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RESEARCH ARTICLE

Variation in male body size and reproductive allocation in the leafcutter ant *Atta colombica*: estimating variance components and possible trade-offs

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Abstract Remarkably little is known about the traits that determine reproductive success of males in eusocial insects. Their window for mate choice decisions is very short, the actual mating process is very difficult to observe, and their small body sizes have likely prevented systematic studies in many species. In 2008 and 2009, we revisited a Panamanian population of *Atta colombica* leafcutter ants to partially repeat and complement a study of more than 15 years ago. We compared within- and between-colony variation in male body size (mass and width of head, mesosoma and gaster) and sperm characteristics (length, number and survival after exposure to saline buffer with and without added accessory gland secretion). We also measured the size of accessory glands as the main contributor of seminal fluid and the accessory testes containing all mature sperm, but we found few correlations between these variables. We also obtained little or no evidence for expected trade-offs between sperm number and sperm length and between mesosoma mass and sperm complement, although this could be due to limited sample size and unknown variation in larval resource allocation that was beyond our control. However, we found an interestingly bimodal distribution in broad-sense heritabilities (intra-class correlations) among the variables that we measured. Low heritabilities suggest that mesosoma size (mass and width), accessory testes size, sperm viability (measured as % survival in saline) and probably also accessory gland size are traits directly correlated with reproductive success. However, the much higher heritabilities for total body mass, gaster mass, head width, sperm length and sperm number suggest that these traits are less likely to make direct contributions to male fitness.

Keywords Sperm number · Accessory gland size · Sperm length · Sperm viability · Fungus growing ants

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Introduction

The males of ants and other eusocial Hymenoptera have phenotypes that are highly specialized for optimal performance during a swarming period that often lasts only a few hours, after which they die. This suggests that the characteristics of their morphology and sexual functioning have been shaped primarily by sexual selection, rather than natural or kin selection (Baer, 2010), but explicit studies of the possible trade-offs involved in maximizing ant male reproductive success have been scant (Boomsma et al., 2005). Exceptions are a recent study on honeybees showing male size to be an important predictor of reproductive success (Couvillon et al., 2010), as well as studies on *Cardiocondyla* male ants, which are peculiar because they are long-lived and spermatogenesis continues throughout adult life (Heinze and Hölldobler, 1993; Heinze and Schrempf, 2008) in contrast to all other known ants where sperm production ceases during the pupal stage (Hölldobler and Bartz, 1985).

Several studies have shown that body size and caste in social insects are determined both genetically and by larval provisioning (including environmental and social effects) (Hughes et al., 2003; Fjerdingstad, 2005; Smith et al., 2008) but does not change after eclosion. These studies have all been carried out on female workers; however, it can be assumed that the same components contribute to male body size. The maximal number of sperm cells that males mature is set at the larval stage as spermatogenesis ceases shortly after eclosure and a male's total sperm complement is gradually transferred to the accessory testes (ATs) (Hölldobler and Bartz, 1985; Boomsma et al., 2005). However, eclosed males may be fed by their worker sisters during maturation, so that fresh masses may also reflect the nutritional status of the colony in which males mature before leaving for their mating flight (Hölldobler and Wilson, 1990; Chown and Gaston, 2010).

Fjerdingstad and Boomsma (1997) performed a first study on male traits in the leafcutter ant *Atta colombica*. They found that body and gaster fresh masses of males varied significantly among colonies, but that other variables including sperm number in males did not. In addition, their data indicated that flight ability might be traded-off with sperm complement. The present study revisits the same *A. colombica* population in Gamboa, Republic of Panama and investigates a number of additional variables that are likely to affect male reproductive success: sperm length, sperm viability (measured as % survival in a saline diluent), and the relative sizes of the accessory glands (AGs) and the accessory testes (ATs). These variables have all been shown to be important in recent studies on social insect males (Baer and Boomsma, 2004; Den Boer et al., 2008; Den Boer et al., 2010), and have also revealed high heritabilities and significant genetic variations in other insect species (Simmons and Kotiaho, 2002; Moore et al., 2004; Baer et al., 2006a, b).

We present data collected across 2 years (2008 and 2009), although not all variables became available for both years. Collecting data across years allowed us to test whether colony-level variation in reproductive traits differs across years, which strengthens any overall inferences about trait variation. Some of these measures could be compared with those reported by Fjerdingstad and Boomsma (1997). In addition to statistical analyses of within- and between-colony variation in body-size and genital traits of males, we also provide estimates of broad sense heritabilities and correlation analyses within and across colonies.

