0.0206	4, 73.6	1.24	0.3010	4, 128	0.43	0.7883
Drone line*Rearing date	4, 7.1	0.24	0.9041	4, 18.2	1.49	0.2467	4, 55.4	0.46	0.7659	4, 128	1.18	0.3217
Drone age	2, 450	3.94	0.0202	2, 16.5	2.89	0.0839	2, 153	0.64	0.5275	2, 128	0.31	0.7364
Drone line* Drone age	2, 450	0.73	0.4835	2, 15	4.13	0.0371	2, 152	0.67	0.5128	2, 128	0.59	0.5567
Rearing date* Drone age	8, 450	1.93	0.0535	8, 15.7	2.97	0.0313	8, 152	1.76	0.1814	6, 128	0.89	0.5059