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# Identification of predicted seminal fluid proteins in *Tribolium castaneum*

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## Abstract

**In several insect species, seminal fluid proteins (SFPs) have been demonstrated to be key regulators of male and female fitness through their ability to alter female physiology and behaviour. *Tribolium castaneum* is an economically important pest species and a model system for sexual selection research, but little is known about SFPs in this insect. To create a foundation for the study of *T. castaneum* SFPs, we used mass spectrometry to identify putative SFPs by comparing proteins detected in the male reproductive glands with those found in the reproductive tracts of virgin and mated females. Fourteen putative SFPs, thirteen with male biased expression, were identified through this approach. We also used reverse transcription PCR (RT-PCR) to examine expression levels across different tissue types. We found strongly male-biased expression in 13 genes, nine of which were expressed only in male accessory gland tissue. This represents the first proteomic-based method of identifying putative SFPs in any coleopteran species, and is the first study in this species to identify putative SFPs that are likely transferred to the female. This work could lead to functional analyses of the role of SFPs in sexual selection, sexual conflict and potential control of a pest species.**

**Keywords: seminal fluid proteins, male accessory glands, gene expression, *Tribolium castaneum*.**

## Introduction

Although the primary function of copulation is gamete transfer, male ejaculates contain numerous other substances produced by secretory tissues in the reproductive tract (Leopold, 1976; Chen, 1984; Gillott, 1996, 2003; Simmons, 2001; Poiani, 2006). Recent work in arthropods has identified many specific nonsperm components of male ejaculates (reviewed in Gillott, 2003; Poiani, 2006; Wolfner, 2007; Avila *et al*., 2011). These substances not only assist in storing and provisioning sperm (Poiani, 2006), but also have diverse physiological and behavioural effects on females. In insects, seminal fluid proteins (SFPs) have been implicated as the principal seminal component responsible for inducing many of these post-mating responses (Gillott, 2003; Wolfner *et al*., 2005; Wolfner, 2007; Avila *et al*., 2011).

The most extensive characterization of SFPs has been accomplished in *Drosophila melanogaster* (reviewed in Wolfner, 2002, 2007; Ravi Ram & Wolfner, 2007), although some characterization of SFPs has occurred in other species of insects (tephritids: Davies & Chapman, 2006; mosquitoes: Dottorini *et al*., 2007; Sirot *et al*., 2008; Rogers *et al*., 2009; honeybees: Collins *et al*., 2006; Baer *et al*., 2009; crickets: Braswell *et al*., 2006; Andres *et al*., 2008 and bedbugs: Reinhardt *et al*., 2009). The *D. melanogaster* studies demonstrated that SFPs influence many processes in females, including oviposition and egg production, sperm utilization and storage, female re-mating rates and even female life span (reviewed in Eberhard & Cordero, 2003; Gillott, 2003; Chapman & Davies, 2004; Ram & Wolfner, 2007; Wolfner, 2007; Chapman, 2008). SFPs have also been implicated as being crucial mediators of sperm competition (Harshman & Prout, 1994; Prout & Clark, 2000; Wigby *et al*., 2009) thereby influencing fertilization success amongst rival males. Thus, the complex cocktail contained in male ejaculates and transferred to females during mating is likely to have profound fitness implications for both sexes, influencing evolutionary processes ranging from sexual conflict to reproductive isolation in addition to aspects of a species' mating system, such as the degree of multiple mating and sperm competition. SFPs are amongst the most rapidly evolving proteins (Swanson *et al*., 2001; Swanson & Vacquier, 2002; Clark & Swanson, 2005; Andres *et al*., 2006; Clark *et al*., 1995, 2006; Panhuis *et al*., 2006), with attempts to identify homologues of *D. melanogaster* SFPs outside of *Drosophila* being met with limited success, most likely

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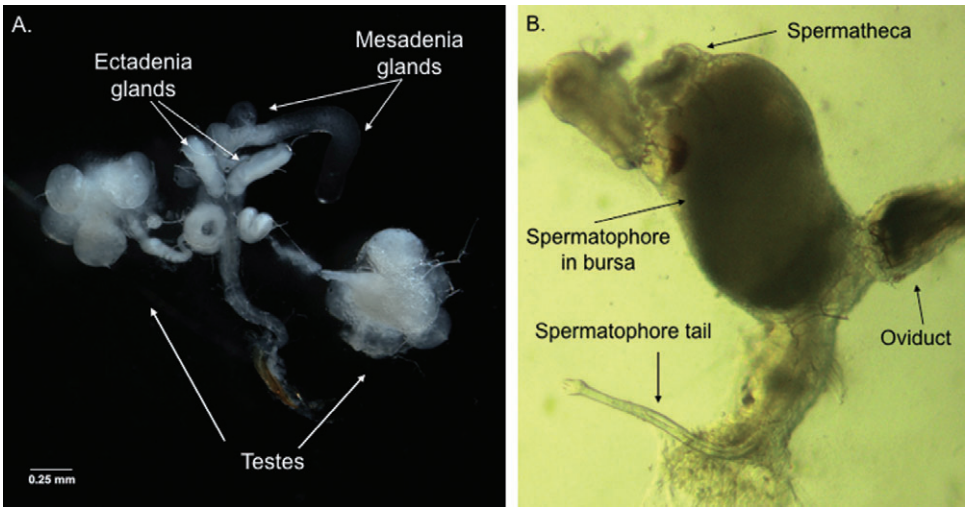