Materials and methods

Following Fjerdingstad and Boomsma (1997) we measured the sizes of several body parts for each male (Fig. 1a), the width of the head (Fig. 1b), the width and mass of the mesosoma, containing the wings and the flight muscles (Fig. 1c), and the mass of the gaster, containing the genitalia. We also dissected and measured the sexual organs, i.e. the area of the AGs as the main contributor of seminal fluid and the AT size as illustrated in Fig. 1d. Finally, we measured the total number, average length and viability of sperm for each male. Males were collected from mature *A. colombica* colonies that we excavated in Gamboa, Republic of Panama in May 2008 and 2009. We sampled males from each colony and kept them together with workers and fungus garden material in plastic containers prior to any further investigation. We sampled different colonies between years and only used sexually mature males, which we defined as males with degenerated testes and the presence of sperm within the accessory testes (Hölldobler and Bartz, 1985; Baer et al., 2009). We used four colonies in 2008 and five colonies in 2009, and sampled 10 males from each colony.

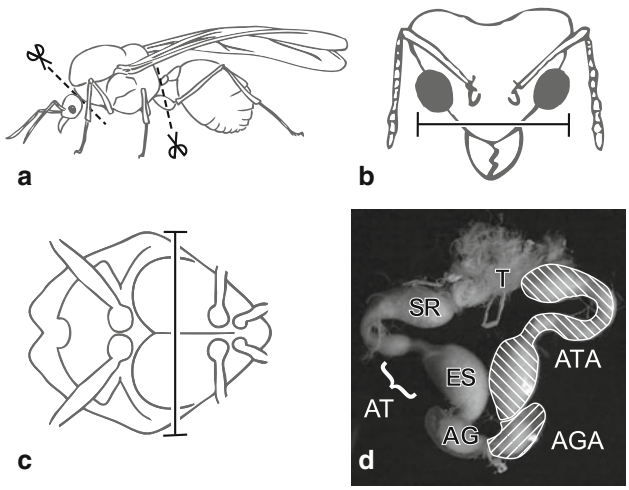

Fig. 1 Measurements of *Atta colombica* male traits. **a** Body parts as they were separated in this study. **b** Dorsal head width and **c** ventral mesosoma width measurements. **d** The reproductive organs of an *A. colombica* male, with the degenerated testes (*T*), the sperm-filled accessory testes (*AT*) made up of the ejaculatory section (*ES*) and sperm reservoir (*SR*), and the accessory glands (*AG*) with transparent AG secretion and the somewhat denser products that will form mating plugs. On the *right hand side* are examples of how the AG area (*AGA*) and AT area (*ATA*) were measured from such photographs

Body size and reproductive organ measurements

To obtain phenotypic measures of body size we anaesthetized males with CO_2 and determined their total body mass to the nearest 0.1 mg using a Sartorius electronic balance. To obtain individual measurements for different male body parts we separated the gaster from the mesosoma at the junction between the propodeum and petiole and the head

from the mesosoma as shown in Fig. 1a. The gaster and mesosoma were then weighed separately. All mass measurements done only in 2009 were fresh masses as we needed to preserve reproductive organs intact to obtain digital photos and sperm counts, so using dry mass was not possible. Additionally, fresh masses represent the weight the males have to lift during a nuptial flight and hence might reflect fitness-relevant covariances in weight measures of the body parts containing wing muscles versus reproductive organs. Head and mesosoma widths were measured to the closest 0.01 mm as indicated in Fig. 1b and c, by taking photographs of each body part with a Leica (DFC 420) camera connected to a Leica MZ12 dissecting microscope at 12.5–25× magnification. Digital images were measured using the software program Leica Application Suite 2.5.0 R1.

To obtain morphological measurements for individual parts of the reproductive organs as indicated in Fig. 1d, we dissected males in Hayes saline (9 g NaCl, 0.2 g $CaCl_2$, 0.2 g KCl, and 0.1 g $NaHCO_3$ in 1,000 ml H_2O). Digital photographs of the sexual organs were taken and analyzed using Image J (freely available at http://rsbweb.nih.gov/ij/). For each male, the two-dimensional area was measured for both AGs, except for 2 of the 50 males sampled in 2009 that only had a single gland measured (because the other gland was not completely level in the photograph, which would have affected the area measurement). We also measured the area of the accessory testes (AT) as the combined area of the ejaculatory section and the sperm reservoir, excluding the associated degenerated testes and AGs (see Fig. 1d). Sixteen males were not included in this 2009 AT series as we were unable to accurately measure AG and AT area concurrently. These excluded males were distributed evenly across the colonies.

