Accessory gland size increases with sperm competition intensity in *Cataglyphis* desert ants

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In many species, females have multiple mates, whose sperm compete for paternity. Males may subsequently invest in the increased production of sperm and/or seminal fluid. The latter is a complex mixture of proteins, peptides, and other compounds generated by the accessory glands (AGs) and is transferred to females along with a male's sperm. Seminal fluid is known to be a key determinant of competitive outcomes among sperm, and its production may trade off with that of sperm. We show that AG size—a proxy for seminal fluid production—has a positive and phylogenetically robust correlation with both sperm competition intensity and sperm production in nine species of *Cataglyphis* desert ants. These results indicate a lack of trade-off between sperm production and seminal fluid production. They underscore that sperm competition may strongly shape sperm traits and could drive reproductive performance in eusocial hymenopterans.

Introduction

Within species, female promiscuity often fosters competition among males, whose spermatozoa vie for fertilisation opportunities (Parker, 1970). This competition extends sexual selection beyond mating and can significantly affect male fitness. Extensive theoretical and empirical research has shown the evolutionary impacts of sperm competition on sperm traits, particularly (i) number (*e.g.*, Gomendio et al., 1998; Gage and Morrow, 2003), (ii) size (*e.g.*, Gomendio and Roldan, 1991; Immler et al., 2011; Tourmente et al., 2011), and (iii) velocity (*e.g.*, Anderson et al., 2007; Firman et al., 2013; Nakanishi and Takegaki, 2018).

Investment in the seminal fluid is another powerful mean through which males can enhance their reproductive success (Ramm, 2020). Typically transferred to females at the same time as sperm, seminal fluid is a complex combination of secretions produced by the sex accessory glands (AGs) (Chapman, 2001; Manaskova et al., 2002; King et al., 2011; den Boer et al., 2015; Santiago-Moreno and Blesbois, 2020), and it contains diverse organic compounds, including proteins, lipids, and sugars (Owen and Katz, 2005; Avila et al., 2011;

Gorshkov et al., 2015; Lu et al., 2016). Post ejaculation, seminal fluid can influence sperm performance (den Boer et al., 2009; Smith and Stanfield, 2012), competitive outcomes among rival sperm in the female reproductive tract (den Boer et al., 2010), and female reproductive behaviour and physiology (Simmons and Gwynne, 1991; Wolfner, 2002).

There thus exist two potential, mutually exclusive relationships between sperm production and seminal fluid production (Ramm, 2020). First, there may be a positive correlation if seminal fluid boosts male reproductive success by enhancing sperm function. Second, there may be a negative correlation if a trade-off exists between sperm production and seminal fluid production. For instance, if seminal fluid has effects that are independent of sperm (e.g., the fluid generates a plug that prevents or interferes with further female mating), then a greater investment in seminal fluid production may relax selection pressure on sperm production. To date, findings are mixed: the production of sperm and seminal fluid appears to be positively correlated in mammals (Dixson, 1998; Ramm et al., 2005), fishes (Mazzoldi et al., 2005), and some insects (Morrow and Gage, 2000) but negatively correlated in fungus-growing ants (Baer and Boomsma, 2004).

The mating system of eusocial hymenopterans (ants, social bees, and wasps) imposes unique selective pressures on male ejaculates that are rarely, if ever, found in other animals (Boomsma et al., 2009; Boomsma, 2013). First, males produce sperm only once in their lives. Spermatogenesis mainly occurs during the pupal stage and is usually completed shortly after males emerge. Their testes then deteriorate, and sperm are stored in the accessory testes until mating. At ejaculation, the semen and seminal fluid are combined. Males die shortly after copulation, by exhaustion or predation. Second, mating occurs during a single nuptial flight at the beginning of adult life for both sexes. During this short window of time, female reproductives copulate with one or several males, amassing a lifetime supply of sperm in their spermatheca. In some species, females live for decades, generating offspring from stored sperm. Third, polyandry has evolved repeatedly in ants, bees, and wasps (Hughes et al., 2008), creating conditions conducive to sperm competition in multiple species. In the few studies looking at how sperm competition influences sperm traits, it has been observed that sperm competition may select for (i) enhanced sperm production (ants: Baer and Boomsma, 2004; Aron et al., 2016; Degueldre and Aron, 2023), (ii) increased sperm size (bumble bees: Baer et al., 2003). Furthermore, seminal fluid can promote male fitness by (i) enhancing sperm viability (honey bees: den Boer et al., 2009); (ii) incapacitating rival sperm when females have multiple mates (ants and honey bees: den Boer et al., 2010); and (iii) reducing female promiscuity by either generating a mating plug (ants: Baer, 2011; bumble bees: Duvoisin et al., 1999; Brown et al., 2002) or by negatively affecting the eyesight of females during the mating flight (honey bees: Liberti et al., 2019).

