Seminal fluid of honey bees contains multiple mechanisms to combat infections of the sexually transmitted pathogen *Nosema apis*

Yan Peng[1a], Julia Grassl[1a], A. Harvey Millar[1], & Boris Baer[1]

[1] *Centre for Integrative Bee Research (CIBER) and ARC Centre of Excellence in Plant Energy Biology, Bayliss Building (M316), The University of Western Australia, Crawley WA 6009, Australia. Yan Peng: dryanpeng@gmail.com, Julia Grassl: julia.grassl@uwa.edu.au, Harvey Millar: harvey.millar@uwa.edu.au, Boris Baer: boris.baer@uwa.edu.au.*

[a] These authors contributed equally to the paper

[b] Corresponding Author

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ABSTRACT

The societies of ants, bees and wasps are genetically closed systems where queens only mate during a brief mating episode prior to their eusocial life and males therefore provide queens with a lifetime supply of high quality sperm. These ejaculates also contain a number of defence proteins that have been detected in the seminal fluid but their function and efficiency has never been investigated in great detail. Here we used the honey bee *Apis mellifera* and quantified whether seminal fluid is able to combat infections of the fungal pathogen *Nosema apis,* a widespread honey bee parasite that is also sexually transmitted. We provide the first empirical evidence that seminal fluid has a remarkable antimicrobial activity against *N. apis* spores and that antimicrobial seminal fluid components kill spores in multiple ways. The protein fraction of seminal fluid induces extracellular spore germination, which disrupts the lifecycle of *N. apis*, whereas the non-protein fraction of seminal fluid induce a direct viability loss of intact spores. We conclude that males provide their ejaculates with efficient antimicrobial molecules that are able to kill *N. apis* spores and thereby reduce the risk of disease transmission during mating. Our findings could be of broader significance to manage honey bee diseases in managed honey bee stock in the future.

INTRODUCTION

Sex is the most widespread mode of reproduction in animals and has a number of well documented advantages compared to clonal reproduction [1, 2]. However it can also incur substantial costs, such as for example the need for a species to produce two types of sexes [3]. Furthermore, in species engaging in reproductive behaviours involving close physical contact such as copulation, parasites can hitchhike on the mating process and be transmitted between sexes or to offspring [4]. To achieve this, sexually transmitted diseases contaminate ejaculates and use them as vectors for the transfer to females [4]. To counter this, males are expected to evolve adaptations to reduce the risk of infecting their mates or offspring and ejaculates are indeed known to contain molecules with antimicrobial activity [5, 6]. Host-parasite interactions in reproductive tissues and secretions has received relatively little scientific attention in insects but have been studied in vertebrates [7], suggesting that proteins with antimicrobial functions within the ejaculate are of central importance for male fitness because they supress microbial growth [8, 9], and affect sperm motility [10]. Antimicrobial proteins in ejaculates have also been reported in insects [5, 6, 11-13], and antimicrobial activity of seminal fluid in the bedbug *Cimex lectularius* reduces sperm mortality in vitro [13, 14]. However, the functioning of antimicrobial molecules in ejaculates or the female's sexual tract or their influence on male and female reproductive success in insects has not been studied in detail.

The males of hymenopteran social insects (the eusocial ants, bees and wasps) are under strong selection to provide females (queens) with high quality ejaculates [12, 15, 16], and components such as seminal fluid [5] have been identified as the key determinants of male fertility and fitness [17-19]. Proteomic analyses of seminal fluid have provided detailed

insights into the complex biochemical networks that both support sperm [6, 20] and target ejaculates of rival males to reduce their sperm viability and reproductive success [17]. These studies have also identified a number of antimicrobial and defence-related proteins, which could counter potentially sexually transmitted diseases [6].

Male social insects are known to be more susceptible to parasites because their immunity is often reduced compared to workers and queens [21-23]. Two different explanations have been put forward to explain compromised male immunity in hymenopteran social insects. The haplodiploid susceptibility hypothesis states that haploidy in social insect males reduces genetic variation in their immune system genes and consequently increases their disease susceptibility [24]. Alternatively, the trade-off hypothesis states that social insect males reallocate immunity-related investments to maximise fertility in order to satisfy the exceptional high demands on sperm numbers and quality [25]. In this case, antimicrobial and defence proteins within the ejaculate represent key long-term investments of males into high quality ejaculates, as it is not in the interest of an infected male to transfer diseases to a queen. The two hypotheses therefore make different predictions about the efficiency of antimicrobial systems within ejaculates. The haplodiploid susceptibility hypothesis predicts that social insect males have very limited opportunities to compensate for a genetically determined inferiority of their antimicrobial defence. On the other hand, the trade-off hypothesis predicts that males invest into efficient antimicrobial defence in their ejaculates to minimize infection and fitness costs to their mates and offspring. However, to date, empirical work has been lacking that quantifies the biological activity, efficiency or the molecular mechanism of these defences.

Here, we used the European honey bee *Apis mellifera*, where queens mate with 25 or more males [26, 27] and thereby increase a queens risk of acquiring pathogens through

ejaculates [4]. A number of honey bee pathogens have been detected both in ejaculates and in queens that were artificially inseminated with infected semen; for example acute bee paralysis virus and deformed wing virus [28-30]. Furthermore, spores of the widespread fungal pathogens *Nosema apis* and *Nosema ceranae* have recently been identified in honey bee semen and are able to infect queens if transferred during mating [31, 32]. Because some of the antimicrobial and defence-related proteins identified in the seminal fluid of honey bees have predicted antifungal activities [6], we hypothesised that these proteins reduce the viability of *Nosema* spores and the risk of disease transfer during mating.

MATERIAL AND METHODS

Seminal fluid collection

Mature *A. mellifera* drones were collected at the entrance of hives while returning from their daily mating flights in an apiary at the University of Western Australia. Up to 200 drones were placed in wooden cages and kept in foster colonies prior to the experiment. To collect ejaculates, we used a method developed earlier to artificially inseminate honey bees [33]. In short, we allowed drones to fly in a cage for 10 min before inducing ejaculation by anaesthetising them with chloroform. We manually squeezed the males' abdomen between two fingers and collected ejaculates appearing at the tip of the endophallus with a pipette. Our previous work indicated that this mode of semen collection does not result in major contaminations, as indicated by the absence of highly abundant tissue or haemolymph proteins in our seminal fluid samples [6]. To separate sperm from seminal fluid we used a previously developed protocol [6, 20]. In brief, we centrifuged pooled semen samples for 25 min at 18,500 x *g* at 4° C. The supernatant containing the SF was collected and centrifuged

again for 10 min at 18,500 x *g* in 4° C to remove remaining sperm and was then stored at -80° C.

