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Protein-Level Interactions as Mediators of Sexual Conflict in Ants

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ABBREVIATIONS:

AEBSF: 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, serine protease specific inhibitor

AT: accessory testes

AGs: accessory glands

ANOVA: analysis of variance

DIGE: differential in-gel electrophoresis

EDTA: Ethylenediaminetetraacetic acid, specific inhibitor of carboxypeptidases

ESI-Q-ToF: electrospray ionization Q-TOF

FA: formic acid

FDR: false discovery rate

GLM: generalized linear model

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

HMW: high molecular weight

h: hours

IDGF4: imaginal disk growth factor 4

IEF: isoelectric focussing

LMW: low molecular weight

min: minutes

PEBP: phosphatidylethanolamine-binding protein

RT: room temperature

s: seconds

SF: seminal fluid

SpF: spermathecal fluid

ABSTRACT

All social insects with obligate reproductive division of labour evolved from strictly monogamous ancestors, but multiple queen-mating (polyandry) arose *de novo* in several evolutionarily derived lineages. Polyandrous ant queens are inseminated soon after hatching and store sperm mixtures for a potential reproductive life of decades. However, they cannot re-mate later in life and are thus expected to control the loss of viable sperm because their life-time reproductive success is ultimately sperm-limited. In the leaf-cutting ant *Atta colombica,* the survival of newly inseminated sperm is known to be compromised by seminal fluid of rival males and to be protected by secretions of the queen sperm storage organ (spermatheca). Here we investigate the main protein-level interactions that appear to mediate sperm competition dynamics and sperm preservation. We conducted an artificial insemination experiment and DIGE-based proteomics to identify proteomic changes when seminal fluid is exposed to spermathecal fluid, and then a mass spectrometry analysis of both secretions that allowed us to identify the sex-specific origins of the proteins that had changed in abundance. We found that spermathecal fluid targets only seven (2%) of the identified seminal fluid proteins for degradation, including two proteolytic serine proteases, a SERPIN inhibitor, and a semen-liquefying acid phosphatase. *In vitro* and *in vivo* experiments provided further confirmation that these proteins are key molecules mediating sexual conflict over sperm competition and viability preservation during sperm storage. Exposure to spermathecal fluid reduced the capacity of seminal fluid to compromise survival of rival sperm in a matter of hours and biochemical inhibition of these seminal fluid proteins largely eliminated that adverse effect. Our findings indicate that *A. colombica* queens are in control of sperm competition and sperm storage, a capacity that has not been documented in other animals, but is predicted to have independently evolved in other polyandrous social insects.

INTRODUCTION

When females mate with multiple males, i.e. are polyandrous, conflicts over paternity continue after mating if sperm from different males compete for storage and egg fertilisation, a process referred to as sperm competition. Due to conflicting reproductive interests between the sexes, female reproductive tracts have evolved discrimination mechanisms to secure appropriate mate choice and reproductive success in spite of sperm competition constraints (1, 2). The phenotypic manifestations of these sexual conflicts have been well studied (2-4), but the underlying molecular processes mediating them remain largely unknown. Resolving these mechanisms is becoming increasingly feasible (5, 6) and has the potential to provide key insights into the homology and possible convergence of genes and proteins mediating sexual conflict (7-9).

Seminal fluid (SF) is a glandular secretion that males provide to sperm. It contains proteins that are important determinants of male reproductive success (10), as is particularly well documented in *Drosophila* where the sex peptide has a range of effects on female reproductive physiology and behaviour (11). These interactions may serve joint reproductive interests, but they tend to primarily favour male interests while reducing future reproductive success of females (12). Such sexual conflicts are often influenced by dynamic co-evolutionary arms-races, where molecular mechanisms are difficult to disentangle particularly when rates of re-mating are unpredictable. Females affect the outcome of sexual conflicts via defensive sexual strategies, but they are unlikely to gain ultimate control when re-mating continues to expose them to new male ejaculates (12, 13).

The mating systems of *Atta* leaf-cutting ants offer unique opportunities to study male-female protein interactions during insemination and sperm storage. Queens and males have large body sizes and ejaculates are transferred directly to the spermatheca, the queen's sperm storage organ, where they encounter both rival ejaculates and queen-derived secretions (14). These characteristics imply that artificial insemination is a feasible experimental approach for emulating natural insemination (15). Because queens never re-mate later in life (16), selection can produce both female enhancement mechanisms of sperm motility after insemination (14) and mechanisms to terminate sperm competition if it compromises future female reproductive success (17). Directly after mating, *Atta* queens must individually dig a burrow to establish a new colony. Thus, *Atta* queens face a series of trade-offs when allocating metabolic resources to control sexual conflict and maximize survival during solitary colony-founding under significant constraints of resource availability and disease pressure (17, 18).

We previously found that SF of *Atta colombica* males reduces survival of rival sperm *in vitro*, a process known as sperm incapacitation, and that the queen's spermathecal secretions terminate this process of competitive elimination (19). Here we conducted a series of experiments to unravel the molecular basis of these male-male and male-female interactions. We first show that the *in vitro* incapacitation effects of seminal secretions on sperm are due to proteins (20) and that the interactions between SF and spermathecal fluid (SpF) are protein-based. This implies that proteins play crucial roles in the phenotypic expression of sperm competition and its termination. In a second step, we used a quantitative proteomics approach by mixing SF with SpF to identify the proteins that are targeted for degradation using Differential in Gel Electrophoresis (DIGE). In a third step, to assess whether focal proteins originated from male or female secretions, we conducted a full proteome analyses of both SF and SpF, which allowed us to identify the SF proteins that are degraded by SpF. Finally, we

performed an *in vivo* artificial insemination experiment and follow up *in vitro* trials with general and specific protease inhibitors to obtain phenotypic confirmation for these SF proteins being molecular agents and counter-agents of sperm competition. The experimental procedures and results are presented in this order in the sections below.

EXPERIMENTAL PROCEDURES

Collection of virgin queens and males

All animal sampling and fieldwork was done in May (2008-2016) at the Smithsonian Tropical Research Institute in Gamboa, Republic of Panama. Male and virgin queens of *A. colombica* were dug out of specific colonies around Gamboa. Male sexual maturity was confirmed by dissecting subsamples of individuals to verify that testes had degenerated and all sperm had been transferred to the accessory testes (ATs), which are enlarged sections of the vas deferens used for the storage of mature sperm prior to ejaculation (21). Virgin queens were assumed to be sexually mature when found in fungus-garden chambers close to the surface and when their cuticles had become dark brown. All dissections were conducted using Inox 5 watchmaker forceps in Hayes saline solution (9 g NaCl, 0.2 g $CaCl_2$, 0.2 g KCl, and 0.1 g $NaHCO_3$ in 1000 ml H_20) as used for our previous studies (22).

The effects of spermathecal fluid on sperm incapacitation activity of seminal fluid

An overview of the experimental procedures of this section is given in Fig 1. To obtain seminal fluid, the accessory glands (AGs) of 60 males originating from two different *A. colombica* colonies were dissected in 200 µl Hayes, ruptured with forceps and vortexed for 3 min before centrifugation for 3 min at 13,500 g. The supernatant of all AGs was pooled and 48 ml of Hayes was added to create a 60 ml stock solution and stored at -20 ºC. This solution represented the

contents of approximately 2 AGs/ml, which seemed a reasonable approximation because: 1. Protein concentrations in natural ejaculates are unknown, and 2. Males can inseminate multiple queens and therefore allocate some unknown fraction of their total complement of SF to each ejaculate. The concentrations that we used are therefore most likely to be lower than in natural ejaculates, so the effects we quantified can be regarded as conservative estimates of the protein-level interactions as they occur in nature.