However, it remains largely unknown whether there is a correlation between the production of sperm and seminal fluid in eusocial hymenopterans. To date, a single study on fungus-growing ants has revealed that, in species with singly mated females, males have small accessory testes and large AGs, while the opposite is true

in species with multiply mated females (Baer and Boomsma, 2004). It has been suggested that males exploit AG compounds to produce mating plugs and prevent further mating by females (Baer and Boomsma, 2004; Baer, 2011; den Boer et al., 2015). Consequently, if mating plugs became incapable of serving this function, males might redirect their investment from seminal fluid (i.e., large AGs) to sperm production (Baer and Boomsma, 2004).

Desert ants of the genus *Cataglyphis* are well suited to explore the association between seminal fluid production and sperm production. Indeed, (i) the degree of polyandry varies almost 10-fold across species (range of the mean number of male mates: 1.07 – 8.5; Aron et al., 2016) and (ii) sperm production increases with sperm competition intensity (Aron et al., 2016; Degueldre and Aron, 2023). Using phylogenetically controlled analyses, we explored the relationship, and potential trade-off, between accessory gland size—a proxy for seminal fluid production— and sperm production in nine species of *Cataglyphis* ants experiencing various degrees of sperm competition.

Methods

Sampling

During regional periods of sexual reproduction, we sampled colonies of nine *Cataglyphis* species across several countries: *C. cursor* in France; *C. hispanica* and *C. velox* in Spain; *C. bombycina*, *C. emmae*, and *C. viatica* in Morocco, and *C. livida*, *C. niger*, and *C. savignyi* in Israel (Table S1). Specifically, we collected male pupae and returned them to the laboratory, where they emerged as adults. Estimates of seminal fluid production and sperm production were obtained from the same males.

Accessory gland size

Because gland volume usually correlates with production (Widdicombe and Wine, 2015), we used AG size as a proxy for seminal fluid production (see also Baer and Boomsma, 2004). Between 8- and 10- days post emergence (i.e., depending on species maturation times), males were decapitated and dissected under a Leica MZ6 stereomicroscope (Leica Microsystems, Wetzlar, Germany) in semen diluent (188.3 mM sodium chloride, 5.6 mM glucose, 574.1 nM arginine, 684.0 nM lysine, and 50 mM tris [hydroxymethyl]aminomethane, pH 8.7; Paynter et al., 2014). All the males had degenerated testes; sperm were found in the accessory testes, indicating their complete maturation.

Each male's AGs were properly isolated, and carefully placed on a grid in the dissection plate with a drop of semen diluent. They were positioned to measure their greatest length. AGs were photographed using light microscopy (50x magnification) and then measured with a precision of 0.01 mm using IMAGEJ (version 1.8). In all our analyses, absolute AG size was controlled by male body size using analyses of covariance with maximum head width (including eyes) as a covariate (García-Berthou, 2001).

Maximum head width is a reliable proxy of body size in *Cataglyphis* ants (Aron et al., 2016; Lecocq de Pletincx et al., 2021). Males were decapitated and their head measured using the same method as AGs.

Sperm production

Sperm production data for the nine study species were obtained from Degueldre and Aron (2023). Briefly, both accessory testes from each male were dissected in semen diluent. They were placed in 1 ml semen diluent, their membrane was removed and the resulting sperm stream carefully mixed with the diluent. 150 μl of this sperm solution was transferred to an empty 1.5 ml vial; 850 μl of sperm diluent was added to obtain a final volume of 1 ml. This step was repeated to obtain two technical replicates per male. For each sample, cells were quantified by flow cytometry. Sperm populations were identified based on characteristic forward and sideward scatter, enabling total sperm production to be quantified. We used the average of the two technical replicates to estimate sperm production for each male. A square root transformation was used to ensure normality and homoscedasticity of the data.

Paternity frequency

For each species, sperm competition intensity was inferred from paternity frequency (i.e., the number of males that father the offspring of a queen; Aron et al., 2016; Degueldre and Aron, 2023). Data on paternity frequency were obtained from previous studies in which microsatellite loci were used to determine patriline number. For each species, AG size and sperm production were measured from males collected in the same location as that in which paternity frequency was estimated (Table S1).