N. apis spore collection

We took advantage of the fact that Western Australia is currently free of *N. ceranae*, a pathogen that only recently switched from its original host, the Asian honey bee (*Apis ceranae*) to European honey bees [34]. Consequently we were able to study interactions of antimicrobial molecules in the honey bee seminal fluid with *N. apis* in the absence of possible confounding effects of *N. ceranae*. To collect *N. apis* spores we used a technique previously developed [31]. In brief, we caught honey bee workers at the entrance of hives with confirmed *N. apis* infections and killed them at -20° C. We dissected and pooled the intestines of 20 workers, added 1 mL of distilled water and homogenised the sample by adding a 3 mm tungsten bead (Qiagen, Australia). After manually shaking each sample for 2 min we used 0.5 mL of the homogenate and layered it onto 1.5 mL of 100% Percoll (Sigma-Aldrich, Australia), followed by centrifugation at 18,000 x *g* for 60 min at 4° C. The pellet containing *N. apis* spores was washed with 1.5 mL DDI water, briefly vortexed and centrifuged again at 20,700 x *g* for 5 min at 4° C. This procedure was repeated three times before the spore pellet was finally resuspended in 100 µL of DDI water and the spore concentration quantified as described by Cantwell [35]. The final sample was diluted to a concentration of 10^9 spores/ml, before freezing it at -80° C prior to any further experiments. Our previous experiments have confirmed that this method of collection and freezing does not impact the viability of the spores [36].

N. apis spore viability in seminal fluid

To quantify the effect of seminal fluid on *N. apis*, we incubated spores in undiluted SF or SF diluted 1:2, 1:10, 1:100, and 1:1000 as well as in Hayes semen diluent (0.15 M NaCl, 1.80 mM $CaCl_2$, 2.68 mM KCl, 1.19 mM $NaHCO_3$) as a control. To do this we used 10^6 spores in 10 µL of each treatment and incubated all samples at room temperature for 5 min, 4, 6, 8, and 24 hours in the dark. For each treatment and incubation time we used three independent biological replicates of seminal fluid that we collected prior to the experiment as described above. Spore viability was quantified using a flow cytometry method developed earlier [36]. In brief, we used fluorescent nucleic acid dyes and stained spore samples for 90 min in the dark on ice with 5 µM SYTO (16) green and 0.02 µM SYTOX red (Invitrogen, USA) which allowed us to distinguish live and dead spores [36]. To increase spore concentration in samples, we centrifuged them at 20,800 x *g* for 10 min at 4° C, and discarded 680 µL of the supernatant before resuspending the spores in the remaining supernatant. We then used a BD FASCalibur flow cytometer with CellQuest Pro version 5.1™ (Becton Dickinson, USA) to quantify *N. apis* spore viability in our samples. A 488 nm (blue) laser was used to excite SYTO green (emission collected using 530/30 BP filter) and a 635 nm (red) laser was used to excite SYTOX red (emission collected using 670/30 BP filter). All parameters were recorded using logarithmic amplifications. In the forward scatter (FSC) and side scatter (SSC), we set the flow cytometer to capture 10,000 spores, but smaller particles were also gated which we will referred to as debris. We used FlowJo Version 10 for Windows (TreeStar, USA) for gating and analysis of the raw data. We used spore solutions with known viabilities to set live and dead spore gates. Within the spore gate, the spores were sub-gated into live and dead spores within the FL1 and FL4 channels and the percentage of live (Q4+Q3) and dead (Q1+Q2) spores was calculated for each sample. The debris was sub-gated into general and fluorescent debris, which is made up of particles exceeding a fluorescent intensity of 5 in SYTO green stained particles. The fluorescent debris was

quantified as a ratio of the total count of particles passing the flow cytometer. Each biological sample was measured twice and averages of technical replicates were used for statistical analyses.

Nosema apis spore germination

Our microscopic and flow cytometry work indicated that seminal fluid induces germination-like rupture of *N. apis* spores, which is the first step to establish an infection and characterised by the protrusion of a polar tube that will penetrate a host cell to transfer nuclear material of the parasite [37]. To confirm that some of the debris observed in our flow cytometry data resembles that derived from germinated *N. apis* spores, we artificially induced spore germination as described by De Graaf [37]. To do this we used 10^6 *N. apis* spores and added 0.5 M sodium chloride, 0.5 M sodium hydrogen carbonate, pH 6.0 (0.1 M orthophosphoric acid) and incubated the sample for 15 min at 37° C. Microscopic confirmation of successful germination was carried out using a Olympus BX53 microscope with a PlanN (UIS2) lens under DIC. Digital photos were taken using the UC50 camera and on the LabSens software (Olympus, Japan). Spore viability and debris was then measured using flow cytometry as described above.

Size exclusion separation to isolate the active component in seminal fluid

To measure the biological activity of different fractions of seminal fluid, we split seminal fluid samples into a protein fraction, containing molecules >3 kDa and a non-protein fraction, containing small molecules <3 kDa, using Amicon® Ultra-0.5 mL 3 kDa molecular weight cut-off centrifugal filters (Millipore Corporation, America). After centrifugation of 100µl of seminal fluid for 20 min at 14,000 x g in 4° C, the flow-through was recovered and

100 µL of Hayes was added to the protein fraction of seminal fluid and centrifuged again for 20 min at 14,000 x g in 4° C. This procedure was repeated 5 times to ensure maximal removal of the non-protein part of seminal fluid before the protein fraction was collected by reversing the cartridge and centrifuging for 10 min at 1000 x g in 4° C. Both fractions were reconstituted back to the original volume of 100 µL using Hayes solution, the washes were kept separately. We collected a total of four independent biological replicates of seminal fluid for this experiment and quantified the antimicrobial activity of both fractions on *N. apis* spores using flow cytometry as described above. To visualise the success of our separation we used SDS-PAGE and ran 30 µl of the non-protein and 3 µl of the protein fraction, as well as 3 µl of non-fractionated seminal fluid and 30 µl Hayes as controls. To do this we used a 4–20% Mini-PROTEAN® TGX™ Precast Protein Gel (Bio-Rad), run at constant 200V, running a Dual Xtra LMW standard (250-2 kDa) (Bio-Rad), the gel was stained with Coomassie. As shown in Supplemental Figure 1 the SDS-PAGE protein separation showed comparable protein banding patterns for the protein fraction and non-fractionated seminal fluid. As expected, no protein or peptide bands are visible in the non-protein fraction and the Hayes control lane. Consequently our centrifugation protocol successfully separated seminal fluid into a protein and non-protein fraction, which is consistent with results we published earlier [19].

The fractions were furthermore visualised by spectrophotometry, using a Nano-drop (Thermo Scientific, Wilmington, USA). To do this, we analysed 2 µL in protein mode and visualised absorbance between 220nm - 350nm.