Figure 1

To obtain SpF, we collected 30 virgin queens from four different colonies and artificially inseminated (i.e. transferred using artificial insemination techniques) 20 µl of Hayes saline into the spermathecae of each queen as described previously (15). The queens were sacrificed after 60 min, dissected, and their spermathecae ruptured in Hayes saline. The samples were then vortexed for 3 min, followed by centrifugation for 3 min at 13,500 g to separate SpF from tissue residues. The supernatant from all 30 queens was pooled, after which half of this SpF stock solution was transferred to a new Eppendorf tube and suspended in Hayes resulting in a final concentration of ca. 1 spermatheca fluid complement per ml, i.e. a comparable protein concentration as in the AG samples. The second half of the SpF stock solution was separated into a high molecular weight (HMW) fraction, mainly containing protein molecules >3 kDa, and a low molecular weight (LMW) fraction, most likely containing peptide and metabolite molecules <3 kDa, using a centrifugal filter device (AMICON Ultrafree Centrifugal Filters 3 kDa, Millipore) for 30 min at 13,500 g. Both fractions were resuspended in Hayes saline to reach the original volume of 15 ml (Fig 1).

An additional 20 males were collected from a single *A. colombica* colony that was not used for any of the reproductive fluid collections described above (Fig 1). This new colony allowed us

to obtain five 1 µl samples of sperm dissected from the ATs of each of these males, which were diluted in: (T1) 1 ml of AG suspension of the same male collected as described above, (T2) a 1 ml aliquot of the AG stock suspension (from 60 rival males from one of the previous colonies), (T3) a 0.5 ml aliquot of the AG stock suspension and 0.5 ml of the SpF stock solution (collected from 30 queens), (T4) 0.5 ml AG stock suspension and 0.5 ml of the LMW fraction of the SpF stock solution, and (T5) 0.5 ml AG stock suspension and 0.5 ml of the HMW fraction of the SpF stock solution.

A 5 µl aliquot of each of the treatment samples was used to estimate sperm survival with a Live/Dead sperm viability kit (Molecular Probes) and using an earlier developed protocol (22). A minimum of 300 sperm cells per treatment were scored as live or dead using a fluorescence microscope (Olympus CX41, EXFO X-Cite 120, filter cube CX-DMB-2 and 800x magnification). Counting was performed blindly in relation to treatment and data analysis was performed in JMP, version 11.0.0 (SAS Institute, USA), for Windows using *Generalized Linear Models* (GLM) with a binomial error structure and logit link function. Sperm origin (male identity) was included as a random factor, number of live sperm as dependent variable and the total number of sperm counted per replicate as binomial denominator.

Changes in abundance of seminal fluid proteins after exposure to spermathecal fluid using DIGE

To identify the proteins involved in sexual conflict, we used DIGE to detect protein abundance changes when SF encounters SpF in the experimental design summarized in Fig 2. DIGE compares protein abundances between multiple proteomes of interest while maintaining their key properties such as isoelectric point (pI) and molecular weight (MW). In order to minimize experimental variation, samples are labelled with different fluorescent dyes before being

separated by 2D-PAGE. An internal standard, labelled with a separate fluorescent dye, allows comparison between samples run on different gels. Although DIGE has limited detection efficiency for proteins with extreme isoelectric points, unusual molecular weights, post translational modifications, and non-standard amino acid sequences (23), it is a well-established method for obtaining quantitative data on changes in abundance of intact and fragmented proteins, which was the main objective of our study.

Figure 2

Sample collection and preparation

Four biological replicates of SF were collected from four colonies by squeezing a male's abdomen between two fingers to induce ejaculation and collecting 2-4 µl of outflowing semen per male (15, 20). Each biological replicate originated from a separate colony and the SF was collected from approximately 350 males per colony. Each replicate was divided into 100 µl aliquots, and assigned to one of the following treatments: (1) un-treated SF controls, (2) SF transferred to the spermatheca using artificial 'insemination' and retrieved after 30 min, and (3) SF transferred to the spermatheca in the same way and retrieved after 12 h (Fig 2). Because leaf-cutting ant queens never re-mate later in life to replenish stored sperm (20), we hypothesised that queens should have been under strong selection to quickly intervene in male-driven sperm incapacitation. The 30 min time point is a reasonable estimate for the duration of naturally occurring mating flights of *Atta* queens when they are inseminated by 2-5 males with a median of 2-3 (18). Our selection of 12 hours as second time point was also purposefully considered because by that time queens will have terminated all mating activities and finalised digging a nest burrow to initiate a new colony.

In each of the two different treatments, we transferred 10 µl of SF to the spermathecae of 10 queens and allowed them to recover for 30 min or 12 h before dissecting them to obtain the spermathecal content as described above. Recovered spermathecal contents (transferred SF plus SpF) were then pooled per treatment and replicate, resulting in a total of 12 samples (3 treatments with 4 biological replicates, see Fig 2). Proteins were precipitated by adding 4x volume of ice-cold acetone to each sample for 4 h at -20°C and centrifuging at 14,000 g for 10 minutes, after which supernatants were discarded and the protein pellets were stored at -80°C until further use.

Differential in-gel electrophoresis (DIGE) experiment

For DIGE we prepared all 12 samples as described in Bogaerts et al. (24) and performed minimal labelling as described in Grassl et al. (25). Each sample, containing 150 µg of protein, was re-suspended in 100 µl of DIGE buffer (7M urea, 2M thiourea, 4% CHAPS, 20mM Tris, 1% DTT) and cOmplete Protease Inhibitor Cocktail (Roche), sonicated four times for 30 s and placed on ice for 1 min, followed by centrifugation at 14,000 g for 10 min at 4°C. Samples were desalted using the PlusOne Mini Dialysis Kit (GE Healthcare) according to the manufacturer's protocol, and dialysed against DIGE buffer. IPG strips (24 cm Immobiline Drystrips pH 3-10 NL; GE Healthcare) were rehydrated overnight with DIGE buffer (DTT replaced by 1.2% DeStreak Reagent and 0.5% IPG buffer pH 3-10 (both GE Healthcare). Each protein sample was minimally labelled with DIGE Cy3 fluorescent dye, and an internal standard containing equal amounts of each sample was labelled with Cy5 dye (GE Healthcare) according to manufacturer's instructions. Using cup loading, we loaded 100 µg proteins (50 µg per sample plus 50 µg of internal standard) in 150 µl of DIGE buffer using isoelectric focusing.

Isoelectric focussing (IEF) was performed with an IPGphor Manifold (GE Healthcare) at 20°C allowing a maximum of 50 µA per strip using the following setup: 4 h at 300V, 3 h gradient to 600V, 2 h gradient to 1000V, 2 h gradient to 3500V, 3 h gradient to 8000V, 5 h at 8000V (total 60000 Vh). Strips were stored at -80°C and thawed at room temperature (RT) prior to SDS-PAGE separation. Each strip was equilibrated twice at RT by incubation for 15 min in equilibration buffer (6M urea, 50 mM Tris-HCl (pH 8.8), 30% glycerol, 2% SDS) containing either 1% w/v DTT (reduction step) or 4% w/v iodoacetamide (alkylation step, in the dark). The second dimension was separated by polyacrylamide gel electrophoresis (1 mm thick, 12%T, 2.6%C) and using a Hoefer SE90010NA at 10°C for 1 h at 7 mA per gel followed by overnight at 50 mA per gel. All gels were scanned using a Typhoon fluorescence scanner (GE Healthcare) and the resulting images were imported into Delta2D software (v 4.34, Decodon GmbH) for spot analyses.