Phylogenetic analyses

Using species-level data, we evaluated whether paternity frequency was correlated with AG size. To account for potential trait similarities resulting from shared ancestry among species, we utilised phylogenetic generalised least squares (PGLS) regression (Pagel, 1999). In this approach, maximum likelihood ratio tests are used to estimate the degree of phylogenetic association (λ) within the data. When λ values are closer to 0, it is more likely that traits evolved independently. We performed these analyses using the pgls function in the caper package (Freckleton et al., 2002) and employed the *Cataglyphis* phylogeny (Figure S1; from Lecocq de Pletincx et al., 2021), which was handled using the ape package (Paradis et al., 2004). All analyses were performed in R (v. 4.2.1; R Core Team, 2020).

Results and discussion

We measured AG size for 117 males across the 9 study species (mean number of males per species ± SD = 13 ± 5.79, range: 8–23; mean number of colonies per species ± SD = 2.56 ± 1.01, range: 1–4; mean number of males per colony ± SD = 5.17 ± 2.87, range: 2–16; Table S1). Absolute AG size ranged from 1.17 to 2.23 mm.

Across species, AG size was positively associated with paternity frequency (covariance PGLS: model coefficient = 0.07; R^2 = 0.7, $p < 0.01$; Figure 1). Similarly, AG size was positively correlated with sperm production (partial correlation: $\rho = 0.64$, $p < 0.001$; Figure 2).
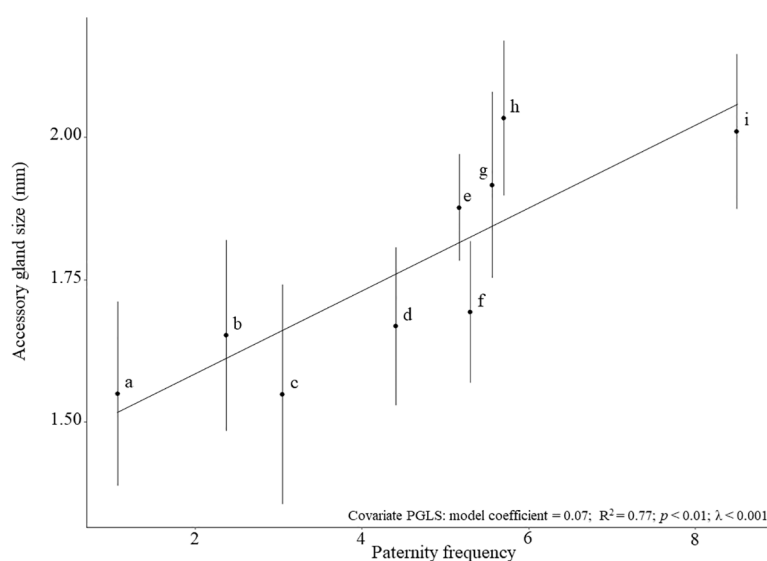

FIGURE 1
Relationship between paternity frequency and mean AG size (± SD) in nine species of *Cataglyphis* ants. The linear regression line is shown for AG size. (a) *C. hispanica* (paternity frequency [Mp] = 1.07; Leniaud et al., 2012); (b) *C. velox* (Mp = 2.37; Eyer et al., 2013); (c) *C. emmae* (Mp = 3.05; Jowers et al., 2013); (d) *C. livida* (Mp = 4.41; Timmermans et al., 2010); (e) *C. niger* (Mp = 5.17; Leniaud et al., 2011); (f) *C. cursor* (Mp = 5.3; Pearcy et al., 2009); (g) *C. viatica* (Mp = 5.56; Aron et al., 2013); (h) *C. bombycina* (Mp = 5.7; Leniaud et al., 2013); and (i) *C. savignyi* (Mp = 8.5; Leniaud et al., 2011).
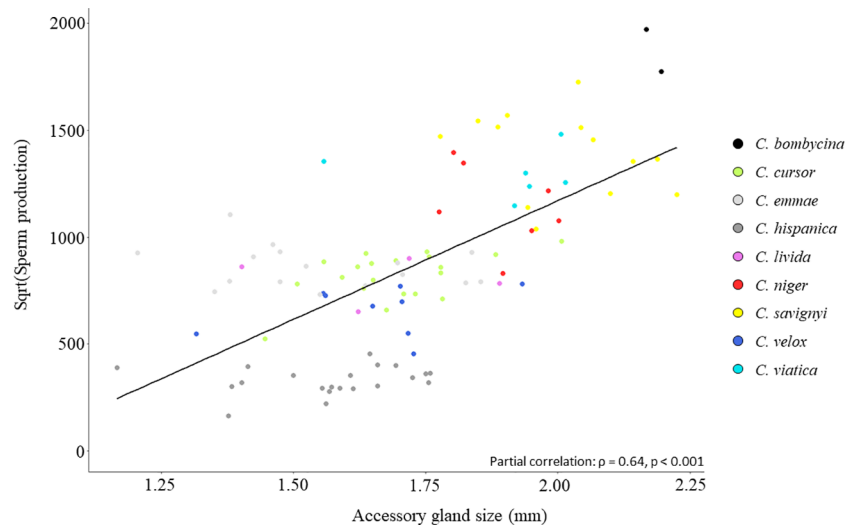
FIGURE 2
Relationship between sperm production and AG size across all nine *Cataglyphis* study species. The linear regression line is shown.

