

Genetic caste polymorphism and the evolution of polyandry in *Atta* leaf-cutting ants

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Abstract Multiple mating by females with different males (polyandry) is difficult to explain in many taxa because it carries significant costs to females, yet benefits are often hard to identify. Polyandry is a derived trait in social insects, the evolutionary origins of which remain unclear. One of several leading hypotheses for its evolution is that it improves division of labour by increasing intra-colonial genetic diversity. Division of labour is a key player in the ecological success of social insects, and in many successful species of ants is based on morphologically distinct castes of workers, each with their own task specialisations. *Atta* leaf-cutting ants exhibit one of the most extreme and complicated forms of morphologically specialised worker castes and have been reported to be polyandrous but with relatively low mating frequencies (~2.5 on average). Here, we show for the first time that there is a significant genetic influence on worker size in *Atta colombica* leaf-cutting ants. We also provide the first estimate of the mating frequency of *Atta cephalotes* (four matings) and, by analysing much higher within-colony sample sizes, find that *Atta* are more polyandrous than previously thought (approximately six to seven matings). The results show that high polyandry and a genetic influence on worker caste are present in both genera of leaf-cutting ants and add weight to the hypothesis that division of labour is a potential driver of the evolution of polyandry in this clade of ants.

Keywords Division of labour · Caste · Social evolution · Attini · Morphology

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Introduction

The phenomenon of multiple mating by females (polyandry) is taxonomically widespread across animals but is often hard to explain because it is costly to females in terms of exposure to sexually transmitted parasites, risk of predation, and energy expended. It is particularly interesting in the social Hymenoptera (ants, bees, and wasps) because, unlike in most animals, polyandry is relatively rare (two thirds of social hymenopteran species are obligately monandrous; Hughes et al. 2008), it can reach extremely high levels (e.g. *Apis dorsata* queens mate with over 100 males; Wattanachaiyingcharoen et al. 2003), and many of the most commonly identified benefits in other animals do not apply (e.g. direct material benefits or trading up; Crozier and Fjerdingstad 2001). The costs of polyandry may also be relatively high in social insects because the mating flight, where the queen leaves the nest to mate, is the riskiest period of a queen's life as she is not protected by workers (Baer et al. 2006; Fowler et al. 1986; Weber 1972).

The occurrence of polyandry in the social Hymenoptera in spite of its costs suggests there must also be benefits (Crozier and Fjerdingstad 2001). The three best-supported hypotheses are that polyandry dilutes genetically incompatible matings (Simmons 2001; Zeh and Zeh 1997), increases the disease resistance of offspring colonies (Baer and Schmid-Hempel 1999; Hamilton 1987; Hughes and Boomsma 2004; Sherman et al. 1988; Tapy 2003), and improves division of labour in colonies (Mattila and Seeley 2007; Jones et al. 2004; Oldroyd and Fewell 2007). If genotypes vary in their propensity or ability to carry out particular tasks, then the increased intra-colonial genetic diversity generated through polyandry may enable colonies to efficiently adapt their task allocation to changing conditions (Oldroyd and Fewell 2007).

It is notable that seven of the ten known occurrences of extreme polyandry are in derived taxa with large societies and complex division of labour (Hughes et al. 2008; Chappell et al. unpublished data). In two of these (army ants and leaf-cutting ants), division of labour is determined primarily by the morphological caste of workers. Although environmental factors were long assumed to be the sole determiner of caste differentiation (Oster and Wilson 1978; Robinson 1992), it is now clear that genetic effects can also be important. The propensity to develop into different worker castes has been shown to vary between patriline in *Acromyrmex* leaf-cutting ants (Hughes et al. 2003), and evidence of similar genetic influences on worker castes have also been found in harvester ants (Rheindt et al. 2005; Wiernasz et al. 2004), wood ants (Schwander et al. 2005), army ants (Jaffé et al. 2007; reviewed in Schwander et al. 2010), and honey bees (Frumhoff and Baker 1988; Kolmes et al. 1989). In fact, intra-colonial genetic diversity and species with caste systems exhibiting worker polymorphism appear to be positively correlated across ant taxa (Fjerdingstad and Crozier 2006). This is possibly because the high genetic variation generated through polyandry, combined with a genetic influence on worker caste determination, increases homeostasis in systems with complex division of labour because having multiple worker genotypes improves the colony's response to changing environmental conditions (Crozier and Page 1985; Fjerdingstad and Crozier 2006; Hughes and Boomsma 2007; Kraus et al. 2011; Oldroyd and Fewell 2007).