Experimental Design and Statistical Rationale

Each spot on the DIGE gels was inspected manually and only spots present on at least 3 of the 4 gels per treatment were kept for further analyses. Principal Component Analysis was used to exclude one outlier gel belonging to the 30 min treatment and ANOVAs were used to test for significant differences in spot intensity between treatments, based on log-transformed ratios. Finally, we applied Student's *t*-tests for pairwise *post hoc* comparisons among treatments using the "p-values based on permutations – all permutations" and the "False Discovery Rate (FDR) – number of false positive spots should not be exceeded" corrections available in the Delta2D software. Protein spots with significant abundance differences ($P < 0.05$) between treatments and fold-change differences larger than 2 were selected for protein identification. To do this, we silver-stained one gel per treatment-group using a MS-compatible protocol (26), then de-stained spots by incubating them in 15 mM $K_3[Fe(CN)_6]$ and 50 mM $Na_2S_2O_3$, and rinsed them

in water until fully de-stained. Spots were then dehydrated in 100% ACN, followed by drying in a speed-vac and in-gel protein digestion with trypsin (Promega, 20 ng/µl in NH_4HCO_3, pH 8.4) overnight at 37°C. Tryptic peptide extraction was done in 70% ACN, 5% FA in 10mM NH_4HCO_3 (pH 8.4) with sonication repeated three times. All supernatant extracts were dried in a vacuum centrifuge and stored at -20°C.

In order to identify the spots selected for mass spectrometry (MS), each tryptic digest was resuspended in 5% ACN, 0.1% FA, and filtered with Ultrafree Centrifugal Filters (Durapore, PVDF 0.2 µM, Millipore). The individual digests were analysed with LC-MS/MS using a Q-TOF mass spectrometer (Agilent 6550 with a HPLC-Chip Cube and a large capacity C18 chip), coupled to an Agilent 1200 series HPLC and a 20 min gradient (5-35% ACN, 0.1% FA). Peak lists were generated using MassHunter Quantitative Analysis B.07.01. Data files were analysed using a Mascot server (v. 2.5.1) against the *A. colombica* total proteome (16,519 sequences; 7,007,606 residues, Acol_1.0, downloaded October 2016) (27) using the following settings: MS error tolerance ±100 ppm, MS/MS error tolerance ±0.5 Da, trypsin digestion, maximum missed cleavages tolerated as 1, peptide charge as 2+, 3+ and 4+, and the instrument selected ESI-Q-TOF setting allowing for variable modifications: Carbamidomethyl (C) and Oxidation (M). We accepted protein identifications when at least three different peptides were detected with an overall MOWSE ion score greater than 30. We report all protein matches from each spot with a comparable emPAI score.

Obtaining seminal fluid and spermathecal fluid proteomes

To distinguish whether proteins identified in our DIGE analysis were of male or female origin, we obtained proteomes of both SF and SpF, using a pooled SF sample of 25 males from five colonies as described above by squeezing their abdomens to collect 2-4 µl ejaculate per male

(15, 20). The SF supernatant was collected from the pooled ejaculates after two centrifugations for 10 min at 13,500 g to separate sperm from SF, before storing the final supernatant at -80ºC. SpF was obtained from 4 different colonies by collecting 10 virgin queens per colony and transferring 10 µl Hayes into their spermathecae using the same methodology as for artificial insemination. All 40 queens were allowed to recover for 12 h before retrieving the content of their spermathecae as described above.

The SF and SpF samples went through reduction/alkylation as described above before trypsin digestion (1:20 enzyme: protein ratio, Promega) at 37ºC overnight. Samples were acidified to pH 3 with formic acid and separated by high pH C18-RP fractionation (28). We performed a concatenated, high pH reversed phase fractionation off-line using an Agilent 1200 series HPLC configured with two J4SDS-2 guard columns (PolyLC) and a Xbridge™ C18 column (particle size 3.5 µm, ID 4.6 x 250 mm (Waters)). Peptides were fractionated using a gradient of 5 - 60% ACN (v/v) at a flowrate of 1 ml / minute in 10 mM ammonium formate, pH 10 / NH_4OH. Fractions were collected in 1 min windows over 96 min in a 96 deep-well plate. The first and last 12 fractions were discarded and the remaining fractions were pooled for each plate column, resulting in 12 fractions. These were dried in a vacuum centrifuge and processed as described above before MS analysis, also conducted as described above except that the peptides were separated across a longer gradient of 60 min (5-35% ACN, 0.1% FA). Data files of the 12 fractions were combined in Mascot Daemon (Matrix Science) and searched as described above. We accepted proteins with at least two peptide identifications and with a protein MOWSE ion score greater than 30. For all proteins identified by a single peptide we reviewed the MS/MS spectra and removed 106 proteins from the SF list and 74 proteins from the SpF list that had low peptide ion scores and poor b-ion/y-ion series. For all other single-peptide identifications that were retained the fully annotated MS/MS spectrum is provided as supplemental files S1

and S2. We compared the obtained SF proteome of *A. colombica* with published SF proteomes of honey bees (29) and fruit flies (30), looking for homologous amino acid sequences using BLASTp at NCBI.

Effects of exposure time of seminal fluid to spermathecal fluid on sperm incapacitation

To test whether the exposure of SF to SpF impacts the ability of SF to incapacitate rival sperm, we collected a pooled SF sample from ~200 males from several colonies and used artificial insemination methods to transfer 6 µl of SF to 25 virgin queens collected from different colonies. The spermathecal content of five randomly chosen queens was then recovered through dissection 5 min, 3 h, 6 h, 9 h or 12 h after the transfer procedure and pooled to produce five treatment samples. We then collected 25 males from four additional colonies to obtain sperm samples from dissected ATs, and incubated these samples in 500 µl of one of the five spermathecal treatment samples for 60 min before quantifying sperm survival with live-dead fluorescence dyes as described above.

The effect of protease inhibitors on sperm incapacitation

To test whether sperm incapacitation can be reduced through biochemical inhibition of the SF proteins targeted by SpF, we exposed sperm from focal individual males to a mixture of SF from rival males either in the presence or absence of different protease inhibitors (see supplemental Fig S1 for an overview of the experimental design). In an initial experiment, we collected sperm from the AT of one male (22 replicates) and split it into 4 subsamples (1µl of sperm in 200µl of Hayes saline each). Two of these sperm subsamples were exposed for 2 h at RT to either: 1 µl rival SF (pooled from 250 males from 5 colonies separately) or 1 µl SF + Roche cOmplete Protease Inhibitor cocktail (containing inhibitors for serine proteases, carboxypeptidases and cysteine proteases) at a ratio 1 inhibitor: 2.5 SF sample. The other two

subsamples were exposed to relevant controls of Hayes and Hayes + Roche cOmplete Protease Inhibitor cocktail. The SF samples were incubated with the protease inhibitor for 4 h before they were combined with the sperm subsamples and we compared sperm survival using the Live/Dead kit after 2 h (Fig. S1).

The results showed a significant effect of the inhibitors to neutralize sperm-incapacitation by rival SF. Therefore, in a second experiment we used specific protease inhibitors to explore the identity of the proteases involved in sperm incapacitation (supplemental Fig S1). SF was collected from 250 males (50 males each from five colonies). Each SF sample was split into three sub-samples of equal volume, treated with (a) a serine protease specific inhibitor - AEBSF (100 µM, Sigma Aldrich) (31), (b) a specific inhibitor of carboxypeptidases - EDTA (10 mM, Sigma Aldrich) (32), and (c) Hayes saline as a control. The treated SF subsamples were then incubated at RT for 4 h. We then assessed each treatments' sperm-incapacitation efficiency by splitting a sperm sample from 1 male into 6 aliquots of 1µl sperm in 200µl Hayes and exposing each aliquot to 1 µl of one of the treated SF subsamples (a-c), plus controls of (d) Hayes only, (e) Hayes containing the EDTA, and (f) Hayes containing AEBSF. Sperm survival was then assessed after 2 h using the Live/Dead kit.