**Figure 1.** Reproductive anatomy of male (A) and female (B) *Tribolium castaneum*. Proteins were separately collected from mesadenia and ectadenia glands. Bursal tissue was collected from both virgin and mated females.

because of these rapid rates of evolution. Thus, it is necessary to identify SFPs for each species of interest; however, this has been accomplished for relatively few taxa. For example, SFPs have not been positively identified in any coleopteran, a highly successful insect order that includes several agriculturally important pests. Indeed, the dearth of information about these proteins in such key taxonomic groups currently limits our understanding of their functional significance.

*Tribolium castaneum* (Coleoptera:Tenebrionidae) is an excellent model system in which to investigate SFPs. Adults are highly promiscuous and utilize a variety of mechanisms to bias paternity (Fedina & Lewis, 2008), rendering it an appropriate species for exploring the role of these proteins in influencing the consequences of multiple mating and sexual conflict. Previous work on *T. castaneum* has revealed that last male sperm precedence varies widely, and several peri- and post-copulatory behaviours influence paternity (Bernasconi *et al.*, 2006; Fedina & Lewis, 2006, 2007, 2008). *T. castaneum*'s ease of culture, short generation time, sequenced genome and efficacy of genetic manipulation make it an ideal model organism (Richards *et al.*, 2008; Brown *et al.*, 2009). Finally, *Tribolium* is a worldwide stored product pest species, and understanding how SFPs influence reproduction could potentially open future avenues for pest control.

Several studies in *Tribolium* and related beetles suggest that substances manufactured by male beetles and transferred to the female could be important in determining paternity success (Fedina & Lewis, 2008). *Tribolium* males have two pairs of accessory glands (AGs), the mesadenia (ME) and ectadenia (EC) glands. Both glands are involved in producing a spermatophore that is transferred to the female (Fig. 1). Several previous studies have concluded that these AGs produce not only different proteins, but also large carbohydrate-containing macromolecules (Sevener *et al.*, 1992; Novaczewski & Grimnes, 1996). Additionally, 112 genes that are highly expressed in *T. castaneum* AG have been identified with custom microarrays, and 14 of those genes show biased expression in AG (Parthasarathy *et al.*, 2009). Furthermore, RNA interference knockdown of genes involved in juvenile hormone synthesis decreased expression of these AG genes, lowered sperm production and lessened male mating vigour. Females mated to those knockdown males produced fewer eggs and progeny than females mated to control males (Parthasarathy *et al.*, 2009). Taken together, these results suggest that SFPs might play a role in explaining variance in male reproductive performance and fitness in *T. castaneum*.

Although studies of gene expression in male reproductive glands are useful for identifying potential SFPs, recent studies have shown that not all SFPs show enriched expression in the male reproductive glands (Findlay *et al.*, 2008, 2009). Proteomic analyses have allowed the direct identification of proteins produced in the male accessory glands and transferred to females during mating (Findlay *et al.*, 2008, 2009). Here, we adopt a proteomic approach for identifying SFPs in *T. castaneum*. We used mass spectrometry (MS) to identify putative SFPs by comparing proteins detected in the male reproductive glands with those found in the reproductive tracts of virgin and mated females. This MS-based approach has proven successful in identifying putative SFPs in other taxa (eg Collins *et al.*, 2006; Sirot *et al.*, 2008; Reinhardt *et al.*, 2009). Using reverse transcription PCR (RT-PCR), we then determined the presence/absence of expression of the genes coding for these identified proteins in female and male tissues.

This represents the first characterization of putative SFPs in *T. castaneum* using direct identification of proteins through mass spectrometry.

## Results and discussion

### *Identification and expression patterns of putative SFPs*

We conducted mass spectrometry on a subset of bands of proteins separated through one-dimensional gel electrophoresis that showed patterns suggestive of SFPs (ie presence in male reproductive glands and reproductive tracts of mated females, but not in reproductive tracts of virgin females; Fig. 2). Through mass spectrometry of proteins from male ME (Fig. 1) and virgin and mated female reproductive tracts, we identified 14 distinct proteins that were detected in the ME and the mated female, but not in the virgin female reproductive tracts (Table 1). Of the proteins identified via mass spectrometry, 12 are newly identified putative reproductive proteins and two, TC005744 and TC010066, were previously identified as expressed in the AGs (Parthasarathy *et al*., 2009). These proteins represent only a subset of the possible reproductive proteins that are produced by both male and female *T. castaneum*.

