# Variation in the size of honey bee males leads to different life history characteristics consistent with distinct mating strategy (#69813)



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# Variation in the size of honey bee males leads to different life history characteristics consistent with distinct mating strategy

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Background Honey bee males (drones) exhibit life histories that enable a high potential for pre- or post-copulatory sperm competition. With a numerical sex ratio of ~10,000 drones for every queen, they patrol flyways and congregate aerially to mate on the wing. However, colonies and in fact drones themselves may benefit from a relative lack of competition, as queens are highly polyandrous because their colonies have an adaptive advantage when headed by queens that are multiply mated. Previous research has shown that larger drones are more likely to be found at drone congregation areas, more likely to mate successfully, and obtain a higher paternity share. However, the reproductive quality and size of drones varies widely within and among colonies, suggesting adaptive maintenance of drone quality variation at different levels of selection. Methods We collected drones from 6 colony sources over the course of 5 days. We paint-marked and individually-tagged drones after taking body measurements at emergence and then placed the drones in one of two foster colonies. Using an entrance cage, we collected drones daily as they attempted flight. We collected information on 2,360 drones, collecting emergence data on 207 drones and dissected 565 upon capture to assess reproductive maturity. We measured drone body mass, head width, thorax width at emergence and upon dissection, and further measured thorax mass, seminal vesicle length, mucus gland length, sperm count, and sperm viability from the seminal vesicles. Results We found that drones that were more massive at emergence were larger and more fecund upon capture, suggesting that they are of higher reproductive quality and therefore do not exhibit a trade-off between size and fecundity. However, smaller drones tended to initiate flight at a younger age, which suggests a trade-off not with fecundity but rather between size and developmental maturation. We conclude that smaller drones may take more mating flights, each individually with a lower chance of success but thereby increasing their overall fitness. In doing so, the temporal spread of mating attempts of a single generation of drones within a given colony increases colony-level chances mating with nearby queens,

suggesting an adaptive rationale for high variation among drone reproductive quality within colonies.

# Title

Variation in the size of honey bee males leads to different life history characteristics consistent with distinct mating strategy

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# Abstract

## Background

Honey bee males (drones) exhibit life histories that enable a high potential for pre- or post-copulatory sperm competition. With a numerical sex ratio of ~10,000 drones for every queen, they patrol flyways and congregate aerially to mate on the wing. However, colonies and in fact drones themselves may benefit from a relative lack of competition, as queens are highly polyandrous because their colonies have an adaptive advantage when headed by queens that are multiply mated. Previous research has shown that larger drones are more likely to be found at drone congregation areas, more likely to mate successfully, and obtain a higher paternity share. However, the reproductive quality and size of drones varies widely within and among colonies, suggesting adaptive maintenance of drone quality variation at different levels of selection.

## Methods

We collected drones from colony sources over the course of 5 days. We paint-marked and individually-tagged drones after taking body measurements at emergence and then placed the drones in one of two foster colonies. Using an entrance cage, we collected drones daily as they attempted flight. We collected information on 2,360 drones, collecting emergence data on 207 drones and dissected 565 upon capture to assess reproductive maturity. We measured drone body mass, head width, thorax width at emergence and upon dissection, and further measured thorax mass, seminal vesicle length, mucus gland length, sperm count, and sperm viability from the seminal vesicles.

## Results

We found that drones that were more massive at emergence were larger and more fecund upon capture, suggesting that they are of higher reproductive quality and therefore do not exhibit a trade-off between size and fecundity. However, smaller drones tended to initiate flight at a younger age, which suggests a trade-off not with fecundity but rather between size and developmental maturation. We conclude that smaller drones may take more mating flights, each individually with a lower chance of success but thereby increasing their overall fitness. In doing so, the temporal spread of mating attempts of a single generation of drones within a given colony increases colony-level chances mating with nearby queens, suggesting an adaptive rationale for high variation among drone reproductive quality within colonies.

# Introduction

Like their sister workers and queens, honey bee (*Apis mellifera* L.) drones have little control over their rearing conditions, with colony-level factors such as population, resource availability, and season all impacting the likelihood for a colony to rear drones or reject them (Boes, 2010; Seeley & Mikheyev, 2003; Smith et al., 2014). For adult drones, fecundity measures (e.g., sperm count and viability) and size measures (e.g., wing length, body mass) are almost always positively correlated (*reviewed in* Metz et al., 201[illegible] Further, body size is a major determinant of mating success, with larger drones tending to be more successful at mating, delivering more spermatozoa, and obtaining a higher proportion of worker paternity (Couvillon et al., 2010). It would therefore seem that larger drones are of higher reproductive quality, which would suggest directional selection for large drones and therefore low variation among males within a colony. This, then, raises the question: *why do drones vary widely in their size and reproductive quality?*

Early examination into the size variation of drones explored the potential for honey bee workers to lay male eggs. As a haplodiploid organism, unmated individuals may still realize direct fitness gains through the production of sons, despite their smaller size (Gençer & Kahya, 2011). Worker production of males varies widely among social insects, scaling with higher intracolony relatedness (*reviewed in* Wenseleers & Ratnieks, 2006). However, in the highly polyandrous honey bees (Estoup et al., 1994; Withrow & Tarpy, 2018), worker-laid drones represent only a tiny fraction of those produced (Visscher, 1989), likely owing to policing of worker reproduction (Pirk et al., 2004).