RESULTS

Our results confirm that the key interaction effects between male SF and queen SpF is driven by the protein containing (HMW) fraction of these secretions (Fig 3A), and that the proteomic interactions between these secretions are essential for understanding the molecular mechanisms of sexual conflict in *A. colombica*. This fundamental result prompted our comparative DIGE experiment to identify changes in the sex-specific proteomes when SF interacts with SpF. The

results of the DIGE experiment then triggered out efforts to obtain the SF and SpF proteomes so we could determine the sex-specific origin of the proteins with altered abundances after the male and female secretions interact (Fig. 4), and to do validation experiments to show that serine proteases have key roles in the expression and regulation of sexual conflict (Fig 3 B-D).

The SF and SpF proteomes included 311 and 396 identified proteins, respectively, and showed that less than half of these proteins (135) were shared (Fig 4, supplemental Fig S2, supplemental Table S1). Gene Ontology further showed that a large number of proteins were present either in SF or SpF, but not in both secretions. In particular, proteins involved in DNA replication, amino acid biosynthesis/metabolism, ATP-binding and metal ion binding were identified in greater number in the SpF proteome than in the SF proteome (Fisher's exact probability test, two tailed, $P<0.05$), whereas proteins involved in glycan processing, signalling and carbohydrate turnover were identified in greater number in the SF proteome (supplemental Table S2). These results also showed that the *A. colombica* SF proteome is of comparable size to that of honeybee drones (29), but considerably larger than that of fruit flies (30). The SpF proteome that we obtained extends the resolution depth by an order of magnitude compared to a previous study that identified proteins in the spermatheca of another species of *Atta* leaf-cutting ants (33).

Figure 3

Figure 4

The DIGE experiment (Fig 2) enabled us to identify a total of 471 protein spots, 418 of which did not show any significant change in abundance. Of the 53 spots that changed in abundance, 39 significantly increased and 14 significantly decreased after SF and SpF interacted. These abundance changes accumulated over time after insemination, with 26 spots having changed in intensity after 30 min and 49 after 12 h. However, only four spots had changed after 30 min and not after 12 h (supplemental Table S3), suggesting that almost all changes are initiated shortly after insemination. Because we found no evidence for any overall changes in protein abundance in 89% (418 out of 471) gel spots, we concluded that no overall degradation of proteins had occurred during the experiment. This inference was reinforced by our finding that the average amount of protein retrieved per inseminated queen did not differ across experimental groups (32.2 ± 10.6 µg in controls, 24.1 ± 6.0 µg in the 30 min treatment and 29.2 ± 13.4 µg in the 12 h treatment; ANOVA, $F = 0.4597$, $df = 2$, $P = 0.645$).

Using mass spectrometry, we identified 44 protein spots where abundances were significantly changed on the DIGE gel. No significant protein identifications were found in 9 spots but we identified a total of 22 unique proteins in the remaining 35 spots (Table 1, supplemental Table S3). Some of the SF proteins that changed in abundance after contact with SpF were identified in multiple spots across the gels (supplemental Table S3 and supplemental Figs S3 and S4), suggesting these spots only contained protein fragments, also because molecular weight and pI were substantially different from the intact protein predictions in Uniprot. Such indications of protein fragmentation were found for a total of 24 spots. Three of these proteins (serine protease inhibitor B10 (SERPIN B10), acid phosphatase, and the uncharacterised protein Acol_04113), showed a decreased abundance in the spots representing the expected molecular for the intact proteins and increased abundances in spots representing lower molecular weights consistent with these being fragments (Table 1 and supplemental Fig S3). For all spots that were identified

as representing multiple proteins, we compared the emPAI scores and report all significant identifications (supplemental Table S3).

We identified 22 unique proteins from the 2D-DIGE gels, of which seven showed a decrease and 15 an increase in abundance (Table 1, Fig 4). Of the seven SF proteins with reduced abundance, four (serine protease: snake, acid phosphatase, the uncharacterized protein Acol 04113, and hydroxyacid-oxoacid transhydrogenase (HOT)) were found in the SF proteome only, and three (serine protease: easter, SERPIN B10, and carboxypeptidase B) were present in both the SF and SpF proteomes (Table 1, supplemental Tables S1 and S3, and supplemental Figs S3-S4).

Of the 15 proteins that increased in abundance after transfer of SF to the spermatheca, seven were found in both proteomes (chitinase-like protein IDGF4, superoxide dismutase, glutathione S-transferase, phosphatidylethanolamine-binding protein (PEBP), arginine kinase, actin, and transferrin (Table 1)). The remaining eight proteins were identified only in the SpF proteome, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta-glucoronidase, transketolase, catalase, hexamerin, transgelin, peptidyl-prolyl cis-trans isomerase, and nucleoside diphosphate kinase (Table 1). Because no intact proteins were exclusively found in the SF increased in abundance after contact with spermathecal fluid, we inferred that all observed increases in abundances were the result of proteins that were introduced by the SpF (Table 1).

SF proteins that decreased in abundance after contact with SpF predominantly belonged to the proteolysis functional category (serine proteases: easter and snake, SERPIN B10 and carboxypeptidase B). It is noteworthy that three of the proteins that changed in abundance after

contact with SpF showed a decreased abundance in spots with a molecular weight of the intact protein; while some smaller fragments increased in abundance (SERPIN B10, acid phosphatase and the uncharacterised protein Acol_04113). This suggests that the intact protein was fragmented, consistent with these proteins being specific targets of SpF proteins directly after insemination (Table 1).

Most of the proteins that increased in abundance are involved in energy metabolism (GAPDH, beta-glucoronidase, transketolase and arginine kinase) and oxidative stress responses, including redox and immunity (catalase, superoxide dismutase and glutathione S-transferase) (Table 1, Fig 4) (see supplemental Table S3 for further details on all proteins). Of the eight SpF-specific proteins that we did not identify in the SF, five appeared only on the gels where SF was exposed to SpF for 30 min and 12 h. The remaining three SpF-specific proteins plus all seven proteins shared between SF and SpF were all found in higher abundances after the SF was exposed to SpF (Table 1), suggesting that increased abundances of SF proteins resulted from the mixing with SpF proteins.

As already mentioned, the seven SF proteins that decreased in abundance after exposure to SpF (Table 1) included two serine proteases (easter and snake), SERPIN B10, and a carboxypeptidases B. Snake appeared to be SF-specific, whereas easter and SERPIN B10 were found in both SpF and SF. Carboxypeptidases have a rather broad array of biological functions (32), but SERPINs can target serine proteases involved in sperm-egg recognition (see Discussion). We detected a total of five different SERPIN proteins in the SpF proteome, three of which were also present in the SF proteome. Our results thus provide several lines of evidence that serine proteases and SERPINs are targeted by SpF to terminate mutual sperm incapacitation in *A. colombica*. We also detected a fragment of SERPIN B10 in SF after *in vivo*

exposure to SpF (Table 1 and supplemental Fig S3). Interestingly, although we identified a total of three different SERPINs in the SF proteome, only SERPIN B10 was found to be targeted by SpF for degradation, suggesting that SpF targets individual proteins with high specificity.