Sperm measurements

To estimate sperm number and sperm length, we transferred both accessory testes to 1.5 ml Hayes saline, ruptured them briefly with watchmaker's forceps to facilitate the outflow of sperm, and vortexed the suspension for 30 s. We then obtained a working solution by diluting 5 µl of the original stock solution in 995 µl distilled water. From this final solution we transferred four samples of 1 µl each to a microscope slide and allowed them to air dry. To visualize the sperm cells, we added 3 µl of a DAPI (4′,6-diamidino-2-phenylindole) solution to each sample, obtained by diluting 2 mg DAPI in 1 ml DMSO and further diluting this 500× in Hayes saline. A cover slide was placed over the sample and the total sperm head number for each sample was counted for three out of the four original samples, using a fluorescence microscope (Olympus CX41, EXFO X-Cite 120, at 400–800× magnification). The total number of sperm per male was calculated as the mean of the three individual counts, and multiplied by 300,000 to correct for the dilution factor (Baer et al., 2006a, b; Den Boer et al., 2009a, b). Due to fungal contamination on some of the microscopic slides, sperm counts were not obtained from all males in 2008.

To measure sperm length, we placed a 20 µl subsample of the sperm working solution on a microscopic slide, gently spread out the droplet using a pipette tip and allowed it to air dry (Baer et al., 2009). The slides were then examined under an Olympus CX41 light microscope at 800× magnification, and digital photos of five uncoiled and undamaged sperm were taken using an Olympus C-7070 digital camera attached to the microscope. Sperm length was measured using Image J and was calculated for each male as the mean of the five individual measurements.

To examine differences in sperm viability between individual males and means across males per colony we used five males per colony in 2008 and eight males per colony in 2009. We applied a protocol where males are sedated with CO_2 (to prevent partial ejaculation during dissection in Hayes saline), and sperm survival is measured in vitro in the same saline solution with or without the addition of specific quantities of AG secretion (Den Boer et al., 2008). This protocol has also been shown to be suitable in other species of ants and bees (Den Boer et al., 2010) and although the absolute sperm viability is decreased in the Hayes treatment it allows for informative comparisons of relative sperm viability across groups.

A digital photograph of the sexual organs was taken and the size of the AG was estimated as described above. We then transferred one AG per male to 400 µl Hayes saline and briefly ruptured and vortexed it to facilitate the outflow of AG secretion. The sample was then centrifuged for 2 min at 13,000 rpm to separate the soluble AG secretion from the gland tissue. Two 1 µl sperm samples from the AT's of the same male were either diluted in the AG solution or in 400 µl of Hayes saline only (controls). From each of these two samples 2 µl was used to estimate sperm survival. To do this we used a Live/Dead™ sperm viability kit (L-7011, Molecular Probes), consisting of a membrane-permeant nucleic acid dye that stains live cells green (SYBR 14) and a dye that penetrates dead cells, staining them red (propidium iodide) (Holman, 2009).

To count the number of live and dead sperm per sample, 2 µl of the SYBR 14 working solution (2 µl of SYBR 14 stock in 98 µl Hayes saline) and 1 µl of propidium iodide were added to each sample and incubated on a microscope slide for 10 min in the dark. The number of live, dead and dual-stained sperm cells was counted for a minimum of 300 sperm cells per sample using a fluorescence microscope (Olympus CX41, EXFO X-Cite 120, 400–800× magnification). We detected very few dual stained sperm (0.57%) and as in previous studies (Den Boer et al., 2009a, b;

Den Boer et al., 2010), we discarded these sperm cells from the analyses. From the 2008 males we only determined the sperm survival in 1 µl sperm diluted in 1 ml of Hayes saline and did not add AG secretions to any of the samples.

Statistical analyses

Statistical analyses were performed using SPSS 17.0 and SAS 9.1 for Windows. All morphometric characters were $\log_{10}$ transformed to estimate isometric/allometric relationships between variables. Sperm length measures were also $\log_{10}$ transformed to normalize the distribution of the data. Nested analyses of covariance (ANCOVA) were performed using generalized linear models (GLM) with a normal error structure, with colony and year of collection as random factors (colony nested within year) and size measurements as continuous covariates. Model fitting was carried out by backward elimination of non-significant terms from the full model to leave the minimal adequate model that explained the dependant variable being assessed. To test for the effect of accessory gland secretion and the size of the gland on the percentage of live sperm, we used a GLM with a binomial error distribution and a logit-link function. The sperm viability data were overdispersed, so we corrected for this using a scale parameter estimated from the Pearson χ^2 value of the minimum adequate model.

For all traits measured we estimated the proportion of variance between colonies, as the intra-class correlation t (between colony variance/total variance). Broad sense heritability was then calculated as $h^2 = t/r$, where r is the relatedness (0.5) between brother males. This is a standard full sibling analysis used in quantitative genetics to get a rough estimate of heritability (Falconer, 1981).