Another possibility is queen error; that is, mother queens mistakenly laying drone eggs into worker cells. However, genetic analyses of queen-laid eggs showed that under normal circumstances, the rate of a queen laying unfertilized eggs into a worker cell is extremely low

(Ratnieks & Keller, 1998). Much study into the consequences of size variation in drones has made use of the inherent constraints of honey comb by forcing queens to lay drone eggs into worker cells (Gençer & Firatli, 2005), mimicking these conditions. However, we have previously found that even with queen-laid drone eggs in drone cells, drones vary in size and reproductive quality to the exent found from these manipulations (Metz et al., 2019). As such, honey bees may be sloppy nursemaids. Variation in nurse attention to worker larvae is high and has potential consequences for their fates as adults, leaving them less likely to be selected for queen rearing (Sagili et al., 2018) or less effective foragers (Scofield & Mattila, 2015). To our knowledge nursing variation towards drones has not been observed, thus this proximal cause of drone variation cannot be ruled out.

Drone quality may vary as a function of being haplodiploid individuals more susceptible to colony stresses. Drone reproduction, survival, or maturation are negatively impacted by a host of factors, such as season, temperature stress, pollen deprivation and beekeeper-applied acaricides or farmer-applied insecticides (*reviewed in* Rangel & Fisher, 2019). Further, drones may be *more* susceptible to pesticide (Friedli et al., 2020) or pathogen challenge (Retschnig et al., 2014) than workers, consistent with the haploid susceptibility hypothesis. However, contradictory evidence may suggest that the rather protected adult environment of honey bee drones may have more to do with this than their genetic structure (Cappa et al., 2015). Nonetheless, because of their haploid genetic makeup, drone phenotypes are likely to be constrained by genetic architecture contributing to the worker and queen phenotype; stochasticity in drone development may very well be a consequence of pleiotropy in the queen-worker dichotomy (Rueppell et al., 2006). In familial-social groups with high inclusive fitness, there is potential for conflict between individual and colony-level selection such that traits promoting the

fitness of the colony may come at a cost of those promoting individual fitness or vice versa (Ratnieks & Reeve, 1992; Reeve & Keller, 1999).

While the reproductive variation in the drones that a colony produces may be an incidental consequence of gene-by-environment interactions, there are a few adaptive consequences that might be considered. Drones of varying sizes may represent different colony-level reproductive strategies, favoring the production of more, smaller drones, rather than fewer, larger ones. As such, it is plausible that variation in honey bee drones in a superorganismal parallel to the equivocal intra-ejaculate trade-offs of individuals, but this possibility has not been fully explored (Decanini et al., 2013). In this case, we would expect to see that intercolony variation in drone size would covary with the number of drones reared, something that has yet to be tested. Intracolonial variation of drones may also be adaptive if drones of various size perform different individual-level sexual strategies. The influence of size on male sexual strategy is widespread among animals (Shuster, 2010) with males having less direct competitive ability taking advantage of alternate strategies (*e.g.* Gross, 1996; Nason & Kelly, 2020 *and references therein*).

In an example from solitary bees, Eastern carpenter bee *Xylocopa virginica* males either defend a territory or patroll areas around other males' territories as interlopers (Barrows, 1983) and the likelihood to perform either behavior is based at least partially on size (Duff, 2018). This is simlarly true for the wool-carder bee *Anthidium manicatum* (Severinghaus et al., 1981). *Centris pallida* males alternatively dig in the ground for female emergence sites when larger or patrol the vegetation for females when smaller (Alcock, 2013). Finally, in a stingless bee *Scaptotrigona* aff. *depilis*, smaller individuals remained in mating aggregations for longer periods of time (Koffler et al., 2016). Honey bee drones are produced in extreme numbers as

compared to queens (Page & Metcalf, 1984), with an approximate 11,000:1 mating ratio found at drone congregation areas (N. Koeniger et al., 2005). However, mating takes place without obvious aggression, with drones assembling in a "comet" behind the queen, with one after another darting forward to mate and drones preferring queens that have already been mated (Gries & Koeniger, 1996; G. Koeniger, 1990). Drones reared from worker cells are more likely to fly outside of the times of peak mating activity compared to normal-sized males (Couvillon et al., 2010) suggesting the possibility of different individual-level mating strategy, although this is contradicted by earlier work by Berg et al., (1997)**.** However, smaller drones are less likely to be found at DCAs (Berg, 1991), and there is conflict over whether drone congregation areas are truly sites of mating in a natural setting or are instead convenient location for researchers to find drones (Loper et al., 1992). This may point to drones of varying quality exhibiting differential mating strategies.

When randomly sampling from a flight-restricted population of drones, we found that older drones tended to be smaller, which would suggest that smaller drones live longer (Metz & Tarpy, 2019); but see Czekońska et al. (2018) for a potentially contradictory result). Drones begin taking mating flights approximately 11 days after adult emergence, although drones that initiate flight at younger ages tend to make *more* flights over the course of their lives (Rueppell et al., 2005). However, despite a wealth of information on variation in flight ontogeny in honey bee females (Rueppell et al., 2004) and evidence that drones utilize similar physiological mechanisms (Giray & Robinson, 1996), there is, to our knowledge, no study directly assessing variation in flight initiation of drones as it relates to body size or reproductive measures. Herein, we explore the variation in flight and reproductive ontogeny in drones with the hypothesis that small drones may initiate flight at earlier ages, therefore making potentially more flights,

balancing the relatively lower success chances of any individual attempt with the maximization of mating attempts.