Solid-phase extraction and protein separation of seminal fluid samples

To further quantify the biological activity of different protein fractions of seminal fluid, we used solid-phase extraction (SPE) C18 Macro SpinColumns (Nest Group, USA) according to the manufacturer's instructions. Four biological replicates were used for separation, 300 µL of sample (40 µL of the SF added to 260 µL of DDI water with 0.1% trifluoroacetic acid (TFA)) was loaded onto the cartridge, then the flow through, wash and 20, 30, 40, 60 and 80% acetonitrile (ACN) elutions were collected by centrifugation. Fractions were dried in a vacuum centrifuge (Acid Resistant CentriVap Concentrator, Labconco, US) until dryness, and reconstituted in 20 µL of Hayes. In order to further determine the nature of the active compounds, the protein fractions (>3 kDa) were reconstituted to 300 µL using 0.1% TFA in DDI water and separated using C18 SPE-cartridges (as described above), and eluted with 0, 30 or 80% ACN by centrifugation. Because of the very small amounts of material that became available, we were not able to visualise samples on SDS-PAGE and no chromatograms were available for the individual fractions.

Zone of inhibition assays

To quantify antimicrobial activity of seminal fluid in bacteria, instead of *N. apis*, we performed zone of inhibition assays, using *Arthrobacter globiformis.* Bacteria were grown at 30°C either in Luria-Bertani (LB) broth or on LB agar plates (15 g/litre agar). An overnight culture of *A. globiformis* was prepared in 10 ml of LB broth and incubated overnight at 30°C with shaking at 80 rpm. We then layered 150 µL of the bacterial culture onto LB plates. Droplets of 1 µL of SF or ampicillin as a positive control were added to LB plates and incubated for 48 hours at 30°C. We then checked the plates for signs of reduced bacterial growth, indicated by clear circles around areas where SF or ampicillin samples had been applied. These assays were performed 3 times.

246

247 **Statistics**

248 Statistical analyses were performed using Rx64 2.14.0 software for Window 7 and
249 SPSS for Macintosh. Prior to statistical analysis, we calculated linear regression residuals to
250 determine whether data were normally distributed. ANOVA and Tukey's Honestly
251 Significant Difference post-hoc or Tukey's HSD tests were used to test for differences
252 between treatments and to identify significant differences within treatments. For datasets that
253 we were not normally distributed we used Generalised Linear Models, Kruskal-Wallis and
254 Mann-Whitney U tests.

255

RESULTS

We found that seminal fluid of honey bees is remarkably efficient in killing *N. apis* by significantly reducing spore viability compared to the control treatment (Figure 1, Wald-Chi-square=1857.934, df=5, p<0.001). Furthermore, incubation time had no significant effect on *N. apis* spore viability (Wald-Chi-square=0.416, df=1, p=0.416). Consequently, the antimicrobial effect of seminal fluid occurred rapidly and did not alter spore viabilities afterwards for up to 24 hours. We therefore performed all further experiments using an incubation time of 5 minutes. Post-hoc tests revealed that the spore killing effect was most pronounced in pure seminal fluid, but statistically significant reductions in spore viability were also found in samples diluted up to 10 times (Figure 1). When we analysed our flow cytometry data in more detail (Figure 2A), we found that the amount of fluorescent debris increased significantly in samples with higher concentrations of seminal fluid (Wald-Chi-square=178.598, df=5, p<0.001) but was not influenced by incubation time (Wald-Chi-square=0.068, df=1, p=0.795). When we inspected these samples microscopically, we found that exposure to seminal fluid resulted in the clumping and apparent germination of *N. apis* spores (Figure 2B), while control spores did not show any signs of germination-like rupture and clumping.

When we used flow cytometry to visualise the antimicrobial effect of pure seminal fluid, the protein fraction and the non-protein fraction on *N. apis* spores (see Figure 3 for examples of flow cytometry dot plots), we found fluorescent debris only in samples where spores were exposed to undiluted and the protein fraction of seminal fluid. When we consequently quantified the antimicrobial effect of the non-protein and protein fractions on *N. apis*, we found that both fractions significantly reduced spore viability compared to the

281 control treatment (Figure 4A, ANOVA, F=209.2, df=3, p<0.001). Post-hoc tests revealed that
282 the potency of the non-protein fraction in killing spores was not significantly different from
283 complete seminal fluid (Tukey's HSD, p=0.790), but the protein fraction killed significantly
284 fewer spores compared to complete seminal fluid (ANOVA, F=209.2, df=3, p<0.001). As
285 expected from our visual observations, the amount of fluorescent debris differed significantly
286 between treatments (Figure 4A, ANOVA, F=11.82, df=3, p<0.001) and was lower in the non-
287 protein fraction compared to the protein fraction (Tukey's HSD, p=0.005) or seminal fluid
288 (Tukey's HSD, p=0.024). There was no significant difference in the amount of fluorescent
289 debris between the protein fraction and complete seminal fluid (Tukey's HSD, p=0.806) or
290 between the non-protein fraction and the control sample (Figure 4A, Tukey's HSD, p=0.924).
291
292 When, we analysed *N. apis* spores exposed to germination solution we found a
293 significant reduction in spore viability (Mann-Whitney U-test: N=8, p=0.029) and a
294 significant increase of fluorescent debris (Mann-Whitney U-test: N=8, p=0.029) compared to
295 the Hayes control (Figure 4B). Because these findings were similar to those obtained from
296 spores exposed to the protein fraction of seminal fluid (Figure 4A), we conclude that
297 molecules in the protein fraction induce germination-like rupture of the *Nosema* spore wall
298 whereas the non-protein fraction killed spores without spore wall rupture.
299
300 Furthermore, the absorbance spectra of the different fractions showed three distinct
301 peaks (Figure 4C). Non-fractionated seminal fluid showed 2 peaks, one at 220-235 nm and
302 another at 245-275 nm. When the seminal fluid was separated into the non-protein and
303 protein fractions, the non-protein fraction contained small molecules (<3 kDa) that have an
304 absorbance at 245-275 nm, while a peak consistent with a peptide bond or phenylalanine at
305 220-235 nm is not visible. The protein fraction on the other hand contained molecules that