Results

Body size measurements

Male body mass differed significantly between colonies, due to differences in gaster mass, but not mesosoma mass (Table 1). Mesosoma widths differed across years (GLM, $F_{1,88} = 60.70$, $P < 0.001$), with males being significantly larger in 2009, but not between colonies nested within years (GLM, $F_{7,81} = 0.74$, $P = 0.64$). In contrast, head width did not differ between years (GLM, $F_{1,81} = 1.97$, $P = 0.20$), but only between colonies within years (GLM, $F_{7,81} = 8.80$, $P < 0.001$) (Table 1). Gaster mass and

Table 1 Summary statistics of variation in body size and reproductive traits of *A. colombica* males originating from four (2008) and five (2009) different colonies sampled in Gamboa, Panama

	Year	Mean	*N*	SE	*F*	*P*	h^2
Body size measures							
Body mass (mg)	2009	172.2	50	2.34	5.95	<0.001	**0.66**
Gaster mass (mg)	2009	71.9	50	1.40	11.58	<0.001	**1.03**
Head width (mm)	2009	2.39	50	0.015	5.55	0.001	**0.61**
Head width (mm)	2008	2.46	40	0.017	14.63	<0.001	**1.15**
Mesosoma mass (mg)	2009	93.6	50	1.26	1.40	0.251	0.08
Mesosoma width (mm)	2009	5.44	50	0.036	0.50	0.733	−0.11
Mesosoma width (mm)	2008	5.06	40	0.033	1.22	0.318	0.04
Reproductive traits							
Sperm length (µm)	2009	130	50	1.51	3.17	0.022	**0.36**
Sperm length (µm)	2008	145	40	1.87	11.75	<0.001	**1.04**
Sperm number	2009	174×10^6	50	5.87×10^6	4.05	0.007	**0.47**
Sperm number	2008	146×10^6	27	11.7×10^6	8.65	<0.001	**1.07**
AG size (mm^2)	2009	2.41	50	0.057	6.56	<0.001	**0.72**
AG size (mm^2)	2008	2.88	40	0.08	1.72	0.180	0.13
AT size (mm^2)	2009	9.83	34	0.18	1.22	0.323	0.07
AT size (mm^2)	2008	10.37	40	0.18	2.43	0.081	0.25
Sperm viability (%) AG treatment	2009	78.32	40	1.30	0.27	0.900	−0.20
Sperm viability (%) Hayes treatment	2009	45.04	40	1.78	1.17	0.340	0.04
Sperm viability (%) Hayes treatment	2008	51.05	20	3.62	1.46	0.264	0.17

Values provided are mean, sample size (*N*) and standard errors across colonies. *F* and *P* values are derived from ANOVAs testing for significant differences between colonies. h^2 values are estimate of maximum heritability, where values are expected to be between 0 and 1, but where sampling error may occasionally produce negative values or values somewhat above 1. High heritability values are marked in bold

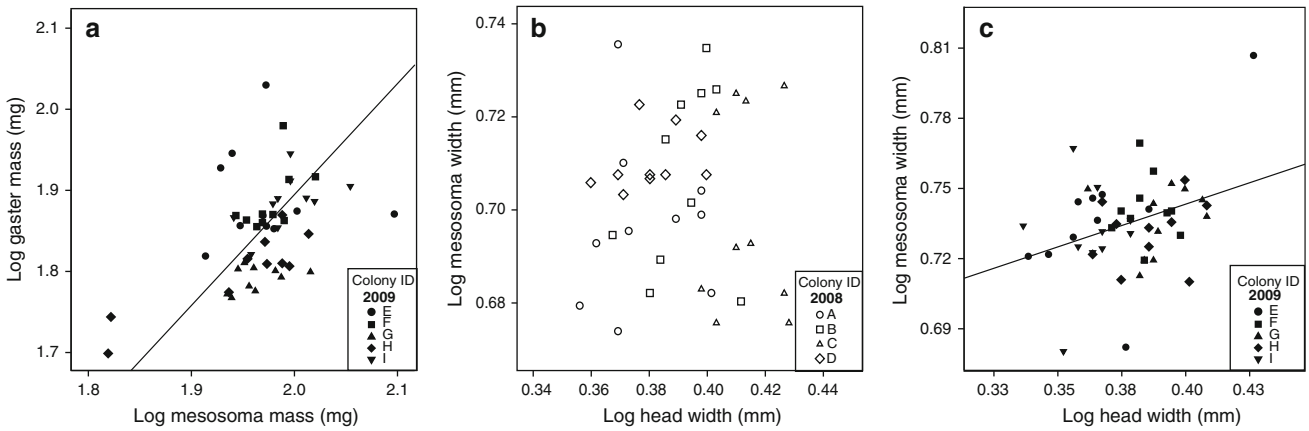

Fig. 2 Double logarithmic plots of body size traits of *A. colombica* males, showing that: **a** mesosoma and gaster mass are positively correlated: the *fitted line* is from a reduced major axis regression (see text for details). **b** Head width did not explain significant variation in mesosoma width in 2008. **c** Head width explained 12% ($P = 0.015$) of the variation in mesosoma width in 2009. *Different symbols* represent colony ID