Of the remaining three SF-specific proteins that were reduced in abundance, uncharacterized protein Acol 04113 was degraded into at least three fragments that all increased in abundance after male and female secretions came into contact (Table 1, supplemental Fig S3), consistent with this protein also being targeted for destruction by SpF. A similar fragmentation effect could be observed for acid phosphatase (supplemental Fig S3) where we detected one fragment spot that increased 21-fold after 12 hours of SpF exposure. Acid phosphatase has fertility reducing effects (see Discussion for further details). The final SF-specific protein identified as being targeted by SpF was HOT, an enzyme that catalyses oxidation-reduction reactions (Table 1). We did not detect any fragments for this protein, but the intact protein decreased in abundance after exposure to spermathecal fluid (supplemental Fig S3).

Five of the 15 proteins that increased in abundance (chitinase-like protein IDGF4, catalase, PEBP, glutathione S-transferase and transferrin) were identified as fragments, but it was not possible to ascertain whether fragmentation occurred before or after SF was put in contact with SpF (Table 1 and supplemental Fig S3). IDGF4 appeared to be already split into several fragments in the male body because six spots were present in the pure SF (369, 371, 380, 386, 397 and 511; Table 1, supplemental Fig S3), but two new degradation products (spots 374 and 517) appeared when exposed to SpF, suggesting some further fragmentation had occurred.

Table 1

Exposure of seminal fluid to spermathecal fluid reduces rival sperm incapacitation very quickly

To confirm the efficiency of SpF in eliminating sperm incapacitation over time, we replicated the experiment of Fig 1 and transferred pooled SF mixtures from six unrelated rival males to the spermathecae of virgin queens, which we then recovered by dissection after time intervals between 5 min and 12 h. These recovered SF samples were then exposed to newly collected sperm of a different male, which showed that sperm survival was higher when the SF had been exposed to SpF for longer periods of time ($\chi^2 = 25.308$, $df = 4$, $P < 0.001$) (Fig 3B).

Protease inhibitors confirm the identity of antagonistic proteins in seminal fluid

We finally tested whether SF proteases are indeed the prime agents of sperm incapacitation by incubating SF mixtures of competing males with or without adding three commercially available cocktails of protease inhibitors. We found that sperm survival increased significantly when general protease inhibitors were present ($\chi^2 = 10.419$, $df = 3$, $P = 0.015$, Fig 3C). We subsequently tested protease inhibitors that are more specifically effective against the two main categories of proteases, carboxypeptidases and serine proteases, as identified in the DIGE experiment. This confirmed that only the serine protease inhibitor AEBSF reduced the incapacitation ability of SF towards rival sperm ($\chi^2 = 62.271$, $df = 5$, $P < 0.001$, Fig 3D). These combined results provide further support that spermathecal secretions have a general and indiscriminate preservation function for the viability of newly stored sperm mixtures (see (19) for phenotypic evidence) by neutralizing those SF proteases that mediate mutual sperm incapacitation between ejaculates.

DISCUSSION

Sexual conflicts are known to continue after insemination when the reproductive interests of females and males are not fully aligned, which is essentially always the case when females mate with multiple males. In the leaf-cutting ant *Atta colombica* male SF proteins incapacitate rival sperm within a brief time window, and queens are known to neutralize hostility among competing ejaculates upon storing sperm, an adaptation that seems logical because they cannot re-mate later in life (17, 19). We here combined proteomic methods with the experimental approaches that are typically used in evolutionary and behavioural ecology to obtain important first insights in the expression and regulation of sexual conflicts in social insects. The SF and SpF proteomes that we obtained are of sufficient size and depth to make meaningful comparisons and we are confident that they enabled us to identify potential proteins of interest that are essential for the expression of sexual conflict in leaf-cutting ants.

We found that proteins secreted by the sperm storage organ, the spermatheca, target a very small subset of male SF proteases and we show that their degradation or biochemical inhibition increases the survival of sperm that would otherwise have died due to exposure to SF of other males. Our results are also consistent with many components of SF having an overall positive effect on sperm survival, similar to what we have reported previously in both ants and bees (17, 19), and that this support is independent of whether SF originates from the same or a different male (19). This is why sperm survival is typically higher in SF from rival males compared to Hayes saline. Phenotypic studies of differential sperm survival in scenarios of sperm competition therefore always require both own-SF and Hayes saline controls.

Our study shows, to our knowledge for the first time, that it is the proteins in the SpF that neutralise the antagonistic effects of rival SF on sperm survival. Our finding that SpF contains a larger number of proteins involved in oxidation-reduction supports the idea that the queen´s spermatheca evolved a highly specific biochemical machinery to keep sperm viable for long periods of time, consistent with *Atta* queens potentially surviving for 20 years and producing in the order of 100 million fertilized eggs (19, 22, 34). Both proteomes appeared to contain a large number of proteins involved in protein degradation and proteolysis, which we were able to functionally link to both the initiation and termination of mutual sperm incapacitation (supplemental Table S2 and Fig S2).

When we inspected the 22 proteins that had changed in abundance after SF had been exposed to SpF (DIGE experiment), we found four that increased in abundance to have known functions in energy metabolism and that three are involved in response to oxidative stress and immunity (Table 1, Fig 4, supplemental Table S3). Both these functions seem logical as adaptations to queen survival and fertility. However, the SF proteins that were significantly reduced in abundance provided direct insights into the expression and control of sexual conflicts in leaf cutter ants. Using a serine-specific protease inhibitor, we were able to reproduce the SpF neutralising effect *in vitro*, which confirmed that serine proteases are directly involved in sperm incapacitation (Fig 3C) and that they are eliminated by SpF proteins. Indeed, four proteins reduced in abundance are involved in proteolysis, including the serine proteases easter and snake.

In *Drosophila*, snake (specific to SF in *Atta*) and easter (present in both SpF and SF proteomes of *Atta*) are part of a proteolytic cascade that ultimately activates the Toll receptor during embryo development (35, 36), which initiates the innate immune system (37) by responding to

the protein spätzle (38). This suggests that there is a connection between the incapacitation of rival sperm and cellular immunity functions in SF, consistent with other insect homologs of easter and snake being involved in the pro-phenoloxidase cascade (39, 40), which determines innate immune defences mediated by self-non-self-recognition. Sperm incapacitation by rival SF could therefore have evolved via modifications of immune proteins that originally functioned to recognise pathogens and minimize disease transmission during mating in the strictly monogamous ancestors of the leaf-cutting ants. Proteins of the innate immune system are also very abundant in the SF of polyandrous honeybees (29) and have been shown to kill sexually transmitted diseases with high efficiency (41).

We detected that fragments of SERPIN B10 and of several serine proteases increased in abundance, indicating that the intact protein was cleaved when SF came into contact with SpF (Table 1). SERPINs are a class of protease inhibitors that irreversibly target serine proteases, which can mediate sperm-egg recognition (reviewed (42, 43)). Serine proteases and SERPINs are further known to affect proteolytic cascades responsible for incapacitating and preserving sperm in mammals and solitary insects by protecting sperm against damage from female immune reactions (42). This is once more consistent with the conjecture that immune proteins might have been precursors for the evolution of sperm competition proteins (42). We detected a total of five different SERPIN proteins in the total SpF proteome, two of which were also present in the SF proteome, including SERPIN B10, the protein we found to be specifically targeted by female secretions. This suggests that female spermathecal secretions specifically eliminate easter and snake (especially the latter, which is SF-specific), possibly with the three SpF-specific SERPINs.

As to the remaining proteins targeted by the SpF (Table 1), carboxypeptidase B, acid phosphatase and HOT have many biological functions including protein digestion and regulating the insect moulting process (32, 44), However, the uncharacterized protein Acol 04113, the most abundant protein *in A. colombica* SF but not detected in SpF, shows strong homology with a family of uncharacterized ant proteins (27) that all share an Armadillo-like domain normally used in protein binding. Uncharacterized protein Acol 04113 also has some degree of homology with mucins, which are glycoproteins belonging to the extracellular matrix, and with the BRO-1 domain involved in protein targeting to the lysozyme. Acol_04113 is degraded into at least three fragments when SF and SpF come into contact (supplemental Fig S3), highlighting its focal importance as target for elimination by SpF.