306 have an absorbance consistent with proteins, showing a peak for aromatic amino acids, such
307 as threonine and tyrosine at 275-290 nm and a peak at 220-235 nm consistent with the
308 peptide bond.
309
310 When we analysed the effect of different reverse-phase C18 SPE separation fractions
311 of seminal fluid we found an overall significant reduction in *N. apis* spore viability (Kruskal
312 Wallis, *Chi-squared*=25.79, *df*=7, $p<0.001$, Figure 5). Post-hoc comparison revealed
313 significant reductions in spore viability in the flow-through (FT), 40%, 60% and 80% (v/v)
314 ACN fractions compared to the Hayes control solution. Furthermore, only the fractions eluted
315 with 40% and 60% ACN produced significantly more fluorescent debris compared to the
316 Hayes control. The flow through fraction killed spores but did not produce more fluorescent
317 debris than the Hayes control. The detected activity of the flow through as well as proteins
318 that bind to the stationary phase therefore provide further evidence for the presence of at least
319 two types of molecules that are capable to kill *N. apis* spores.
320
321 A further separation of the protein fraction (>3kDa) by C18 reverse-phase showed
322 that the molecules retained, killed *N. apis* spores and produced fluorescent debris using the
323 fraction eluted with 80% ACN. We therefore confirmed that a protein fraction compound
324 induced spore wall rupture whereas a non-protein fraction compound had a direct killing
325 effect on *N. apis* spores. Finally we found that the two biologically active compounds must
326 have substantially different biochemical properties, given the different affinity to the C18
327 reverse-phase solid-phase which complements the evidence of different absorbance spectra
328 from the protein and non protein fractions (Figure 4C).
329
330

Finally we found that seminal fluid had no measureable effects on the growth of *A. globiformins* compared to the ampicillin control treatment, as no measurable zones of inhibition were observed in any of the seminal fluid samples tested. Follow up work using two additional microbial species (*Escherichia coli* and *Saccharomyces cerevisiae*) provided the same results.

DISCUSSION

We provide empirical evidence that seminal fluid of honey bees has a remarkable anti-microsporidial activity and is able to reduce spore viability of the sexually transmitted pathogen *N. apis* by over 80% (Figures 1A, 2A, 4A and 5). Our findings imply that males are able to efficiently protect their ejaculates in order to reduce the risk of sexually transmitting the pathogen to the queen during mating. Our findings therefore provide support for the trade-off hypothesis, implying that males maximize immunity in their germ line at the cost of their somatic tissue. This adds further empirical evidence for the trade off hypothesis to what has already been published in ants and bees [15, 25, 38]. Our results are also in line with our previous work, where we found males to be highly susceptible to *N. apis* [31], but able to supress the spread of the infection to their reproductive organs. Contaminations of the ejaculate seem to occur as a consequence of dysentery and/or the rupturing of tissue during the ejaculation process and the disease can consequently be transmitted during mating from the male to the queen [32]. Our findings therefore imply that the anti-microsporidial activity of seminal fluid provides an additional male adaptation to further reduce the risk of sexual transmission, but does not provide complete protection. As we show, seminal fluid is highly efficient in reducing the viability of *N. apis* spores (Figures 1A, 2A, 4A, 5), but a small fraction of spores are able to survive exposure to seminal fluid for up to 24 hours. Experimental follow up work confirmed that these spores are still capable of infecting worker

357 bees (Baer *et al*, in prep). It is therefore likely that the *N. apis* spore samples used for our
358 experiments contained a mixture of spores from several individual strains, as we collected
359 them from workers of different colonies. Consequently surviving spores might represent
360 strains with some level of resistance against the antimicrobial activity within the seminal
361 fluid. More experimental work is required to test the idea that the survival of spores in
362 seminal fluid samples is indeed non-random, and whether seminal fluid of different
363 genotypes of honey bees vary in their effectiveness to kill individual *N. apis* strains. If this is
364 the case, studying antimicrobial activity of seminal fluid against *N.apis* spores offers a new
365 opportunity to unravel host-parasite interactions and the underlying genotype x genotype
366 interactions at the proteomic level.
367
368 Our experiments provide further insights into a remarkable complexity of antimicrobial
369 activity within an insect ejaculate. Firstly, from our analyses of the fluorescent debris data
370 (Figure 3, 4A), microscopic observations (Figure 2B) spectrophotometric absorbance (Figure
371 4C), and the comparison of antimicrobial activity in different subfractions of seminal fluid
372 (Figure 5), we conclude that seminal fluid components kill *N. apis* spores in at least two
373 distinctly different ways. The protein fraction of the seminal fluid induces germination-like
374 rupture of the spore wall whereas small molecules in the non-protein fraction of seminal fluid
375 kills *N. apis* spores directly, implying some form of redundancy within the defence system of
376 the honey bees ejaculate. Secondly, we found that the defence response against *N. apis* also
377 shows a degree of specificity, as our zone of inhibition experiments detected no antimicrobial
378 activity of seminal fluid towards a number of other microorganisms. Such a specific
379 antimicrobial response to a pathogen in the ejaculate has so far only been described for
380 vertebrates [7, 9]. Consequently, finding redundancy and specificity of a immune protection
381 in an insect is quite surprising, and requires further research to unravel the genetic and

382 biochemical mechanisms that underlie the phenotypic interaction between the honey bee's
383 antimicrobial defence and the *N. apis* pathogen, using novel approaches such as evolutionary
384 proteomics [39].
385
386 The antimicrobial activity that we found in the protein fraction of seminal fluid is consistent
387 with a protein or proteins being responsible for the observed antimicrobial effect, based on
388 their observed size, absorbance and chemical properties. Interestingly, we found that the
389 protein fraction of seminal fluid induced the germination of *N. apis* spores. Germination of
390 microsporidia, such as *Nosema* can be caused by a number of factors such as changes in pH,
391 temperature, ionic concentrations, or exposure of dehydrated spores to water [37, 40-42].
392 Regardless of the trigger, spore germination results from an increase of osmotic pressure
393 inside the spore that eventually triggers the expulsion of the polar tube [41-43] but the exact
394 mechanisms are still unknown. Here we conclude that the protein fraction also plays a role in
395 spore germination and thereby killing the microsporidia. Seminal fluid of insects contains a
396 range of proteins with predicted antimicrobial activity including proteases, peptidases [6, 20,
397 44-46] and chitinase in honey bees [6]. Fungal cell walls contain a chitin/protein matrix;
398 rupture requires chitinase and proteases and this is a normal part of the germination process
399 in the lifecycle of fungi that produce spores [47]. Significant work has been undertaken on
400 fungal chitinase and proteases that would act from inside of the spore in model fungi [47].
401 Premature weakening of the wall by exogenous proteases and chitinases from the seminal
402 fluid could therefore lead to rupture of the spore wall due to turgor pressure from the cell and
403 expansion of the spore cell, which would also expose the fungal cell to other forms of
404 antimicrobial attack by the insect defence machinery [48, 49]. The specificity of the effect we
405 detected in our samples is consistent with the known properties of chitinases that appear to be
406 optimised for chitin/protein matrices in different species and to act synergistically with a