# Materials & Methods

## Drone rearing

A single standard-deep frame (243 mm x 480 mm) of emerging drones was collected from each of six colony sources headed by "European" queens (i.e., the commercially available American stock broadly defined here as a mixture of the subspecies *Apis mellifera ligustica* Spinola*, Apis mellifera caucasia Pollmann, and Apis mellifera carnica* Pollmann, selected and interbred for commercially desirable characteristics) at the Lake Wheeler Honey Bee Research Facility in Raleigh, NC. Queens were open-mated with uncontrolled local populations, and colonies were maintained by standard beekeeping practice prior to experimentation. Drone eggs were laid by the queens into selected and measured frames of drone-sized comb. A "mite count" (standard sampling of *Varroa destructor*) was taken by powdered sugar shake using approximately 300 bees based on a ¼ cup volume (Macedo et al., 2002). Mean cell size of each drone frame was estimated by counting three rows of 20 cells and averaging the width. Frames were stored in individual boxes in an incubator at 33 ˚C and ~50% RH along with 100 workers from the brood nest of the source colony to aid in emergence. Each day at 6:00 and 18:00, emerged drones were captured and marked with paint; each drone was marked on the abdomen with a unique color according to its source colony and on the thorax according to its day of emergence. Because prior research suggests that adult colony environment impacts age of first foraging in workers (Winston & Katz, 1982), we transferred all drones from source colonies to separate foster colonies. Drones emerging in the morning and evening were then taken to separate colonies for

fostering to keep the cohort as closely age-matched as possible for the estimation of age of first flight. Drones that emerged from 18:00-6:00 (and placed into the AM colony) were considered to be 12 hrs (0.5 d) older than the drones that emerged from 6:00-18:00 (and placed into the PM colony) on the same day. Marked drones were lightly shaken onto the top bars of the frames of the foster colonies to introduce them. Emergence and marking proceeded from 06/17/18-06/24/18. On 06/22/18 at 6:00 instead of paint-marking, 214 emerged drones were individually number tagged, weighed, and photographed prior to being added to the AM foster colony; only 5 of the 6 source colonies had drones that emerged on this date.

Foster colonies were selected as being similar in size and conditions to each other and the source colonies, and they were placed adjacent to each other within the same apiary. Each colony was housed in a hive consisting of two standard Langstroth brood boxes with a top feeder regularly supplied with 1:1 sucrose:water solution. To estimate age of first flight, we collected drones as they first left the nest. This results in an estimation of flight that may include drones being expelled from the nest and drones taking sanitation or orientation flights prior to mating but ensures that we were highly unlikely to miss focal drones and bias the sample against those drones that successfully mate. Colonies were fitted with a custom-built drone trap consisting of cleaned plywood with queen excluder to prevent large drones from escaping the colony. Drone traps had a 7.6 cm hole on the top and a wire mesh funnel with a 1.3 cm opening leading into a secondary cage made of plywood and queen excluder (Figure 1). Drones that attempted to leave the nest flew up into the cage, were prevented from exiting, and were unable to return to the nest. Drones were collected from the cages daily at 18:00 and immediately transported to the lab for dissection and measurement. Occasionally, drones were found dead in the bottom of the cage; these were not always distinguishable as having flown or having been carried by undertakers,

and they were counted separately but not dissected. Finally, counts of dead drones in the trap bottom were taken every other day in the morning prior to the period of drone flight activity.

## Dissection and measurement

The number of drones captured quickly exceeded our ability to fully analyze their reproductive quality. As such, all paint-marked drones were weighed and counted for age and colony source, but once the number of drones exceeded 30 per trap only one of each age- and colony pairing was dissected and measured as previously reported (Metz & Tarpy, 2019). All individually tagged drones, however, were dissected and fully analyzed. Briefly, each was weighed to the nearest 0.1 mg and its head and thorax photographed. They were then dissected with the mucus glands and seminal vesicles removed, cut free from the testicles and ejaculatory duct, which were also photographed. Finally, the head, wings, legs, and abdomen were cut free from the thorax, which were weighed. The seminal vesicles were immediately ruptured in 1.0 mL Buffer D (Collins & Donoghue, 1999; Makarevich et al., 2010; Metz & Tarpy, 2019) and lightly mixed to homogenize. This obviated the need for the drone to be capable of ejaculating to assess sperm viability. The solution was then dyed using the Invitrogen live/dead spermatozoa staining kit #L7011 (Carlsbad, CA) and read using a Nexcelom Cellometer® Vision Sperm Counter machine (Nexcelom Bioscience LLC; Lawrence, MA, USA) to gain a count of viable sperm. The photographs were then analyzed using ImageJ version 1.51m9 (Schneider et al., 2012) to measure the width of the head and thorax (as measured by the distance between tegulae), as well as the length of the seminal vesicles and mucus glands.

## Statistical Analyses

Experimental methods resulted in multiple populations of drones, for which there was varying levels of information. At the minimum for all analyzed drones was colony source, colony mite count, mean drone cell size, foster colony, emergence date, age when trapped, location trapped, and condition (live/dead). All live drones at capture included their weight as well, and all drones were recorded for estimation of age of first flight based on their know emergence and capture dates. Drones that were captured alive and dissected have additionally reproductive quality and size measures. Finally, the individually marked drones had all the above variables plus their emergence characteristics associated with their age of first flight. Experimenters were not blinded to the colony identity or emergence date of the drones during data collection or analyses. Whenever it is ambiguous which drones were used for analyses, we will use the terms "tallied" for those drones simply marked and counted, "dissected" for those drones dissected but not tagged individually, and "tagged" for those individually tagged drones. In all cases, *tagged* drones were analyzed along with *dissected* and *tallied* drones*,* although the converse is not true. Statistical analyses and visualization were performed using R (version 3.6.0; R Core Team, 2019) with relevant packages referenced and code provided as supplemental information. The dataset used for this publication will be made available upon emailed request to the corresponding author.