One group in which polyandry has been surprisingly little investigated is the *Atta* leaf-cutting ants. The very large colonies of these ants, with millions of workers, exhibit one of the most extreme and complex examples of division of labour, based on worker castes that are morphologically adapted for particular tasks in an agricultural production line (Wilson 1980; 1983). The smallest workers (minors, headwidths of <1.4 mm) do not only mainly work in the nest, caring for brood and tending the fungus garden, but also occur on foraging trails where they patrol for threats, clean incoming leaf material, and reinforce pheromone trails (Evison et al. 2008; Feener and Moss 1990; Griffiths and Hughes 2010; Hughes and Goulson 2001). Medium-sized workers (medias, headwidths of 1.5–3.0 mm) include foragers and ants that remove waste (Hart and Ratnieks 2001). The largest workers (majors, headwidths of >3.0 mm) are usually associated with colony defence (Wilson 1980) and cutting up large materials (Evison and Ratnieks 2007; Helanterä and Ratnieks 2008). This complex division of labour has led *Atta* to becoming ecologically dominant herbivores in the Neotropics (Hölldobler and Wilson 1990). These characteristics make *Atta* one of the preeminent examples of complex division of labour, but the mechanisms responsible for this alloethism have never been investigated.

Atta queens are amongst the most long-lived and fertile insects known (Hölldobler and Wilson 1990). In *Atta colombica* and *Atta sexdens*, 96–100% of females have been reported to mate multiply with one to five males, with average, observed mating frequencies of 2.6–2.8 and effective mating frequencies of 1.89–2.72 (Baer et al. 2006; Fjerdingstad et al. 1998; Fjerdingstad and Boomsma 1998; 2000; Helmkampf et al. 2008). The level of polyandry previously reported in *Atta* is, therefore, significantly lower than that of *Acromyrmex* leaf-cutting ants which have been observed to have effective mating frequencies on an average of >6 and >4, respectively (Ortius-Lechner et al. 2000; Sumner et al. 2004). This difference is surprising given that *Atta* have much larger and more complex societies and that hypotheses for polyandry suggest a positive, rather than negative, relationship with colony size and complexity. Although polyandry in *Atta* has been shown to involve costs (Baer et al. 2006), there have been no previous investigations into the benefits of polyandry in this genus.

Here, we utilise microsatellite genotyping to measure levels of polyandry in *Atta*. We compare the size of workers in different patriline to test the hypothesis that a genetic influence on worker size and caste polymorphism extends to *Atta* leaf-cutting ants, which would then provide the potential for polyandry to improve division of labour. Importantly, we use far higher sample sizes per colony than in previous studies in order to detect rarer patriline and obtain more accurate estimates of mating frequency. We provide revised estimates for *A. colombica* queens and the first report of the mating frequency exhibited by *Atta cephalotes*.

Materials and methods

Ant sampling

Workers of consistent cuticle colouration (to reduce variation in age) covering the full size range, from minors to majors (headwidths, 0.6–3.1 mm), were sampled from the fungus gardens of four monogynous *Atta* colonies. Samples of 96 workers were collected from two mature *A. colombica* colonies (AtcoP and AtcoB) in the field in Gamboa, Panama, in May 2008 and 2009, respectively. Samples of 192 workers were collected in May 2009 from each of two laboratory colonies, one of *A. colombica* (Atco089) and one of *A. cephalotes* (Atce081). Both of these colonies had been collected in Gamboa, Panama, in May 2008 when they were one year old. They were maintained in the laboratory at 27±2°C and 85±5% RH on a diet of privet leaves (*Lingustrum vulgare*). As well as the

sample taken from the fungus garden, an additional sample of 96 workers was collected from the foraging arena of Atco089 in November 2009 to enhance the mating frequency estimate for this colony. All samples were stored individually in 96% ethanol at -20°C .