407 complex network of proteases [50-53]. More work is required to specifically study whether
408 the chitinases and/or proteases present in the seminal fluid are indeed involved in triggering
409 spore germination in *N. apis*. Spore germination ultimately results in the expulsion of
410 genomic material from the spore, and in the case of seminal fluid-treated spores, we find this
411 material to accumulate in the fluorescence debris. Successful *N. apis* infections depend on the
412 parasite delivering its sporoplasm into the cytosol of a host cell [42, 54]. The protein fraction
413 of the seminal fluid therefore provides an efficient pathway to interrupt the life cycle of *N.*
414 *apis* spores and indirectly killing the spores before they could propagate.
415
416 Based on the SDS-PAGE gel (Supplemental Figure 1), the absorbance spectra (Figure 4C)
417 and the chemical properties of active fractions in their interaction with C18, we can conclude
418 that the biologically active molecule in the non-protein fraction of seminal fluid is unlikely to
419 be either a protein or a peptide. Nevertheless, the identity of the antimicrobial molecule(s)
420 and its biological mode of activity will need to be studied in more detail in the future. The
421 non-protein fraction of seminal fluid is able to decrease spore viability without cell wall
422 rupture and does not cause spore germination, implying it can enter the spore directly.
423 Analysis of seminal fluid-treated spores and/or biochemical dissection of seminal fluid
424 samples, using the bioassays of Nosema spore viability or rupture as developed here, could be
425 used in the future to identify the compounds responsible.
426
427

STATEMENT OF AUTHORSHIP

YP and BB designed and YP and JG conducted the experimental work presented in this paper, all authors contributed to the analyses of data and the writing of the manuscript.

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DATA ACCESSIBILITY

The datasets supporting this article are accessible at doi:10.5061/dryad.18bv9.

There is 1 supplemental Figure attached to this paper that can be downloaded at XXX.

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589
590

591

Figure legends

Figure 1

The seminal fluid of honey bees (*Apis mellifera*) is highly efficient in killing spores of the fungal pathogen *N. apis*. Flow cytometry identified two distinct populations of spores (for methodological details see [36]), consisting of live and dead spores. Spore viability was consequently calculated as the percentage of live spores within the total amount of spores counted. Letters above columns indicate significant differences from post-hoc tests between treatments. See main text for statistical details.

Figure 2

A: The effect of seminal fluid and Hayes control solution on the viability of *N. apis* spores (black columns) and the fluorescent debris detected in samples by flow cytometry (grey columns). Germination resulted in the release of nuclear material from spores and consequently increased the amount of fluorescent debris detected by flow cytometry.

B: Differential Interference Contrast image of *N. apis* spores exposed to complete seminal fluid, showing the clumping of spores and filaments protruding from germinated spores (white arrows, see insert for a magnified view of a single germinated spore and its filament).

Figure 3

Dot plots from light scatter (FSC/SSC) analysis of *N. apis* treated spores with the protein fraction (**A**), the non-protein fraction (**B**) and Seminal Fluid (**C**). Dot plots were gated into two regions: spores and debris as high FSC/high SSC and low FSC/low SSC. The debris was further sub-gated into general and fluorescent debris (containing nucleic acid material). The spores were further sub-gated into 2 populations, dead spores (Q1 + Q2) and live spores (Q4 +Q3).

Figure 4

A: The antimicrobial effect of pure seminal fluid, as well as the non-protein and protein fractions of seminal fluid on *N. apis* spore viability (black columns) and fluorescent debris (grey columns). All seminal fluid samples significantly decreased spore viability compared to the Hayes control treatment, but only complete seminal fluid and the protein fraction of seminal fluid resulted in a significant increase in the amount of fluorescent debris.

B: The effect of germination solution (see [37] for details) on *N. apis* was comparable to that of the protein fraction as seen in A. Germination solution significantly decreased *N. apis* viability and increased the amount of fluorescent debris measured by flow cytometry.

C: Absorbance spectra of unfractionated seminal fluid (top panel), the non-protein (middle panel) and protein fraction of seminal fluid. The protein fraction of seminal fluid shows a peak for the aromatic amino acids and peptide bond whereas the non-protein fraction shows a different peak at 260nm. The black and grey lines show data from two biological replicates.

Figure 5

Antimicrobial effect of seminal fluid fractions after C18 (reverse-phase) SPE-separation on *Nosema apis* spore viability (black columns) and fluorescent debris (grey columns). Apart from complete seminal fluid, we also found the flow through (FT), and the 40%, 60% and 80% fractions to significantly reduce *N. apis* spore viability, compared to the Hayes control. This coincided with a significant increase in fluorescent debris detected in complete seminal fluid and the 40% and 60% fractions compared to the Hayes control but not the FT.