# Results

## Collection summary

We collected flight data from a total of 2,360 drones. We individually tagged 207 drones and recovered 70 live for dissection and a tallied a further 77 dead in either the collection cage or trap

bottom. The remainder were lost, likely due to the removal of the number tags. We dissected an additional 495 paint-marked drones and tallied then weighed 887 live drones collected from the flight cage. Finally, we collected 831 dead drones from the cage or trap bottom. Distribution of collected drones is presented in Table 1.

## Age of capture differs by colony source

This analysis included all drones collected in the study. We defined an attempt to fly as drones being found live in the upper cage of the trap, although it is possible that drones were carried there by workers forcibly expelling drones from the nest. Dead drones found in the upper or lower part of the trap were considered censored—meaning they flew at the age collected or older. Tagged drones that were lost were not included in these analyses.

The global median age at which drones were found in the traps was 8.5 days, with drones found in the trap ranging from 0.5 d (trapped the same day as released into the AM colony) to 37 d. An initial Cox proportional hazards model containing colony source (a combination of genetics and rearing environment) and foster colony (adult environment) revealed a significant interaction among foster colony and at least one source colony. We therefore analyzed each foster colony separately. Source was a significant factor in the variation in likelihood to fly in the AM foster colony, only colonies D and E had significantly lower likelihood to fly (and therefore were found in the trap at a significantly older age) than the reference colony A. In the PM foster colony, colonies C-F all had significantly lower risk to fly compared to colony A. Median age of capture for drones from each source are presented in Figure 2a and 2b, and the hazard ratios are presented in Figure 2c and 2d.

## Drone emergence characteristics and age of capture

We measured the subset of drones that were tagged and recaptured to observe effects of different drone size measures at emergence on age of capture and measures at capture. Drones emerged with a body mass of 220.3±26.2 mg, head width of 4.31±0.13mm, and thorax width of 5.46±0.24 mm. We tested first for colony-level differences in emergence properties using univariate ANOVA. We found that emergence size differed among the colonies, with mass ($F_{4,202}$=60.9, p<0.0001), head width ($F_{4,200}$=35.0; p<0.0001), and thorax width ($F_{4,200}$=14.0; p<0.0001) all being significant (Figure 3a-c).

We then tested each emergence measure along with colony source in a cox proportional hazards model, finding a significant effect of colony source (Figure 3d), consistent with the results from the expanded dataset, and emergent body mass such that more massive drones had a slightly smaller risk to fly than their lighter brothers (Figure 3e). Hazard ratios and statistical results are presented in Figure 3e.

## Causes and correlates of drone variation in emergence size & age of capture

To explore potential causes of colony-level variation in drones, the average cell size of drone frames was estimated and a mite count of the source colony was taken when the frame was removed. Mean cell size of the drone frames ranged from 12.88-13.93 mm, and mite counts of drone-source colonies ranged from 3-21. We tested for a relationship between emergence measures and colony characteristics using linear regression. Mite count had a slight but significant effect on drone emergent mass ($F_{1,205}$=8.9; p=0.003; $r^2$=0.037; Mass=234.54-1.06(mites)) but not on head width ($F_{1,203}$=0.40; p=0.52) or thorax width ($F_{1,203}$=0.72; p=0.40). Cell size, on the other hand, had a slight but significant effect on all three measures.

Unexpectedly, this was a *negative* relationship, with larger cells producing slightly less-massive ($F_{1,212}$=23.6; p<0.0001; $r^2$= 0.096; Mass=580.28-27.040(cell)) drones with narrower thorax ($F_{1,210}$=24.9; p<0.0001; $r^2$=0.10; Thorax=8.79-0.25(cell)) and head widths ($F_{1,210}$=38.26; p<0.0001; $r^2$=0.15; Head=6.59 -0.17(cell)). Mite count and cell size were not significantly correlated (Spearman's Rho=-0.029; p=1).

To confirm the change in drone characteristics from emergence to capture, we then calculated the difference in body mass, head width, and thorax width and used a t-test to determine significance from zero. We found that drones lost an average of 21.51 ± 2.28 mg ($t_{69}$= 9.44; p<0.0001) body mass, gained 0.029±0.011 mm ($t_{69}$= -2.65; p=0.001) in head width, and gained 0.174 ± 0.188 mm in thorax width ($t_{69}$= -9.27; p<0.0001) from emergence to capture. These differences were all significantly correlated with capture age such that drones captured at older ages lost more body mass (Spearman's Rho= 0.35; p=0.0033) and gained more head (Spearman's Rho= -0.28; p=0.0204) and thorax width (Spearman's Rho= -0.26; p=0.03).