The acid phosphatase, which has also been found in moth testes (45), cleaves phosphate groups from other proteins and has been shown to liquefy human ejaculates, where it has consistently negative effects on male fertility in conjunction with the serine protease kallikrein (46). Finally, *Atta* SpF reduced the abundance of HOT in SF, an enzyme that catalyses several oxidation-reduction reactions including the interconversion of 2-hydroxyglutarate and 2-oxoglutarate, which are key intermediates in the TCA cycle and amino acid metabolism (47). Further research will be required to further resolve the complex molecular functioning of these less well studied proteins in more detail, but our results suggest that sexual conflict over sperm storage is mediated by very few proteins and that the known characteristics of these proteins are consistent with them being instruments of antagonism between male and female reproductive interests (Table 1).

Our follow-up experiments generated several lines of independent evidence that the proteins we identified are indeed instrumental in the dynamics of sperm competition and sperm

preservation, even though further details remain to be studied in the future. Increased exposure time of SF to SpF *in vivo* increased the survival of rival sperm, and a cocktail of general protease inhibitors and a specific serine protease inhibitor both terminated the harmful effect of SF on rival sperm. These results corroborate the idea that spermathecal secretions have a general and indiscriminate preservation function for the viability of newly stored sperm mixtures (see (19) for phenotypic evidence) by neutralizing hostile SF proteins. In particular, serine proteases from SF seem to have an important role and will likely be gratifying targets in follow-up studies.

A mechanistic understanding of sexual conflict does not only require understanding of the interactions between male and female proteomes, but also depends on the morphological traits that determine the efficiency by which the sexes can impose and control their respective interests. Ants and many other evolutionary derived hymenopteran social bees are peculiar in that males are unable to physically enhance their individual interests because they either die shortly after copulation or continue to seek copulations with other queens (16). This provides newly inseminated queens with considerable opportunities to control the sperm storage process in ways that are maximally favourable for female reproductive success. Consistent with this logic, dissection of artificially and naturally inseminated *A. colombica* queens revealed that spermathecae are highly muscular and able to contract continuously between the two spermathecal lobes (supplemental Movie S1). These contractions are likely instrumental for queens to mix newly stored sperm with spermathecal gland secretions, and their existence is consistent with queens rapidly gaining control over hostile interactions between rival ejaculates.

Comparisons between our results and those obtained for honeybees (29, 48) and fruit flies (30, 49) showed that the *A. colombica* SF proteins targeted by SpF are not expressed in the SF of these other insects (supplemental Table S4), even though homologs are universally present in their genomes. This mutual exclusiveness is consistent with the independent evolution of polyandry in these insect lineages (50), but also suggests rapid evolutionary dynamics and continuous recruitment (over evolutionary time) of novel proteins for attack and defence during insemination. In a more general comparative sense, the 24% overlap between SF and SpF proteomes in *Atta* (Fig 4) was similar to the 20% overlap reported in honey bees (48). This overall proportional similarity may reflect functional evolutionary convergence across social insect lineages that independently evolved obligate polyandry from ancestral ants and bees that were monandrous (50). These similarities across taxa are consistent with SF of polyandrous social insects being universally selected to enhance short-term sperm survival and to express proteolytic activity to reduce the survival of rival sperm as shown phenotypically in previous studies of *Atta* leaf cutter ants and honeybees (19). In contrast, female secretions are expected to have evolved molecular mechanisms to sustain long-term sperm storage and to neutralise hostile SF proteins, but there is no compelling reason to expect that ants and bees recruited the same proteins for their sexually antagonistic arms races.

Taken together, the results of our study underline that complete lack of re-mating later in life imposes strong selective pressure on polyandrous ant queens to prevail in evolutionary arms races with their mates to secure maximal storage of both viable and genetically diverse sperm. Whether queens are capable to discriminate between sperm from different males during this process is unknown, but any such cryptic female choice would have to be based on mechanisms that are more complex than self-non-self-recognition, which appears to drive the interactions we document here. In this context, it might be of interest that the SF-specific protein

Acol_04113 sharply decreases in abundance in the spermatheca after insemination. The partial homology of this protein with mucins, which are glycoproteins known to mediate complex recognition processes via differential glycosylation (51), suggests that Acol_04113 could mediate preferential recognition and discrimination between variants of the same protein molecules. Interactions of this kind have been shown to be important for male fertility in humans because they affect sperm binding to oocytes (52, 53). Further research in this area will be required to understand the mechanisms that inseminated queens might use to discriminate between ejaculates.

To our knowledge, we provide the first study that documents proteomic interactions at a high level of detail in any social insect. The insights that we obtained match predictions from evolutionary theory and underline the unique opportunities that ants and other social insects provide for studying the dynamics of sexual conflict. The interactions that we elucidated appear to involve a limited set of male and female proteins as molecular agents or counter agents (either alone or as key parts of molecular cascades) that mediate differential sperm competition and sperm survival during insemination and sperm storage. The involvement of rather few specific proteases and protease inhibitors will facilitate future studies of the molecular dynamics that induce or avoid harm to sperm cells during the sperm storage process in *Atta* and possibly also other polyandrous social insects such as the honeybee and some of the vespine wasps (50).

SUPPLEMENTARY INFORMATION

Seven additional files are available as supplement of the online version of this paper, including four supplemental Figures, four supplemental Tables, two supplemental Files and a supplemental Movie.

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DATA AVAILABILITY

The datasets supporting this article have been submitted to PRIDE (PXD011306 for the Seminal Fluid and Spermathecal Proteomes and PXD011320 for the DIGE experiment) and will be made accessible upon publication. Eleven additional files are available as supplement of the online version of this paper, including four supplemental Figures, four supplemental Tables, two supplemental Files and a supplemental Movie.

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Table 1: Proteins identified in DIGE spots with significant abundance changes after exposure of seminal fluid to spermathecal fluid: Columns provide protein names, biological functions, presence in male seminal fluid (SF) and female spermathecal fluid (SpF), spot number on DIGE gels, expected *vs* observed molecular weight and abundance changes after exposure to SpF. Protein abbreviations: HOT - hydroxyacid-oxoacid transhydrogenase, GAPDH - glyceraldehyde-3-phosphate dehydrogenase, SOD - superoxide dismutase, GST - glutathione S-transferase, PEBP - Phosphatidylethanolamine-binding protein, PpiC-type - peptidyl-prolyl cis-trans isomerase, NDP - nucleoside diphosphate kinase. See supplemental Tables S1 and S3 for details.