Figure 1

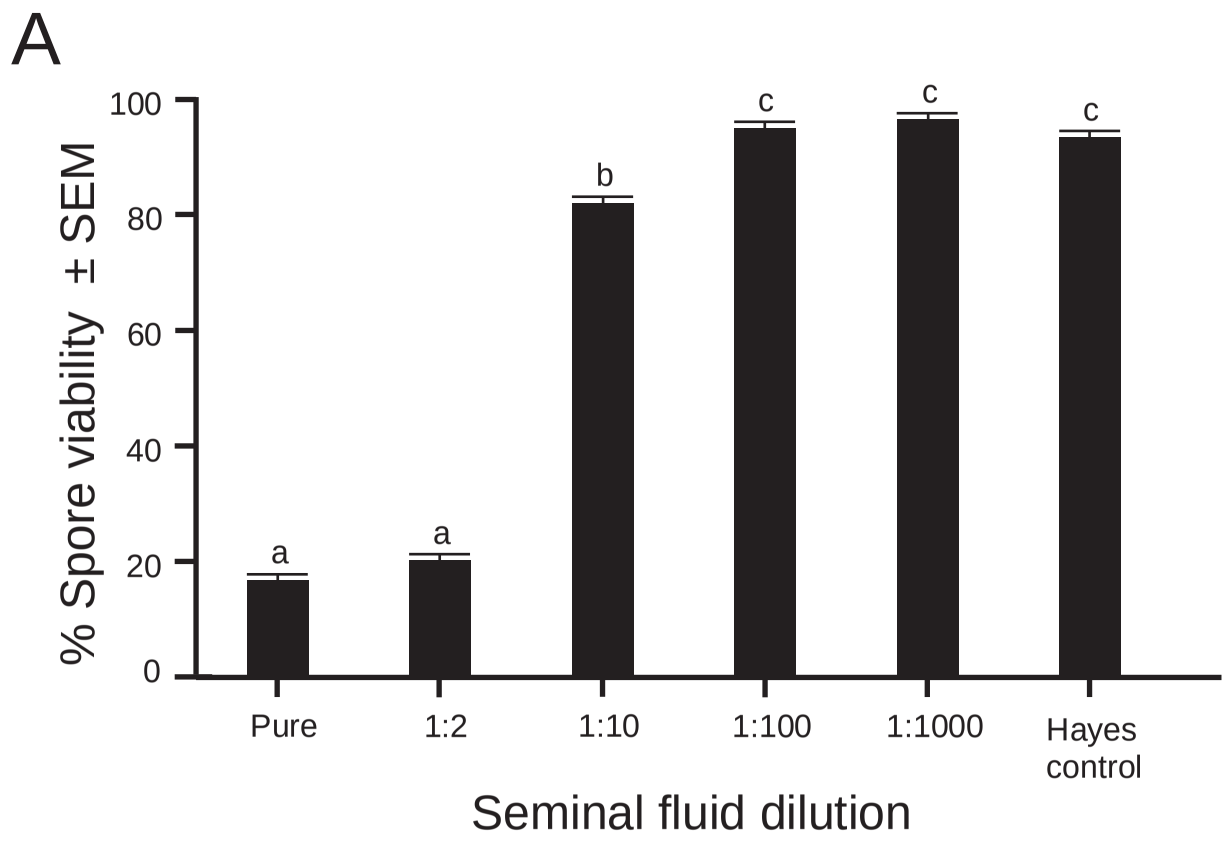
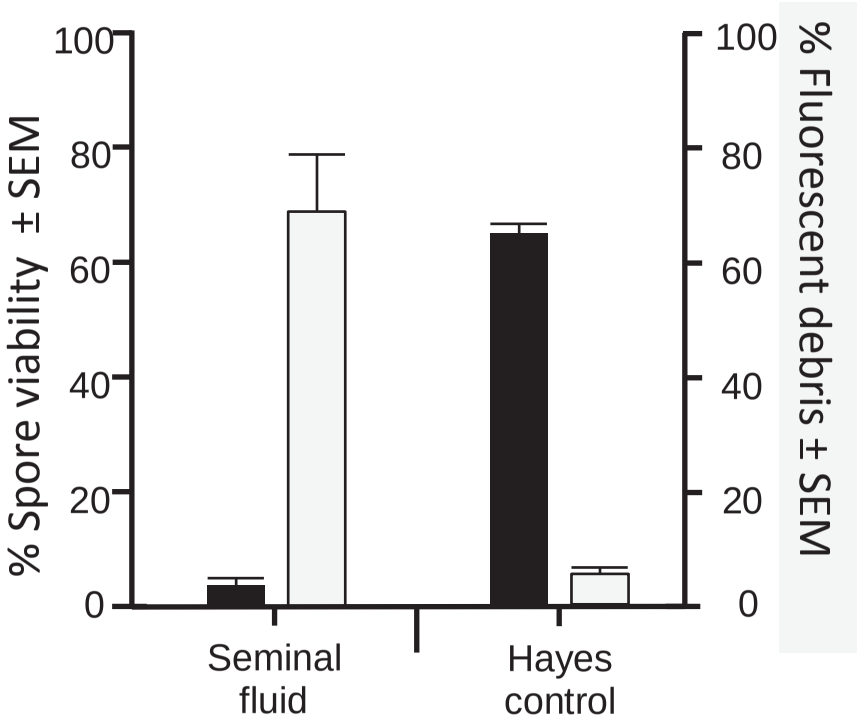

A
% Spore viability ± SEM
100
80
60
40
20
0
a
a
b
c
c
c
Pure
1:2
1:10
1:100
1:1000
Hayes control
Seminal fluid dilution

Figure 2

A


100
80
60
40
20
0
% Spore viability ± SEM
100
80
60
40
20
0
% Fluorescent debris ± SEM
Seminal fluid
Hayes control