Finally, we tested whether emerged properties, source colony, and capture age was related to variation among the reproductive characteristics of drones, which are themselves subject to age-based ontogeny (Metz & Tarpy, 2019). We used a main-effects multiple regression model to test for effects on the variance in total sperm count (log-transformed), sperm viability (arcsine-transformed), and mucus gland length (mm). We found a whole-model significance for sperm count ($F_{8,60}$= 5.05; p<0.0001; $r^2$= 0.32) with a significant effect of capture age ($F_{1,60}$= 11.19; p=0.001), source colony ($F_{4,60}$= 5.37; p=0.001) and emerged body mass ($F_{1,60}$= 5.27; p=0.025), but no significant effect of emerged head or thorax width (Figure 4). For sperm viability, the whole model was again significant ($F_{8,60}$= 2.16; p=0.01; $r^2$= 0.17) with capture age ($F_{1,60}$= 9.26; p=0.003) and colony source ($F_{4,60}$= 2.54; p=0.049) being the only significant

294 factors. Finally for mucus gland length, the whole model was significant ($F_{8,38}$= 3.22; p=0.007;
295 $r^2$= 0.28) with capture age ($F_{1,38}$= 8.02; p=0.007) and colony source ($F_{4,38}$= 2.76; p=0.042) being
296 the only significant factors.

# 297 Discussion

## 298 Age of capture differs by colony source

299 Drone age of first flight differed by both colony source and foster colony, with a positive
300 interaction between source and foster colony. Drones from colony A were captured a median of
301 3-5 days earlier than the other colonies (Figure 2a and b), suggesting that like for workers,
302 genetics and rearing environment—which we cannot disambiguate in this study—play a part in
303 behavioral ontogeny. Because there was a source-by-foster colony interaction, we could not
304 directly observe the effects of foster colony and therefore adult environment. However, the
305 difference in median flight age between the AM and PM colonies was on average 1 day, with
306 drones from colony F being captured a median of 0.5 days *earlier* in the PM than the AM, and
307 drones from colony D being captured a median of 3.5 days *later* in the PM compared to the AM.
308 This suggests that the 12 hr difference in introduction time between the two foster colonies is not
309 sufficient to explain the variation in flight time from one colony to the other. It is therefore likely
310 that, similarly as in workers, both larval and adult colony environment elicits an effect on
311 behavioral maturation (Winston & Katz, 1982). Our median likelihood for age of flight was 8.5
312 days, about 2 days earlier than that reported in previous study (Rueppell et al., 2005). In addition
313 to probable genetic and environmental variations based on evidence shown here, we also used a
314 looser definition of "flight," since we included any drone that attempted to exit the hive entrance.
315 Any drone capable of flight that was expelled from the nest could also likely be counted in the

316 trap, as could drones attempting defecation and orientation flights, all of which would decrease
317 our average age of first flight. However, since the initiation of orientation flights, while
318 occurring prior to full reproductive maturity, remains an indicator of being upon the precipice of
319 mating attempts, there is no reason to assume that small drones take more orientation flights than
320 larger drones. It has been previously found that drones of varying size are subjected to
321 differential treatment by nestmates (Goins & Schneider, 2013); such variation in treatment might
322 serve as a mechanism for the variation in flight ontogeny.

## 323 Drone emergence characteristics, age of capture, and fecundity

324 Drone emergence size differed significantly among the source colonies with head width,
325 thorax mass, and body mass, all significantly smaller particularly for colony C than the other
326 colonies, with colony F intermediate (Figure 3a-c). However, only drone body mass at
327 emergence was significantly related to age of first flight, such that drones that were less massive
328 upon emergence were more likely to fly at a younger age, even when accounting for source
329 colon Body size broadly trades off with the rate to achieve sexual maturity in a number of
330 species (Morita & Fukuwaka, 2006; Stearns, 1992), and while we are not aware of a study
331 specific to males, honey bee development to maturity differs among subspecies consistent with
332 their overall adult size (*reviewed in* Nunes-Silva et al., 2006). Our results are consistent with the
333 idea that the smaller individuals leave the nest earlier.

334 Smaller drones were also less fecund upon capture (Figure 4 rones often initiate flight
335 prior to full reproductive maturity (Witherell, 1971). Our measurement paradigm—specifically
336 measuring sperm parameters from the seminal vesicles rather than upon ejaculation—is intended
337 in part to obviate the problems of sampling somewhat immature drones. As a consequence, we
338 do not know if there is a variation in whether drones of differing size initiate flight at different

reproductive states. However, because the positive correlation between size and fecundity persists from emergence (Metz & Tarpy, 2019), we can broadly assume that the relationship between drone reproductive development and flight ontogeny are similar despite body size and that smaller drones are initiating flight at approximately the same state, regardless of the temporal difference.

## Causes and correlates of drone variation in emergence size & age of capture

In attempting to find an environmental cofactor, we measured colony-level mite loads and the mean cell sizes from which the drones emerged. Mite loads elicited a small but significant effect in the expected direction, with colonies with higher mite counts producing less massive drones. Confusingly, however, cell size elicited an effect in the opposite direction, with smaller drones emerging from colonies with larger diameter cells. Despite the numerous examples of worker-cell produced drones in the literature and manipulated small drones (*e.g.,* Gençer & Kahya, 2020), it appears that "natural" variation in drone cell size has little bearing on drone morphology. It is possible that wall thickness rather than cell width *per se* is a better correlate with drone size, but we did not directly measure this.