mesosoma mass were positively correlated after double log transformation, with a major axis correlation slope of 1.37 (95% CL 0.76–2.47) $r = 0.48$, indicating that the relationship between these variables is likely to be isometric (Fig. 2a). When analyzing these mass measurements from 2009 in a GLM with gaster mass as dependent variable, colony as random factor and mesosoma mass as predictor variable we found a significant main effect of colony ($F_{4,44} = 12.30$, $P < 0.001$), and a significant positive relationship between gaster mass and mesosoma mass ($F_{1,44} = 10.76$, $P = 0.002$). The mesosoma × colony interaction was non-significant (GLM, $F_{4,40} = 1.7$, $P = 0.17$), which indicates that the relationship between gaster mass and mesosoma mass is consistent across colonies, but that colonies differed in their mean gaster mass.

Head width was positively related with mesosoma width in 2009 (GLM, $F_{1,48} = 6.38$, $P = 0.015$, colony interaction n.s.), but not in 2008 (GLM, $F_{1,35} = 0.49$, $P = 0.488$) (Fig. 2b, c). In 2009, the relationship between gaster mass and head width was not significant (GLM, $F_{1,44} = 1.92$, $P = 0.173$), whereas the relationship between head width and mesosoma mass was significant and positive (GLM, $F_{1,44} = 10.39$, $P = 0.002$) and after correcting for variations in head width, colony also became significant (GLM, $F_{4,44} = 3.87$, $P = 0.009$, dependent variable: gaster mass or mesosoma mass, colony as random factor and head width as covariate). Neither of the colony interactions were significant (gaster: $F_{4,40} = 0.86$, $P = 0.50$; mesosoma: $F_{4,40} = 1.80$, $P = 0.15$).

Reproductive organs

We found large differences in sperm complement size in *A. colombica* males, both at the colony-level and across individual males, ranging from 22×10^6 to 270×10^6 (mean $164 \times 10^6 \pm 5.8 \times 10^6$ SE) sperm per male, confirming results from earlier studies in this population (Fjerdingstad and Boomsma, 1997). However, sperm survival varied rather little across males from different colonies (Table 1). AG area showed significant differences between colonies in 2009, but not in 2008, whereas AT area did not display colony differences in either year (Table 1). We found a positive relationship between the size of the AG and the AT in both years (GLM, random factors: year and colony(year), covariate: AT size, $F_{1,71} = 18.07$, $P < 0.001$) an effect that was consistent across colonies as the colony(year) × AT interaction term was not significant ($F_{7,56} = 1.53$, $P = 0.344$, Fig. 3a). When adjusted for variation in AT area, AG areas in 2008 were on average significantly larger than in 2009 (GLM, $F_{1,64} = 11.80$, $P = 0.001$). Interestingly, AG area appeared to increase allometrically with AT area, as the slope of the double-log reduced major axis regression between the two variables was >1 (slope = 1.62, 95% CL 1.04–2.53, $r = 0.49$).

There was no correlation between sperm length and sperm number in either year (Pearson correlation; 2008: $r = -0.25$, $P = 0.21$; 2009: $r = +0.05$, $P = 0.72$). When analyzing these data with a GLM, nesting colonies within years and with sperm number as dependent variable, colony and year as random factors, and sperm length as covariate we also found no significant relationship between sperm length and number either overall (GLM, $F_{1,67} = 1.25$, $P = 0.27$), or within colonies (GLM, $F_{7,59} = 1.30$, $P = 0.27$) (Fig. 3b). Sperm number did not correlate significantly with head width (GLM, $F_{1,51} = 1.12$, $P = 0.29$), mesosoma width (GLM, $F_{1,50} = 0.14$, $P = 0.71$) AT size (GLM, $F_{1,49} = 0.08$, $P = 0.78$), and none of the colony interactions were significant. We found no relation between sperm number and body mass (GLM, $F_{1,44} = 0.61$, $P = 0.44$,