B

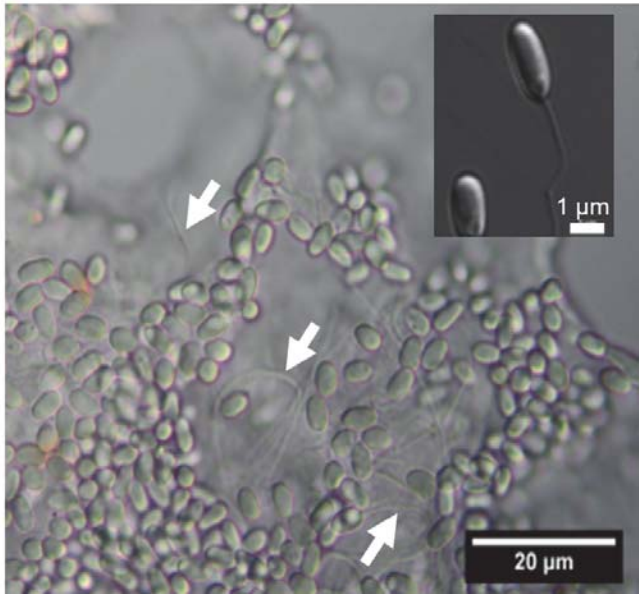

1 µm
20 µm

Figure 3

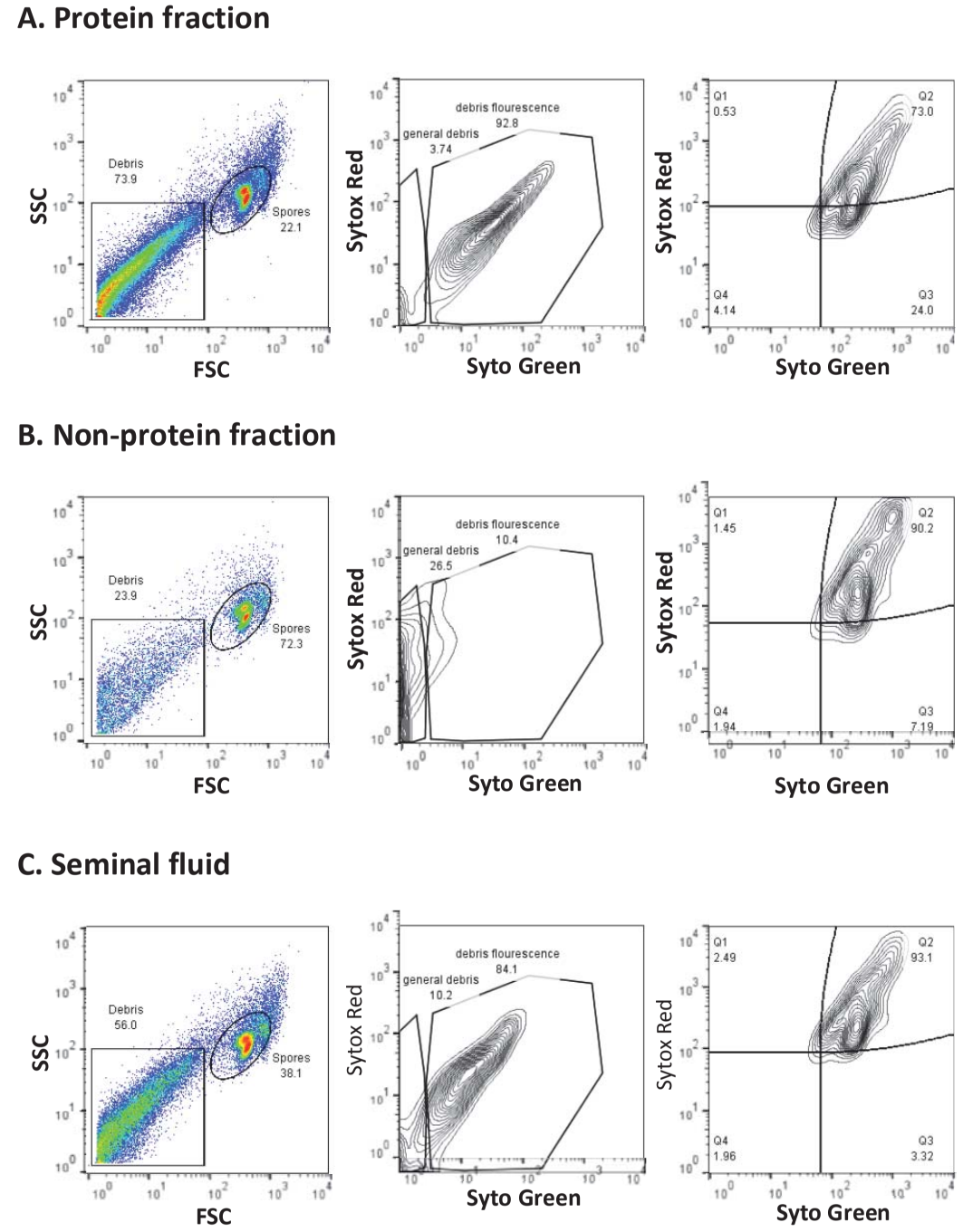
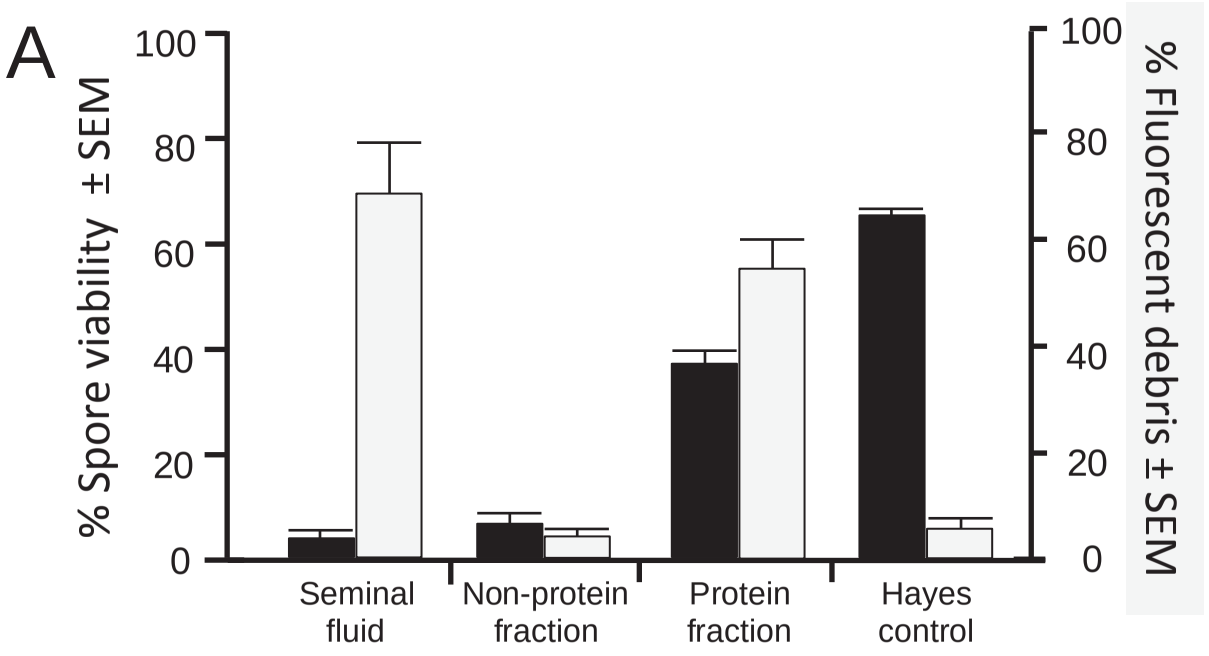
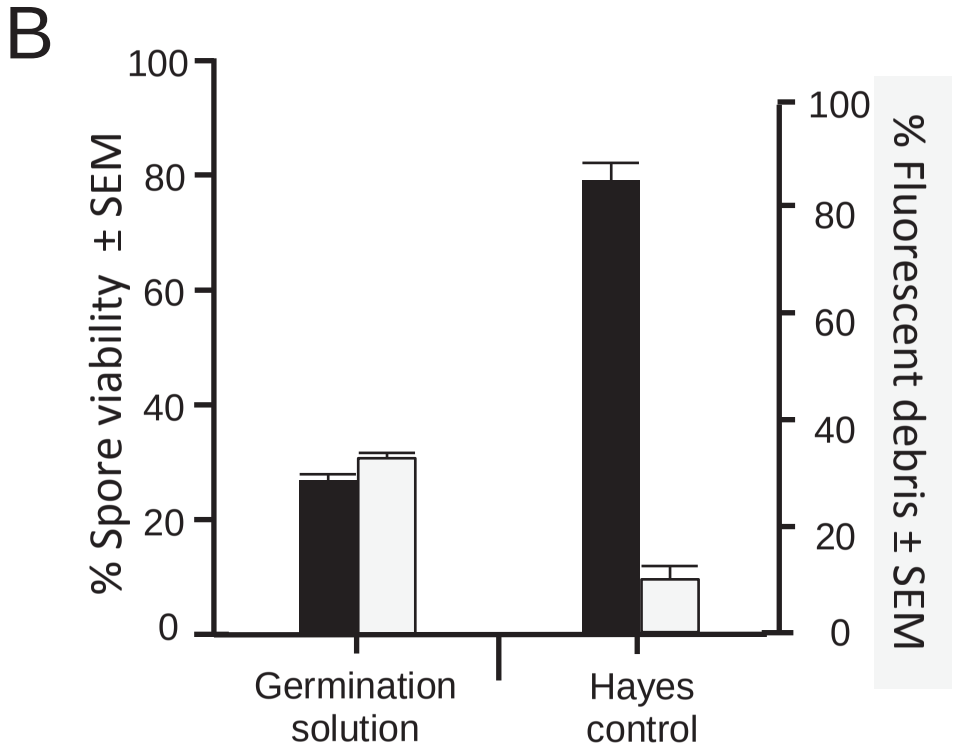