## Evolutionary rationale for variation in drone mating quality

The trait parameters of drones are consistent with the presence of pre-copulatory (N. Koeniger et al., 2005) and post-copulatory competition (Baer, 2005; Liberti et al., 2019), and indeed there appears to be a positive relationship between mating success and drone size (Couvillon et al., 2010). However, any effect of drone competition is likely to be muted by opposing selective pressure at the colony level, specifically the clear benefit to polyandry (Tarpy et al., 2013), the

low likelihood of realized fitness through the production of queens, pleiotropy with worker and queen phenotypes, and colony-level control of larval rearing state and adult sustenance. Combined with prior research, our results suggest that colonies make distinct resource commitments into their drone population and that these commitments have a long-term impact on the developmental ontogeny of their drones. At an individual level, drones of varying sizes may use different lifetime mating strategies (with larger drones making fewer mating flights with a higher likelihood of success but smaller by attempting more flights each with a lower likelihood of success), although this intriguing possibility will require additional empirical investigation.

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# Figure 1

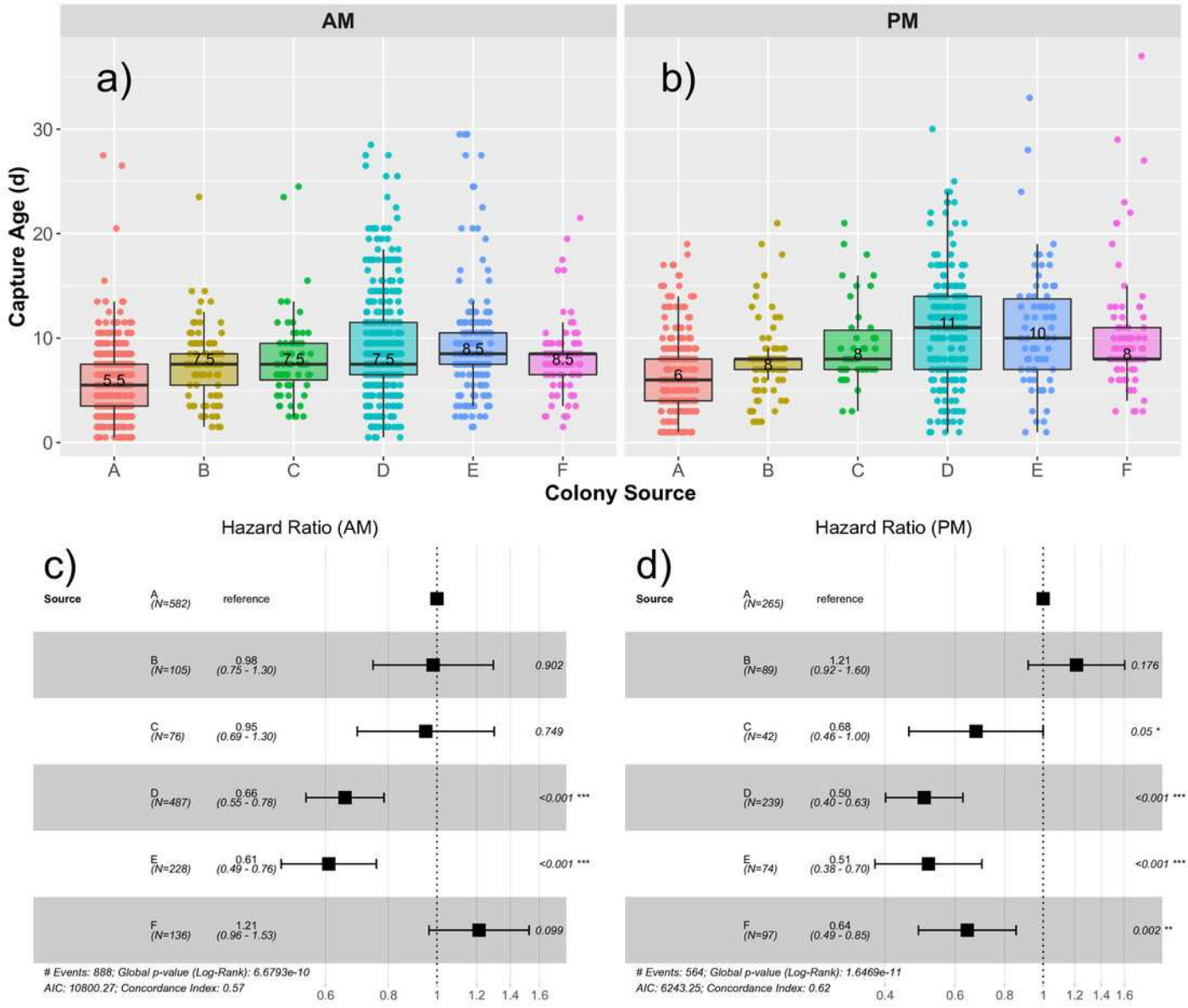
Drone trap construction and placement.

The flight cages used for this study. The bottom part of the trap consisted of a runway and queen excluder material, which restricted drone movement, while the upper consisted of a mesh cone and large face of excluder. Drones, unable to exit through the bottom, were attracted into the top of the trap due to the light and were unable to return into the bottom. Photos taken by JP Milone.

# Figure 2

Drone capture ages by source and foster colonies.