Genetic analysis and morphometrics

DNA was extracted from individual ant legs using 150 μl of Chelex and amplified at four microsatellite loci: *Atco12*, *Atco13*, *Atco15*, and *Ech4225* (Helmkamp et al. 2008; Ortius-Lechner et al. 2000). Reactions were performed in 10 μl volumes with 1 μl of DNA. The DNA was amplified in ABI 3700 thermal cyclers using an initial denaturation step of 94°C for 2 min, followed by 30 cycles of 94°C for 30s, an annealing step (*Atco12*: 66°C ; *Atco13*: 65°C ; *Atco15*: 62°C ; and *Ech4225*: 54°C) for 45 s, an elongation step of 72°C for 2 min, and a final elongation step of 72°C for 7 min. Amplified products were multiplexed and run on an ABI 3130xl capillary sequencer. Allele sizes were scored by comparison with internal size markers using Genemapper[®] software. Multilocus offspring genotypes were used to deduce the genotypes of colony queens and their multiple mates, and the workers were assigned to patriline within their colony with low detection errors (1.4% for *A. colombica* and 7.6% for *A. cephalotes*; Boomsma and Ratnieks 1996). Individuals that could not be assigned to patriline due to failed PCR amplification or because the individual shared the same alleles at a diagnostic locus as its heterozygous mother queen were excluded from the analysis (Atco089, 5% of individuals; AtcoB, 4%; AtcoP, 0%; and Atce081, 58%). Ant headwidths were measured using a stereomicroscope, with a calibrated camera (Moticam 2000, 2.0 Megapixel Digital Colour Camera) attached to one eyepiece.

The effective mating frequency (k_{eff}), which takes into account paternity skew, was calculated following Nielsen et al. (2003) using the equation:

$$k_{\text{eff}} = \frac{(n-1)^2}{\sum_{i=1}^{k_0} \hat{P}_i^2 (n+1)(n-2) + 3 - n}$$

Where n is the worker sample size, p is the proportion of worker paternity of the i th father, and k_0 is the number of different fathers within the worker sample (i.e. the observed mating frequency). The variance for an individual estimate using this estimator is approximately:

$$V[k_{\text{eff}}] = \frac{2k^2(k-1)(n-2)^2(n-1)n(n+1)^2}{(2k + (n-2)(n+1))^4}$$

The estimated mating frequency (k_{est}) was calculated following Tarry and Nielsen (2002) using the equation:

$$k_{\text{est}} = k_0 - \left[k_0 \left(1 - \frac{1}{k_0} \right)^n \right]$$

Differences in headwidths between patriline were tested using Welch's one-way analysis of variance (ANOVA; to take account of the unequal samples sizes between patriline; Welch 1951) on each colony separately (because we found a highly significant difference in headwidths between colonies (ANOVA, $F_{3, 538}=95.41$ and $P<0.001$) but also because we had two species), using R 2.11.1 statistical software (R Development Core Team 2009). Effect sizes (f) were calculated using GPower 3.0.10.

Results

Mating frequency and patriline distribution

All four of the colonies sampled were polyandrous, with the three *A. colombica* colonies containing six to seven patriline, and the *A. cephalotes* colony containing four patriline. A large proportion of individuals within the *A. colombica* colonies were found to belong to two patriline, with 24–36% of workers per patriline, and the remaining patriline were rarer, with 3–16% of workers per patriline (Fig. 1). As a species, *A. colombica* had a mean \pm SE observed mating frequency of 6.33 ± 0.29 , and effective mating frequency of 4.38 ± 0.15 (Atco081, $k_0=7$ and $k_{\text{eff}}=4.343$; AtcoB, $k_0=6$ and $k_{\text{eff}}=4.102$; and AtcoP, $k_0=6$ and $k_{\text{eff}}=4.697$). The *A. cephalotes* colony Atce081 had a different structure with an observed mating frequency of four and an effective mating frequency of 2.27. In this colony, a large number of workers could not be assigned to a specific patriline due to the mother queen and her mates sharing alleles at diagnostic loci, but of the 80 workers that were assigned, four patriline were detected, with one patriline containing 63% of workers and the other three rarer with 9–15% of workers per patriline (Fig. 1). As a result of the large numbers of individuals genotyped and successfully assigned to patriline, the estimated mating frequencies were practically identical to the observed mating frequencies in all colonies. Even in the *A. cephalotes* colony where many individuals could not be assigned to patriline, the mating frequency estimates appear to be robust.