There was a negligible phylogenetic signal for AG size ($\lambda < 0.001$, $p_{(Ho:\ \lambda=0)} = 1$; Figure S2). These results strongly suggest a lack of trade-off between sperm production and seminal fluid production. They are consistent with other studies in rodents (Ramm et al., 2005), fishes (Mazzoldi et al., 2005) and moths (Morrow and Gage, 2000) showing a positive association between sperm competition level and the relative size of accessory reproductive glands. Such a lack of trade-off was also reported in *Drosophila* where testis length is not correlated with accessory gland length (Kraaijeveld et al., 2008).

Although our results are clearly significant, they must be interpreted with caution because our estimates of paternity frequency are based on data from a single population and year for each study species. Past research has documented the existence of biogeographical variation in population-specific paternity frequencies in various ant species (e.g., Sundström, 1994; Boomsma and van der Have, 1998; Suni and Eldakar, 2011), including in the silver ant, *Cataglyphis bombycina* (Leniaud et al., 2015). Here, we measured AG size and sperm production for males that came from the population for which paternity frequency had been estimated. To date, there has been no dedicated work exploring interannual variation in paternity frequencies within populations of desert ants, although preliminary findings suggest that such variation is minimal in *C. viatica* and *C. cursor* (unpublished data). Beyond these results, it remains unknown how variation in paternity frequency interacts with AG size and sperm production.

In *Cataglyphis* desert ants, multiple mating by females could drive males to evolutionarily invest in both functional traits. Our findings contrast with those of previous research on fungus-growing ants (Baer and Boomsma, 2004). This work found that, in species where females have multiple mates, males have small AGs and large accessory testes, suggesting they face intense sperm competition and preferentially invest in sperm production. However, it also noted that, in species where females have a

single mate, males could employ seminal fluid to form mating plugs, suggesting they may experience lower levels of sperm competition and thus decreased selection pressure on sperm production. Despite the strong association between AGs size, sperm production and paternity frequency in *Cataglyphis*, other factors than sperm competition may also influence these reproductive traits, such as the efficiency of spermatogenesis (Johnson et al., 2000) and the queen's sperm storage capacity (Boomsma et al., 2005).

In *Cataglyphis*, AG size and sperm production are positively associated with sperm competition intensity which suggests that seminal fluid boosts sperm function (Ramm, 2020). In the same vein, it has been shown that sperm competition selects for increased sperm quality in *Cataglyphis*: in species with highly polyandrous females, males produce more viable sperm with lower levels of DNA fragmentation (Degueldre and Aron, 2023). Seminal fluid could help maintain high levels of sperm quantity and quality by furnishing (i) antioxidant enzymes that limit oxidative stress (Weirich et al., 2002; Collins et al., 2006; Baer et al., 2009); (ii) substances that sustain sperm metabolism (Blum et al., 1962; Alumot et al., 1969); and (iii) antimicrobial and antifungal enzymes (Baer et al., 2009; Peng et al., 2015; Chérasse et al., 2018; Dávila et al., 2018). Seminal fluid could also improve sperm motility after ejaculation, a trait that is essential for successfully reaching the spermatheca (Pearcy et al., 2014; Liberti et al., 2018; Yániz et al., 2020). In the honeybee (*Apis mellifera*) and leafcutter ants (*Acromyrmex echinatior* and *Atta colombica*), females have multiple mates, and a given male's seminal fluid reduces the survival of sperm from rival males (den Boer et al., 2010), via the action of AG-generated serine proteases (Dosselli et al., 2019). Further research should explore quantitative and/or qualitative differences in the seminal fluid of *Cataglyphis* species facing differing levels of sperm competition.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because my models are desert ants. They are not species requiring ethical approval.

Author contributions

FD: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. SA: Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2024.1336015/full#supplementary-material

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