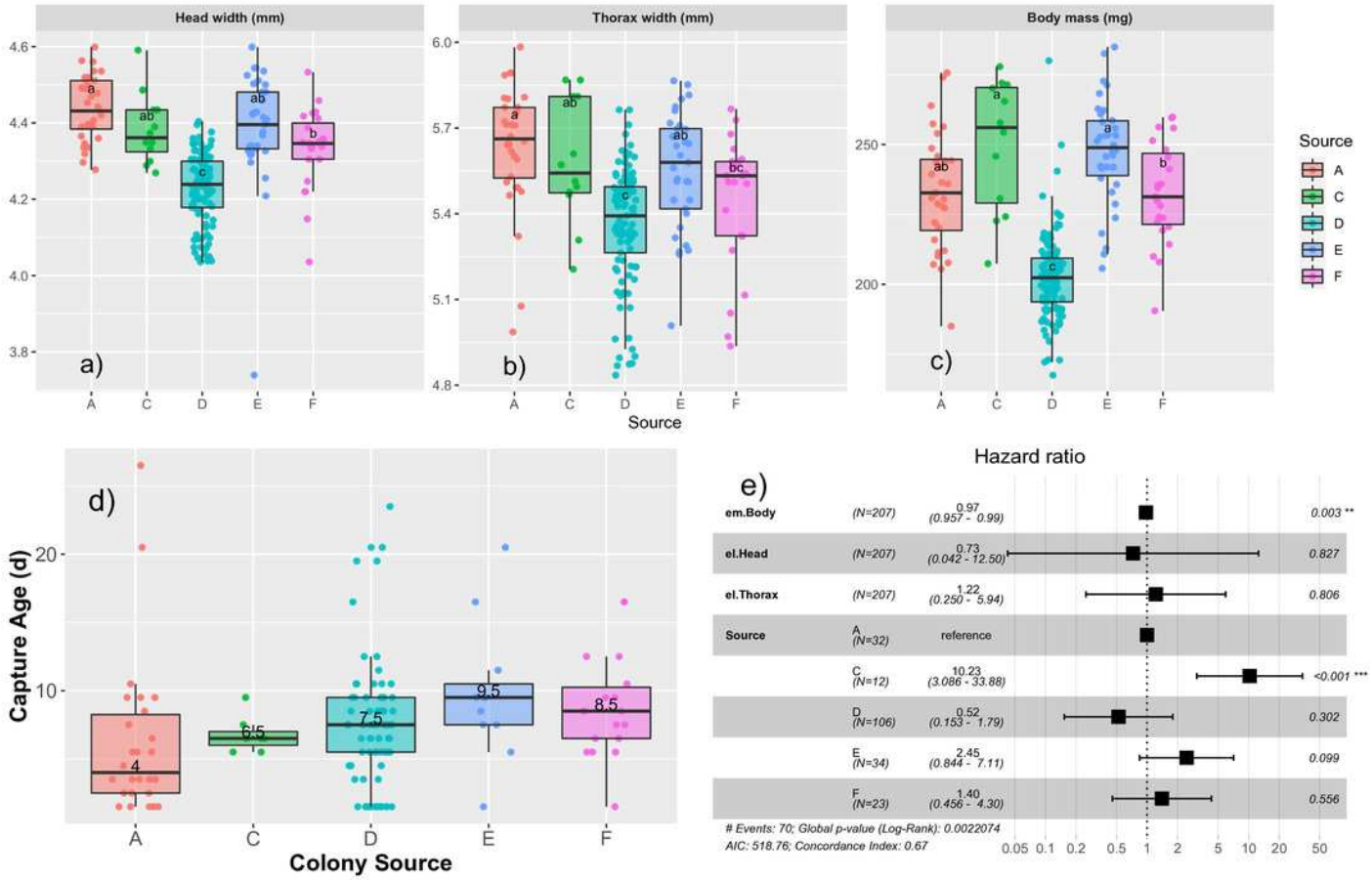
Boxplots showing the distribution of capture ages in the AM (a) and PM (b) foster colony. Median age of capture ranged from 5.5-8.5 d in the AM colony and 6-11 d in the PM colony. Capture age for each source is written over the median of the box for each source colony. Cox proportional hazard ratios are presented in the forest plots for the AM (c) and PM (d) foster colonies separately. In both cases, Colony A was taken as the reference and the relative risks to be captured are presented for each subsequent source colony. Relative risks less than 1 represent a lower risk to be captured and correlate to a higher age of capture.


AM
PM
a)
b)
Capture Age (d)
Colony Source
A
B
C
D
E
F
Hazard Ratio (AM)
Hazard Ratio (PM)
c)
d)
Source
reference
# Events: 888; Global p-value (Log-Rank): 6.6793e-10
AIC: 10800.27; Concordance Index: 0.57
# Events: 564; Global p-value (Log-Rank): 1.6469e-11
AIC: 6243.25; Concordance Index: 0.62

# Figure 3

Relationships among age of capture, larval source colony, and emergent body mass.