A. Protein fraction
Debris
73.9
Spores
22.1
SSC
FSC
debris flourescence
92.8
general debris
3.74
Sytox Red
Syto Green
Q1
0.53
Q2
73.0
Q4
4.14
Q3
24.0
B. Non-protein fraction
Debris
23.9
Spores
72.3
debris flourescence
10.4
general debris
26.5
Q1
1.45
Q2
90.2
Q4
1.94
Q3
7.19
C. Seminal fluid
Debris
56.0
Spores
38.1
debris flourescence
84.1
general debris
10.2
Q1
2.49
Q2
93.1
Q4
1.96
Q3
3.32

Figure 4


A
% Spore viability ± SEM
% Fluorescent debris ± SEM
0
20
40
60
80
100
Seminal fluid
Non-protein fraction
Protein fraction
Hayes control


B
% Spore viability ± SEM
% Fluorescent debris ± SEM
0
20
40
60
80
100
Germination solution
Hayes control

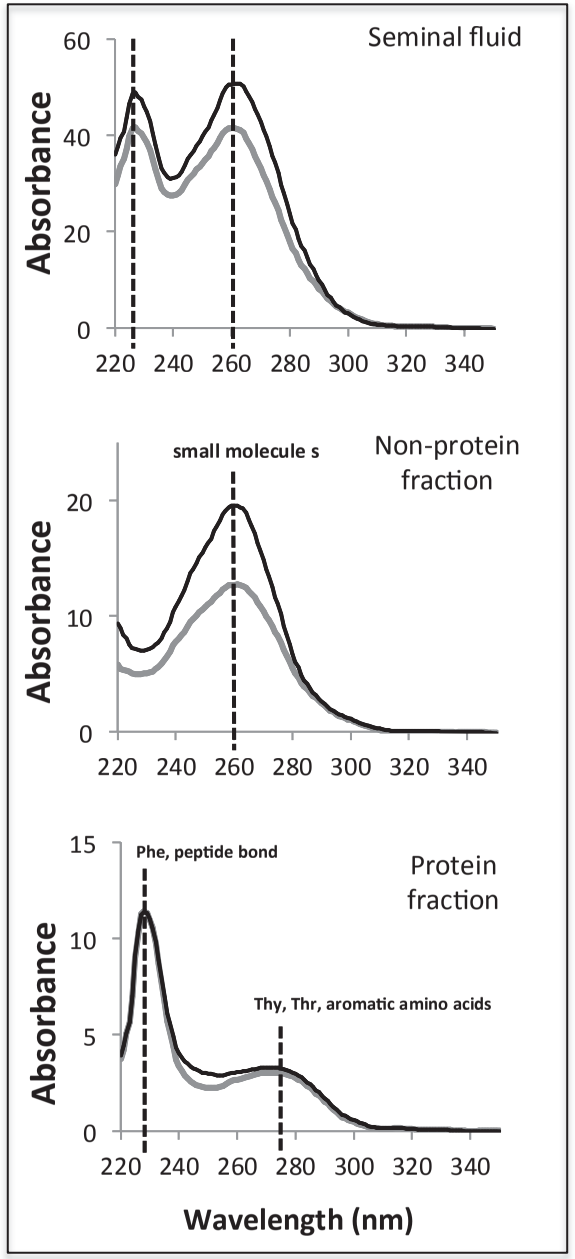
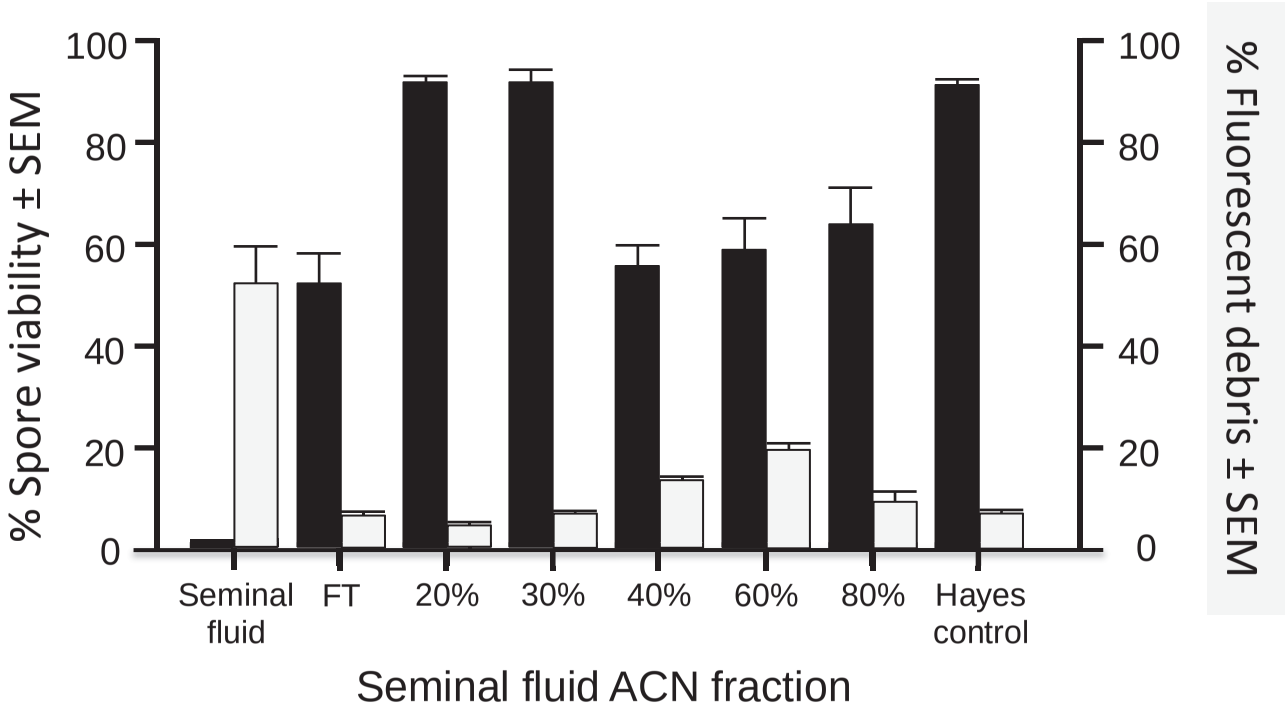

C
Seminal fluid
Absorbance
small molecule s
Non-protein fraction
Phe, peptide bond
Protein fraction
Thy, Thr, aromatic amino acids
Wavelength (nm)

Figure 5


% Spore viability ± SEM
% Fluorescent debris ± SEM
100
80
60
40
20
0
Seminal fluid
FT
20%
30%
40%
60%
80%
Hayes control
Seminal fluid ACN fraction