Protein	Function	SF	SpF	Spot n°	Size	Change
Serine protease Easter (Acol_08100)	Proteolysis	Yes	Yes	365	Fragment	Decreased
				368	Fragment	Decreased
				432	Fragment	Decreased
				436	Fragment	Decreased
Serine protease Snake (Acol_02829)	Proteolysis	Yes	No	421	Intact	Decreased
				436	Intact	Decreased
Serpin B10 (Acol_08024)	Serine protease inhibitor	Yes	Yes	257	Intact	Decreased
				267	Intact	Decreased
				388	Intact	Decreased
				389	Intact	Decreased
				399	Intact	Decreased
				470	Fragment	Increased
Carboxypeptidase B (Acol_07302)	Metallo-carboxyprotease	Yes	Yes	262	Intact	Decreased
Acid phosphatase (Acol_04502)	Dephosphorylation	Yes	No	257	Intact	Decreased
				267	Intact	Decreased
				395	Fragment	Increased
HOT (Acol_07180 HOT)	Energy metabolism	Yes	No	265	Intact	Decreased
				3245	Intact	Decreased
Uncharacterized protein Acol_04113	Unknown	Yes	Yes	87	Intact	Decreased
				192	Fragment	Increased
				198	Fragment	Increased
				551	Fragment	Increased
Chitinase-like protein Idgf4 (Acol_07567)	Morphogenesis	Yes	Yes	374	Fragment	Increased
				386	Fragment	Increased
				369	Fragment	Increased
				371	Fragment	Increased
				380	Fragment	Increased
				397	Fragment	Increased

				511	Fragment	Increased
				517	Fragment	Increased
GAPDH (Acol_07180 GapDH)	Glycolysis	No	Yes	329	Intact	Increased
				346	Intact	Increased
Beta-glucuronidase (Acol_05025)	Carbohydrates metabolism	No	Yes	156	Intact	Increased
Transketolase (Acol_07599)	Pentose phosphate shunt	No	Yes	154	Intact	Increased
				213	Intact	Increased
Catalase (Acol_06379)	Response to oxidative stress	No	No	210	Fragment	Increased
				213	Fragment	Increased
				216	Fragment	Increased
SOD [Cu-Zn] (Acol_09923)	Response to oxidative stress	Yes	Yes	519	Intact	Increased
GST (Acol_15065)	Response to oxidative stress	Yes	Yes	464	Fragment	Increased
Hexamerin (Acol_04072)	Oxygen transport	No	Yes	114	Intact	Increased
				120	Intact	Increased
				2031	Intact	Increased
PEBP (Acol_01467)	Lipid binding	Yes	Yes	464	Fragment	Increased
				467	Fragment	Increased
Transgelin (Acol_06601)	Actin binding	No	Yes	531	Intact	Increased
PpiC-type (Acol_08173)	Protein folding	No	No	552	Intact	Increased
NDP (Acol_07573)	GTP/UTP biosynthesis	No	Yes	562	Intact	Increased
Arginine kinase (Acol_12832)	Phosphorylation	Yes	Yes	320	Intact	Increased
Transferrin (Acol_11379)	Metal ion binding	No	Yes	198	Fragment	Increased
				551	Fragment	Increased
Actin (Acol_04097)	Cytoskeleton	Yes	Yes	213	Intact	Increased

Figure 1.

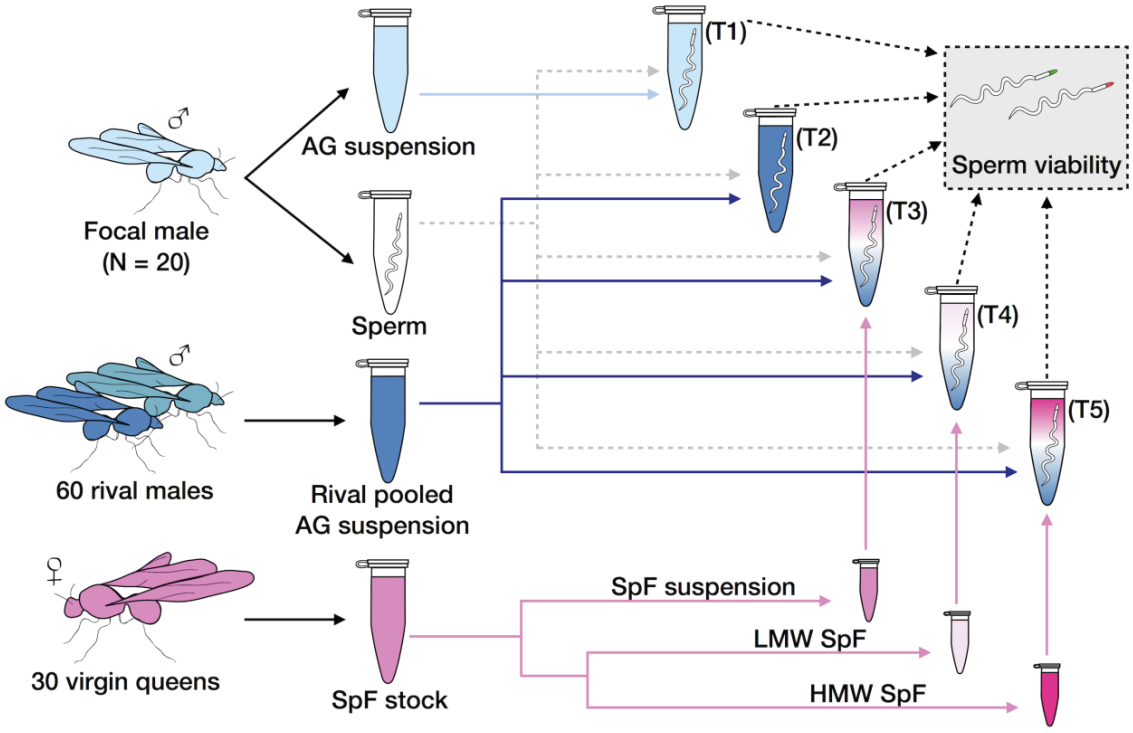

Figure 1. Experimental procedure for quantifying the effect of seminal fluid proteins on mutual sperm incapacitation and the termination of sperm competition by female spermathecal fluid, using unrelated field-collected virgin queens and males of *Atta colombica*. Sperm from a single focal male (N = 20) was exposed to a sample of: (T1) the male's own diluted accessory gland (AG) secretion; (T2) a similar suspension mixture of rival male AG secretion from 60 unrelated males obtained from two other colonies, emulating that queens mate with 2-3 males on average under natural conditions; (T3) this mixture of rival male AG secretion plus queen spermathecal fluid (SpF); (T4) this mixture of rival AG secretion plus the low molecular weight (LMW) fraction of the SpF; and (T5) this mixture of rival AG secretion plus the high molecular weight (HMW) fraction of the SpF. After 30 min, sperm survival was scored under a microscope using live/dead staining.

Figure 2.

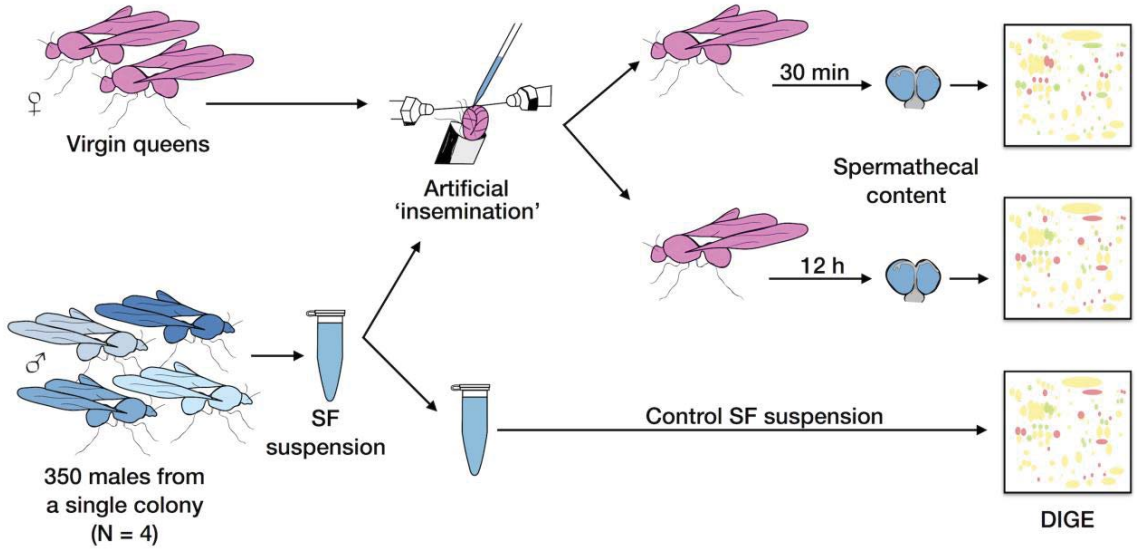

Figure 2. Experimental procedure using artificial 'insemination' (in quotes because no sperm was involved) and DIGE to identify changes in abundance of seminal fluid proteins after exposure to spermathecal fluid *in vivo*. Virgin queens were artificially inseminated with a mixture of seminal fluid (SF) from 350 brother males, which corresponds to the genetic diversity of two unrelated haploid males; after 30 min (10 queens) or 12 h (10 queens) the spermathecal contents were retrieved by dissection, pooled per treatment, and analysed using Difference In-Gel Electrophoresis (DIGE) along with a similar sample of SF suspension that had not been used for artificial insemination. DIGE included four biological replicates (i.e. SF from four sets of 350 brother males), each consisting of a non-inseminated SF control suspension and the two SF 'insemination' suspensions retrieved after 30 min and 12 h.