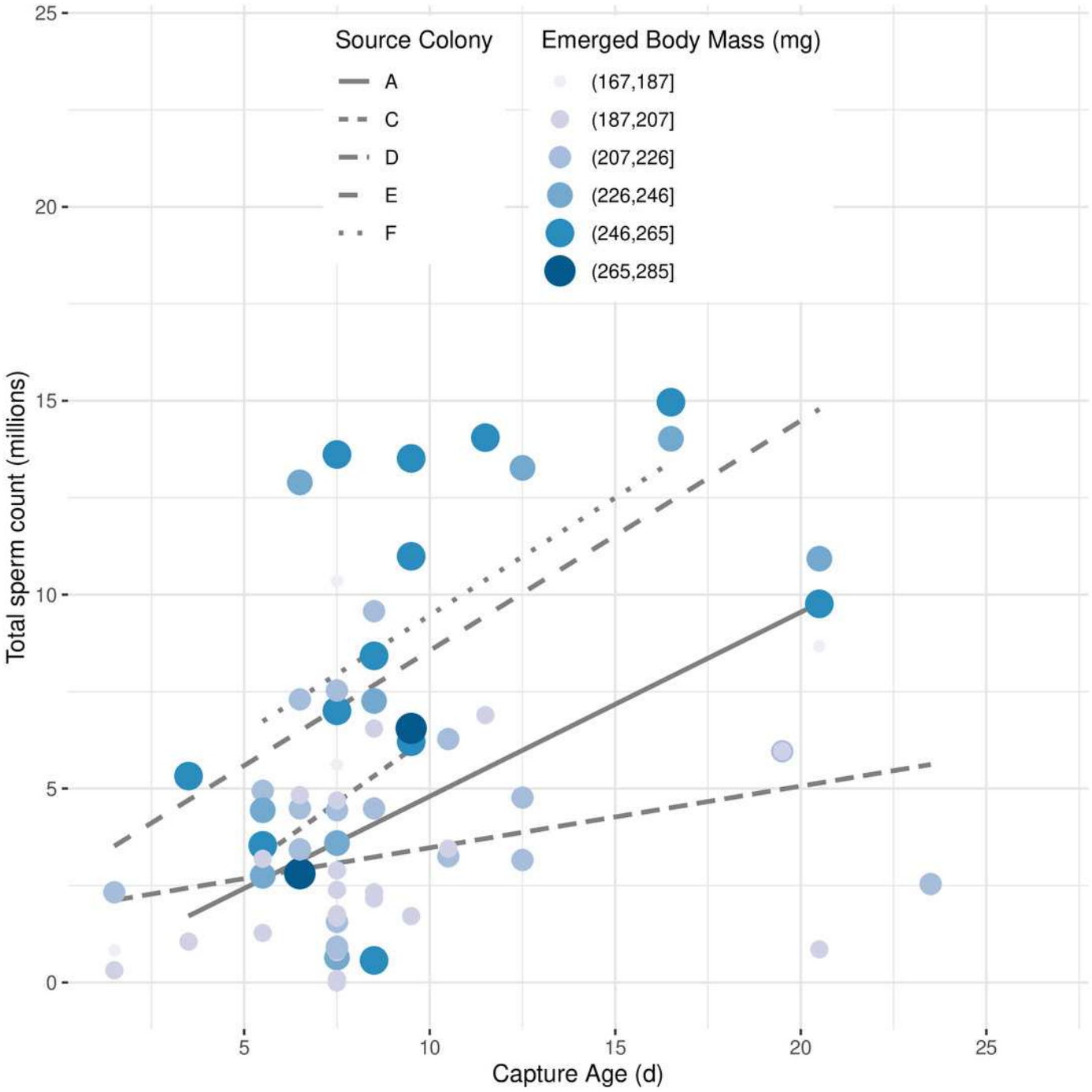
Boxplots of the emergence parameters: head width (a), thorax width (b), and body mass (c) for each source colony. Tukey's significant groups ($p<0.05$) are presented within each box. Note that drone emergence measures were only taken for the AM colony. Median age of capture for each source (d) differs slightly in this subset of data relative to the ages of the expanded population presented in figure 2a and here ranged from 4-9.5 d. Cox proportional hazard ratios (e) show a significantly higher risk of flight for Colony C and a slight, but significant decrease in risk for each unit increase in body mass.



# Figure 4

Impact of age of capture and emerged body mass on captured sperm count.

The impacts of emerged mass and capture age are presented as causal factors in sperm count at capture based on a main-effects, multiple regression model ($F_{8,60}$= 5.05; p<0.0001; $r^2$= 0.32). Age was a significant factor ($F_{1,60}$=11.19; p=0.001). Mass was significant when tested as a continuous variable ($F_{1,60}$= 5.27; p=0.025). Here, mass is binned into six categories for easier visualization with larger, more darkly colored points represent larger drones. Source colony was also significant in this model ($F_{4,60}$= 5.37; p=0.001) with Colony F (dotted line) being significantly different than the others (t=2.83; p=0.006). Line types represent the relationship among capture age and sperm count for each colony separately.


Source Colony
A
C
D
E
F
Emerged Body Mass (mg)
(167,187]
(187,207]
(207,226]
(226,246]
(246,265]
(265,285]
Total sperm count (millions)
0
5
10
15
20
25
Capture Age (d)
5
10
15
20
25

## Table 1(on next page)

Table 1: Collection Summary

**Table 1: Collection Summary**

| Colony Source | Foster Colony | Tagged | Captured | Tallied | Flew* | Didn't Fly** |
|---|---|---|---|---|---|---|
| A | AM | 6 | 210 | 360 | 334 | 249 |
| | PM | | 148 | 117 | 205 | 60 |
| B | AM | | 66 | 39 | 90 | 15 |
| | PM | | 74 | 15 | 81 | 8 |
| C | AM | 7 | 41 | 23 | 61 | 15 |
| | PM | | 31 | 11 | 39 | 3 |
| D | AM | 36 | 272 | 153 | 404 | 89 |
| | PM | | 177 | 62 | 219 | 20 |
| E | AM | 9 | 130 | 71 | 180 | 48 |
| | PM | | 58 | 16 | 66 | 8 |
| F | AM | 12 | 99 | 20 | 122 | 14 |
| | PM | 6 | 76 | 21 | 90 | 7 |

*Drones that were found in the cage top were considered to have flown whether they were collected live or dead.

**Drones found in the trap bottom were censored and considered to have flown at the age collected or older.