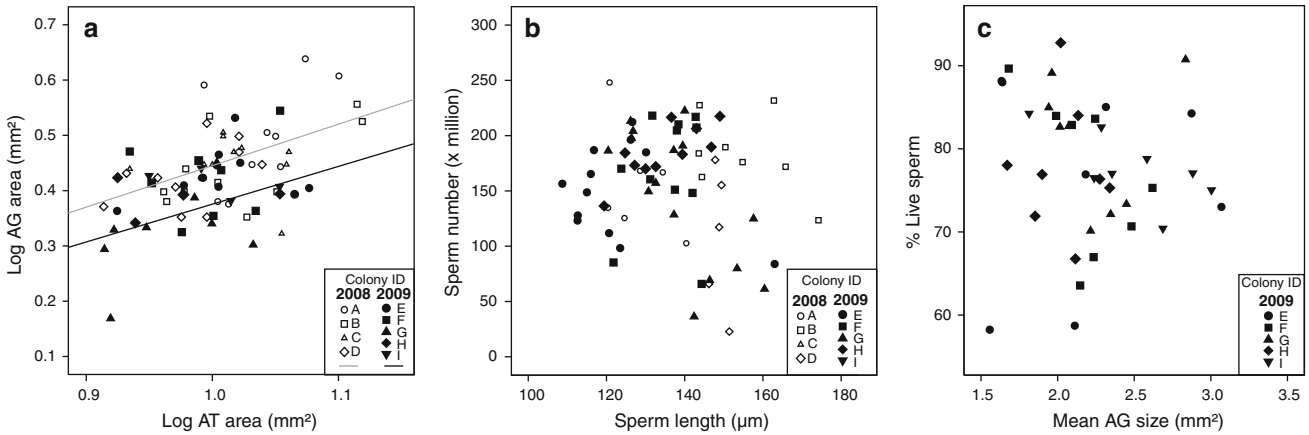

Fig. 3 Plots illustrating variation and co-variation of key reproductive traits of *A. colombica* males. **a** Accessory testes (AT) area is a reasonable predictor of accessory gland area, but the relationship between them differed between years. **b** Sperm length did not explain significant variation in sperm number. **c** AG area did not explain significant variation in the percentage of sperm surviving in the Hayes saline assays. *Different symbols* represent colony ID, *open symbols* 2008, *closed symbols* 2009

colony interaction ns). Across colonies, sperm length was not significantly related with head width (GLM, $F_{1,72} = 0.003$, $P = 0.96$), mesosoma width (GLM, $F_{1,71} = 0.55$, $P = 0.46$) or AT size (GLM, $F_{1,53} = 0.32$, $P = 0.58$) in either year, but the colony (year) interactions were significant for head with ($F_{8,72} = 3.43$, $P = 0.002$) and AT size ($F_{8,56} = 2.16$, $P = 0.045$).

As the average dilution factors of sperm in Hayes saline in 2008 and 2009 were somewhat different, the mean sperm survival percentages could not be compared between years. In the 2009 assays, sperm survival increased significantly after adding accessory gland secretion ($\chi^2 = 7.14$, $df = 1$, $P = 0.008$), confirming earlier results for the same species (Den Boer et al., 2008). In neither of the 2 years did the survival of sperm exposed to Hayes without AG secretion differ significantly between colonies (Table 1). Sperm survival was not correlated with the size of the accessory glands from which seminal fluid was added to the Hayes solution in 2009, after adjusting for colony level differences ($\chi^2 = 0.19$, $df = 1$, $P = 0.664$) (Fig. 3c). Hence, although a male's sperm survival increased when the sperm was accompanied by AG secretion in a saline environment, there was no indication that this effect was stronger or weaker in males with larger glands.

Heritabilities of body size and reproductive traits

Considerable overall differences in broad sense heritabilities were found for the different traits. Head width, gaster mass, and total body mass had high heritabilities, but mesosoma mass and mesosoma width had low heritabilities (Table 1). A similar dichotomy was found for reproductive organ traits, where heritabilities for sperm length and sperm number tended to be high, whereas heritabilities for sperm survival and the size of the accessory testes tended to be low (Table 1). The results for the accessory glands remain inconclusive as we only found colony variation in 2009 but not in 2008.

Discussion

We believe that our study is the first to systematically measure morphological and genital traits of ant males in the same population across different years. Given that excavating mature *Atta* colonies is laborious, our sample sizes remained moderate. Nonetheless, our study allows a number of conclusions and inferences that shed light on the reproductive biology of *A. colombica* and that may stimulate further work on male traits in social insects.