Figure 3.

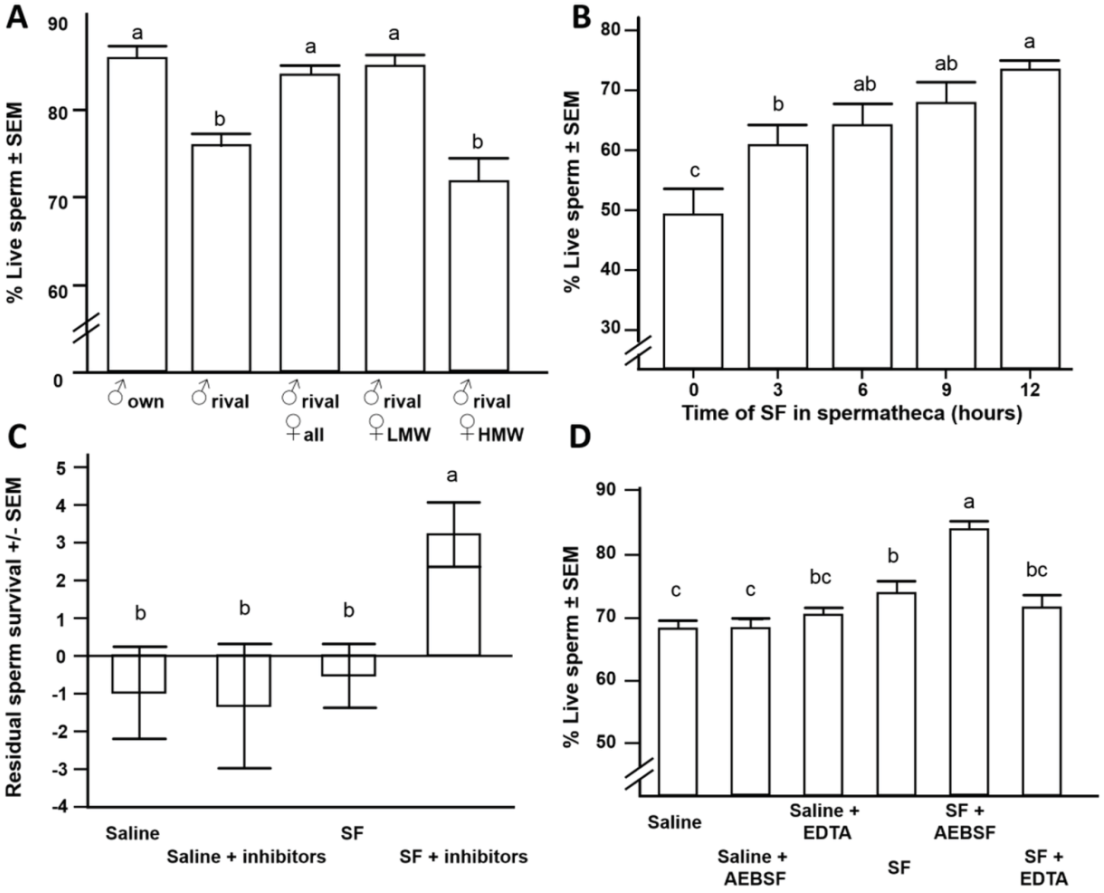

Figure 3. Changes in sperm survival after experimentally manipulating the sperm-incapacitation efficiency of rival males' seminal fluid (SF), by exposure to either spermathecal fluid or synthetic protease inhibitors. Sperm survival after 30 min exposure to: a male's own seminal fluid (♂own; control), rival males' seminal fluid (♂rival), rival males' seminal fluid mixed with female spermathecal fluid (♂rival + ♀all), rival males' seminal fluid mixed with the low molecular weight fraction of female spermathecal fluid (♂rival + ♀LMW), and rival males' seminal fluid with the high molecular weight fraction of spermathecal fluid (♂rival + ♀HMW). The first three bars have previously been published by Den Boer et al. 2010 (18). (B) Sperm survival after exposure to rival seminal fluid (SF; pooled from 350 males), after being artificially inseminated into a queen's spermatheca and recovered at time intervals

between 5 min and 12 h. (C) Sperm survival after 2 h exposure to Hayes saline, saline with a cocktail of synthetic inhibitors of proteases (Saline + inhibitors), rival males' seminal fluid (SF), and rival males' SF with synthetic inhibitors of proteases (SF + inhibitors); we used residuals rather than direct measurements to adjusting the response variable for unexplained variation in overall sperm viability across two subsequent field seasons (see methods and supplemental Fig S1 for details). (D) Sperm survival after 2 h exposure to Hayes saline, saline with synthetic inhibitors of serine proteases (Saline + AEBSF), saline with synthetic inhibitors of carboxypeptidases (Saline + EDTA), rival males' seminal fluid (SF), rival males' SF with synthetic inhibitors of serine proteases (SF + AEBSF), and rival males' SF with synthetic inhibitors of carboxypeptidases (SF + EDTA). See supplemental Fig S1 for procedural details. Bars marked with different letters are significantly different at $P < 0.05$ (contrasts test).

Figure 4.

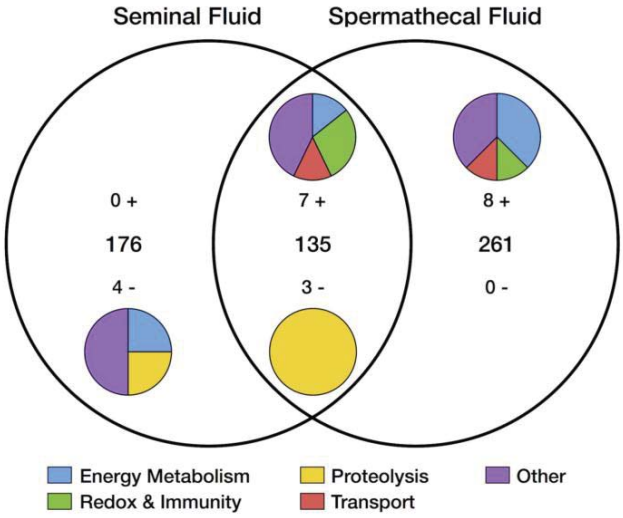

Figure 4. Comparison of the seminal fluid and spermathecal fluid proteomes of *Atta colombica*. The Venn diagram shows the total number of proteins identified from male seminal fluid (SF) and queen spermathecal fluid (SpF), and the number of proteins identified in both proteomes using shotgun proteomics (large font). The pie charts represent functional classes of proteins quantified in the DIGE experiment, showing the class fractions (colors) and numbers of proteins (small font) that increased (+) or decreased (-) in abundance after exposure to SpF (see supplemental Table S2).