Size differences between patriline

All three *A. colombica* colonies showed significant differences in headwidths between patriline (Atco089, $F_{6, 15}=4.28$, $P=$

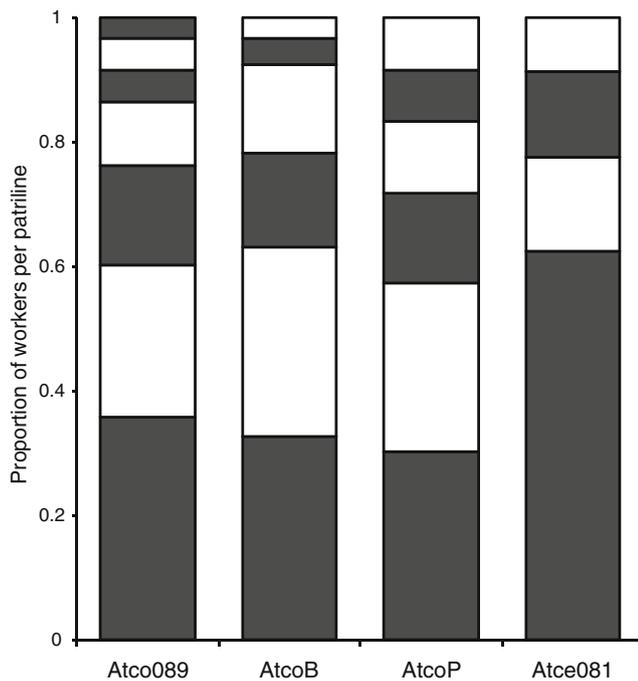
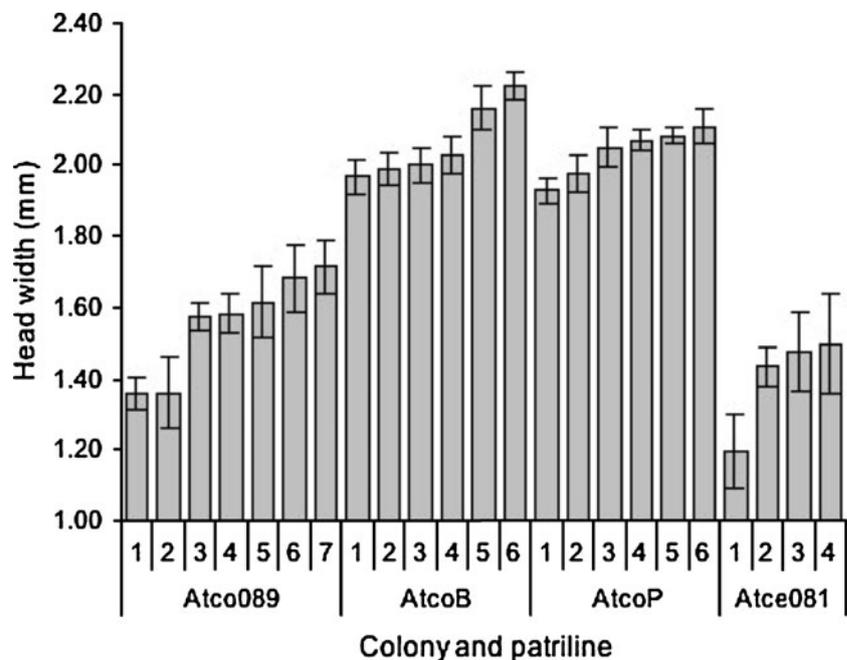