Heritabilities vary across traits and years

There is a general tendency of heritability being low for traits that directly affect fitness, whereas traits that are relatively neutral tend to have high heritabilities (Stearns, 1992). This is consistent with the notion that selection will tend to remove genetic variation for traits in proportion to the degree of differential reproductive success of the bearers of these traits. The bimodal distribution of broad-sense heritabilities that we obtained (Table 1) therefore offers some interesting opportunities for further study. The low heritabilities for mesosoma mass, mesosoma width, the size of the accessory testes, and relative sperm viability (measured as % live sperm in a mildly stressful saline solution; Den Boer et al., 2008, 2009a, b, 2010) suggest that these traits have a long history of stabilizing selection, with only a single optimal state and little genetic variation. This

seems obvious for sperm viability, as strong selection on the production of high quality ejaculates is expected in this species where queens can potentially store sperm for 10–20 years (Boomsma et al., 2005).

The mesosoma result is interesting as it suggests that flight muscle mass (likely a correlate of mesosoma width and mass) in *A. colombica* males also has a single species-specific optimum value. Fjerdingstad and Boomsma (1997) obtained similar results using the same leafcutter ant population. However, in contrast to our results using fresh masses, they found a significant intra-class correlation when dry mesosoma masses were analyzed. This implies that the optimal functioning of flight muscles also depends on nutrients circulating in the hemolymph and/or higher water content related to overall biological activity. In the same study Fjerdingstad and Boomsma (1997) analyzed mesosoma masses of virgin queens and here neither mesosoma fresh mass nor dry mass varied significantly between colonies, suggesting that mesosoma size may be under stabilizing selection in both sexes, and likely correlated with sexual performance. Further studies on other ants would be worthwhile to analyze whether stabilizing selection on body size parameters associated with flight muscle mass is generally found across the sexes in ants that need dispersal by flight before mating. Comparisons between sister species differing in this habit would be particularly interesting, as we would expect that species that have secondarily lost mating flights in one or both sexes should have much higher heritabilities for mesosoma mass (width). In species that produce both winged and wingless queens, the wingless morph often bears the closest resemblance to the worker caste because of similar reductions in mesosoma size following the loss of flight muscles (Peeters, 1991; Peeters and Ito, 2001). This should imply that selection on queen mesosoma size is relaxed, increasing the heritability of this trait. Unicolonial ant species that have adopted intranidal mating and their close relatives would therefore also be promising models to further test this idea (Cremer et al., 2008).

Relatively high broad-sense heritabilities were found for sperm length, sperm number, head width and gaster mass. While this is probably unsurprising for head width and sperm length (known to be highly variable also from previous studies (Fjerdingstad and Boomsma, 1997; Baer et al., 2009), it is striking that a trait such as sperm number has such high heritability as this suggests that sperm number per se is not an accurate predictor of male fitness. However, given we were sampling males from different colonies in the field we here provide broad sense heritabilities that may overestimate the corresponding narrow sense heritabilities (Falconer, 1981) in case of systematic maternal or environmental effects. Such effects could also explain the between year variations we found in heritabilities of some traits such as sperm number and length. High heritability values for sperm length has been reported for other social insects such as bumblebees where a recent study estimating narrow sense heritability of sperm length reported values in the range of 0.2–0.4, i.e. also higher than expected for a trait that is directly correlated with fitness (Baer et al., 2006a, b). Future work will therefore be needed to quantify the magnitude of non-genetic effects on male reproductive traits. It should also be noted that heritability estimates must be interpreted with caution as high trait heritability does not necessarily rule out that the trait is important for fitness. However, this would likely require trade-offs resulting in a bimodal fitness optimum, which does not seem likely as a general explanation.

Ejaculate expenditure has been shown to be condition-dependent in many species (Gage, 1991, 1994; Birkhead et al., 1995) and can be adjusted according to resource availability or body condition in most species. However, this would be unlikely to apply in social hymenopteran males, where resource allocation into sperm production happens at an early stage so that sperm length and sperm number are fixed when males reach sexual maturity. Selection on individual male traits is therefore likely to be complex and may have a substantial colony level component (see also Baer et al., 2006a, b) that optimizes average quality of a large cohort of brothers rather than the fitness of every individual male (Boomsma et al., 2005). As sperm production is determined by larval resource availability, we expected that larger males would also be able to invest more in sperm production than smaller males, creating a positive correlation between sperm number and body size at the individual level, as has been found in honeybees when comparing males from drone cells with males reared in worker cells (Schlüns et al., 2003). We did not find this correlation. Male size did not predict sperm complement size, i.e. larger males did not necessarily produce and possess more sperm, even though such a correlation was previously reported by Fjerdingstad and Boomsma (1997)—albeit they used dry mass and not fresh mass as the present study. Additionally, there was no relationship between sperm length and male body size (head width and mesosoma width) in either year, which contrasts with some bumblebee species where sperm length was positively correlated with body size (Baer, 2003; Baer et al., 2006a, b).

To test for a possible trade off between sperm number and sperm length, we tested for a significant correlation between these two variables. Although we found a weakly negative trend overall (Pearson r all data combined for both years, $r = -0.217$, $P = 0.058$) (Fig. 3b), this trend was non-significant within or across colonies (see "Results"). This suggests that it is not sperm number per se, but sperm in combination with seminal fluid (the amount and potency of accessory gland secretion) that determines male reproductive

success. This would be consistent with recent results by Den Boer et al. (2010), which show that AG secretion is crucial for maintaining high sperm viability during insemination. The high heritability of gaster mass suggests that total genital mass may not be tightly correlated with male fitness.

The allometric rather than isometric relationship between AG area plotted against AT area (Fig. 3a) seems puzzling as such relationships have previously only been found for body size measures such as head widths of ant soldiers (Hölldobler and Wilson, 1990). This indicates that AG volume increased disproportionally with AT sperm content in the male samples that we measured. However, as these males may have differed somewhat in their "readiness to fly" (in spite of all being sexually mature), we refrain from interpreting this allometry until further data would confirm that this relationship is real. The difference in relative AG area across years is also interesting, and suggests that season-specific provisioning during maturation may affect AG volume, whereas the sperm complement in the AT is likely determined in the earlier period of larval growth.

Measuring trade-offs in the wild: intraspecific and interspecific patterns

As outlined by Van Noordwijk and de Jong (1986) it is difficult to infer evolutionary trade-offs (i.e. negative genetic correlations) from phenotypic correlations obtained from field data. The reason is that individuals from a field population have usually experienced very different resource conditions during development and maturation, so that many life-history traits will show positive correlations because of these overall differences that hide underlying negative genetic correlations. Our study may have suffered from such limitations because the colonies that we excavated to obtain our samples are unlikely to be fully comparable in terms of colony size, and size and quality of the foraging territory. Where possible, we therefore compared relative measures, adjusted for differences in colony-specific trait values, but we still found only a weakly negative overall correlation for the possible trade-off between sperm length and sperm number discussed above. Another trade-off that we expected to find was between mesosoma mass and sperm number, as this negative relationship had been reported earlier by Fjerdingstad and Boomsma (1997). However, we were unable to reconfirm this relationship in our data.

In a previous study, Baer and Boomsma (2004) found a negative correlation between AT and AG size across 11 different species of attine ants, whereas the present study reports a positive correlation between these same traits among individuals of a single species. These results do not contradict each other: AG secretions as the main contributors to seminal fluid and mating plug material have likely been under different selection regimes in different species (Baer, 2010). So, while it seems logical that across males of the same population a larger sperm complement requires a larger volume of seminal fluid, this trend can easily be reversed when separate species evolve different mating systems as is known to have happened in the fungus-growing ants (Villesen et al., 2002). When multiple mating evolves, the relative investment in mating plugs may change significantly (Baer and Boomsma, 2004), while also sperm length (Baer et al., 2009) and the time between insemination and final sperm storage (Den Boer et al., 2010) may become adjusted.

Conclusions and perspectives

The data for the Fjerdingstad and Boomsma (1997) study were collected in 1992, i.e. 16–17 years before the data for the present study were collected. Considering an average longevity of *A. colombica* colonies of 8.5 years (Den Boer et al., 2009a, b), this implies that this time period likely represents 2–3 generations. While we confirmed some of the results of this early study, other patterns did not match and we even found differences between some of the male characteristics between consecutive years. This underlines that body size and reproductive traits of *A. colombica* males are likely to be shaped by a complex set of genetic and environmental factors that affect colonies to different degrees and that have different magnitudes over time.

The same complexity that makes it difficult to extrapolate results from a specific year or colony sample also provides a perspective on the possible insights that more systematic studies of social insect males might offer. We already mentioned the potentially interesting contrast in expected heritabilities of specific traits between unicolonial and normally eusocial sister species of ants. It may be of similar interest to compare heritabilities of male traits between species where male life histories differ, such as *Cardiocondyla* ants with their (exceptional) continuing sperm production, and swarming males of army ants and honeybees that die immediately after mating and of whom only a very small fraction will ever copulate (Baer, 2005; Kronauer et al., 2007). Other interesting contrasts could be comparing males from species with male-aggregation and female-calling syndromes (Hölldobler and Bartz, 1985) and comparing males of species with multiply mated queens (i.e. where ejaculates compete) with males of monogamous sister species (Hughes et al., 2008; Den Boer et al., 2010).

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