Fig. 1 Patriline representation in the four *Atta* colonies examined. *Atta colombica* colonies (Atco089, $n=182$; AtcoB, $n=92$; and AtcoP, $n=96$) contained a similar number of patrilines (six to seven) and paternity skew, with two patrilines being more common. The *A. cephalotes* colony (Atce081, $n=81$) contained only four patrilines, with one patriline accounting for more than 60% of workers sampled

0.001; AtcoB, $F_{5, 15}=4.50$, $P=0.01$; and AtcoP, $F_{5, 28}=3.17$, $P=0.02$; Fig. 2). The effect sizes (f) were 0.321, 0.32, and 0.407 for Atco089, AtcoB, and AtcoP, respectively (mean \pm SE=0.350 \pm 0.029), which are medium to large following

Fig. 2 Differences between headwidth of ants from the different patrilines within the three *A. colombica* colonies (Atco089, $P=0.001$; AtcoB, $P=0.02$; and AtcoP, $P=0.01$), and the *A. cephalotes* colony (Atce081, *N.S.*). Bars show mean \pm standard error



Cohen (1988). The *A. cephalotes* colony, Atce081, had one patriline that contained smaller ants on average than the other patrilines, however this was not a significant effect ($F_{3, 19.1}=1.63$, $P=0.216$; Fig. 2), and the effect size f was 0.198.

Discussion

Here, we show a genetic influence on worker size in *A. colombica* leaf-cutting ants, providing further evidence of genetic caste determination in ants in general, and the potential for polyandry to improve division of labour. In addition, we give the first report of the mating frequency of *A. cephalotes*, and a higher mating frequency than previously found for *A. colombica*. The observed mating frequency of *A. colombica* has previously been reported as 2.5 or 2.6 on average (range, 1–5; Baer et al. 2006; Fjerdingstad and Boomsma 1998, 2000; Helmkampf et al. 2008), whereas we found an average observed mating frequency of 6.33 males. Importantly, all of the above estimates, including ours, come from the same population. The observed reproductive skew in paternity may explain the previous underestimates of mating frequency in this species. In previous studies of *A. colombica* genetic structure, either small sample sizes per colony were used for genotyping (e.g. Helmkampf et al. (2008) genotyped workers from 55 colonies but only ten workers per colony), or low numbers of microsatellite loci were used (e.g. Baer et al. (2006) genotyped sperm from the spermathecae of queens but used only two microsatellite loci). Similar differences are seen in the estimates for *A. sexdens*, with one study reporting two to three matings (Fjerdingstad and

Boomsma 2000) and a later study with double the sample size reporting five or six matings (Armitage et al. 2010). The differences found between the estimates highlight the importance of sampling many workers per colony, and using sufficient variable microsatellite loci to detect rarer patriline and determine accurately the observed mating frequency. While rarer patriline contribute relatively few sperm, accurate estimates of mating frequency are essential for understanding the costs of mating and benefits based on behaviourally dominant traits.

Caste determination has been shown to be partially genetically controlled in some species of social insect (reviewed by Schwander et al. 2010). There are rare cases of hard-wired genetic control of caste fate (usually a quantitative trait) such as in the complex hybrid matings of *Pogonomyrmex* harvester ants (Cahan et al. 2002; Cahan and Keller 2003; Julian et al. 2002; Völny and Gordon 2002), the queen polymorphisms of *Hapagoxenus* slave-making ants (Winter and Buschinger 1986), and the parthenogenetic systems of *Cataglyphis* (Pearcy et al. 2004) and *Wasmannia* (Fournier et al. 2005). Then there are the more common cases of plastic genetic control of caste fate, most probably involving genetic influences on the responses to environmental cues, as seen in *Acromyrmex* leaf-cutting ants (Hughes et al. 2003; Hughes et al. 2008), *Pogonomyrmex* harvester ants (Smith et al. 2008), *Eciton* army ants (Jaffé et al. 2007), and *Formica* wood ants (Schwander et al. 2005; see Anderson et al. 2008 and Schwander et al. 2010 for reviews). In this study, we do not only show a genetic link to worker size in *A. colombica* but also that all patriline produced a range of worker sizes and thus castes. Patriline, therefore, influences, rather than determines, the size of worker that an individual will develop into, showing that it is an effect also influenced by environmental cues. This highlights the likely plastic nature of this genetic control over caste determination, as shown in other social hymenoptera that have a genetic influence on caste determination (Hughes et al. 2003; Hughes and Boomsma 2007, 2008; Jaffé et al. 2007; Schwander et al. 2005; Tilley and Oldroyd 1997; Osborne and Oldroyd 1999; Châline et al. 2003; Moritz et al. 2005). Two of the colonies were relatively young (2 years old) with a more restricted worker size range than mature colonies, and all of our samples were focused on the media caste with relatively few minors or majors, so the effect of patriline on size may well be much stronger than we observed. Although differential sperm use over long periods of time (6 months or more) has been reported in *Pogonomyrmex* harvester ants (Wiernasz and Cole 2010), this is unlikely to explain our results. Our sampling protocol minimised variation in age since eclosion, such that differences in absolute age will have been far less than 6 months even if worker sizes of the range in our samples differ substantially in developmental

rates, which has not yet been shown. The smaller sample size for the *A. cephalotes* colony meant that the analysis had lower statistical power, and it is therefore possible that a similar genetic influence on size is present in this species as well.

There are a number of possible explanations for the high polyandry shown by *Atta* leaf-cutting ants (Crozier and Fjerdingstad 2001), of which three non-mutually exclusive hypotheses have some empirical support. Polyandry in *Atta* is most likely to have evolved: (1) to dilute the effects of sexually incompatible matings, which in social Hymenoptera can result in diploid males (Armitage et al. 2010); (2) to improve the resistance of offspring colonies to disease, as is seen in *Acromyrmex* leaf-cutting ants (Hughes et al. 2010; Hughes and Boomsma 2004, 2006); or (3) to improve division of labour, which in the case of the caste-based division of labour in *Atta* would require a genetic influence on worker size. Queens are unlikely to mate multiply simply to gain more sperm (Cole 1983) because the number of sperm stored by an *A. colombica* queen (124×10^6) is very similar to the number carried by a single male (130×10^6 ; Fjerdingstad and Boomsma 1997), and because this would then select strongly for males to evolve sufficient ejaculates to fully inseminate a queen (as they in fact do in most social insects; e.g. Fjerdingstad and Boomsma 1997; Franck et al. 2002; Reichardt and Wheeler 1996; Tasei et al. 1998).

The new estimate of mating frequency in *Atta* is very similar to that for *Acromyrmex*, suggesting that the selective forces for polyandry are similar. All other attines are exclusively monogamous (Villesen et al. 2002). It is important to note that *Atta* colonies are two orders of magnitude larger than *Acromyrmex* colonies and with greater variation in worker size. It also seems unlikely that the costs of mating are lower in *Atta* and *Acromyrmex* than in other higher attines, or that the costs of genetically incompatible matings or pressure from parasites is higher, making these too seem unlikely explanations for the evolution of polyandry in attines. The most notable difference between the *Atta* and *Acromyrmex* leaf-cutting ants and the other attines is their evolution of polymorphic worker castes and more complex processing of fresh leaves, making improved division of labour a likely driver of the evolution of polyandry in the clade.

Considerable interspecific variations in mating frequencies have been found in other clades with high polyandry, with the average observed mating frequencies of 12 to 55 in honeybee species (Tarpay et al. 2004), and 1.3 to 11 in *Pogonomyrmex* harvester ants (Holbrook et al. 2007; Rheindt et al. 2004). This variation highlights the need for more species mating frequency estimates to fully understand the evolutionary biology of polyandry. Moreover, it is likely that the evolution of morphologically distinct castes

and behavioural specialisations seen in many social insects is a complex interaction between environmental and genetic factors promoted through the enigma that is polyandry.

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