The expression patterns of genes encoding 13 of the 14 proteins we identified via mass spectrometry were highly male-biased, with undetectable transcript levels in female tissue (Fig. 3). Nine of the genes produced transcripts that were detectable only within male AGs, with one of those genes, TC015849, expressed only in the EC. Given that proteins from the ME only were submitted for MS identification, this result is surprising and warrants future investigation. It is possible that the protein product could be synthesized in the ME and transferred to the EC, or that the presence of expression in the ME could be detected by more sensitive means, such as quantitative reverse transcription PCR (qPCR). The remaining four genes were found not only in both types of AGs, but also in male body tissue samples (which lacked AGs). Thus, two lines of evidence suggest that the 13 proteins with male-specific gene expression represent putative SFPs: (1) these proteins were identified from both male AGs and the bursa copulatrices of mated females but were not detected from the bursa copulatrices of virgin females; (2) RT-PCR did not reveal detectable expression levels in females. The remaining protein, TC010066, was also identified previously as an AG gene (Parthasarathy *et al*., 2009) but does not have male-specific expression.

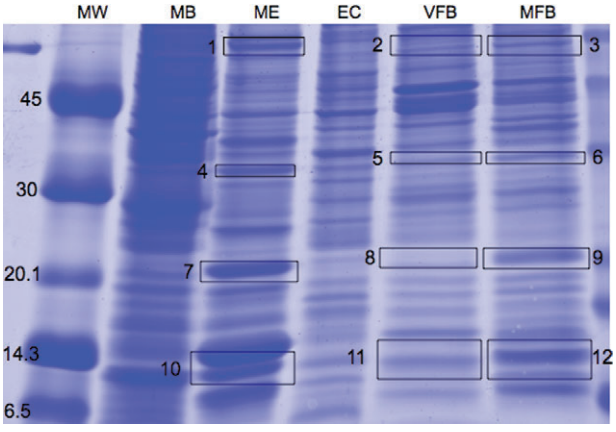


**Figure 2.** Coomassie-stained one-dimensional protein gel of tissue samples from *Tribolium castaneum* male reproductive accessory glands and the bursa copulatrix of virgin and mated females. Numbered boxes indicate protein bands selected for analysis that are present in male accessory glands and mated females but lighter or absent in virgin females. MW, molecular weight; MB, male body; ME, mesadenia glands; EC, ectadenia glands; VFB, virgin female bursa; MFB, mated female bursa. Proteins were separated on a one-dimensional 5–15% gradient sodium dodecyl sulphate-polyacrylamide gel and visualized with SimplyBlue SafeStain.

### *Homology and characterization of putative seminal fluid proteins*

Seven of the 14 proteins we identified had homologues in either *D. melanogaster*, *Aedes aegypti*, *Anopheles gambiae* or *Apis mellifera* (Table 1), and two of these homologues are known or predicted SFPs in these species. The predicted protein classes amongst our putative SFPs are consistent with protein classes that have been identified in the SFPs of other organisms (Mueller *et al*., 2004; Braswell *et al*., 2006; Collins *et al*., 2006; Davies & Chapman, 2006; Dottorini *et al*., 2007; Ravi Ram & Wolfner, 2007; Sirot *et al*., 2008; Walters and Harrison, 2008; Andres *et al*., 2008; Baer *et al*., 2009; Reinhardt *et al*., 2009; Rogers *et al*., 2009). The suite of proteins identified here contains several proteolysis regulators and insect pheromone/odorant-binding proteins. Additionally, several of the proteins that were identified had no conserved protein domains or homologues amongst any of the comparison species, and some of the proteins identified here are novel putative SFP classes. As many SFP sequences are thought to be rapidly evolving (Swanson *et al*., 2001; Swanson & Vacquier, 2002; Clark & Swanson, 2005; Andres *et al*., 2006; Clark *et al*., 1995, 2006), this is not surprising.

Proteolysis-regulating SFPs have been implicated as key modulators of reproductive biology in both males and females of many species. In *D. melanogaster*, these proteins are thought to influence the protein cascade that regulates post-copulatory processes in females such as ovulation and sperm storage (Ravi Ram *et al*., 2006) as well as defence against microbial infections (Khush & Lemaitre, 2000; Mueller *et al*., 2007). *D. melanogaster* SFPs in this class have also been suggested to play roles in mating plug formation (Lung & Wolfner, 2001), hormone cleavage and prohormone protection (Monsma *et al*.,

**Table 1.** Putative *Tribolium castaneum* seminal fluid proteins identified by mass spectrometry with their predicted protein class and homologues in other insects

| Glean number | Predicted size (kDa) | Band number from Fig. 2 | Tissue of expression | Predicted protein class | *Aedes aegypti* homologue | *Anopheles gambiae* homologue | *Drosophila melanogaster* homologue | *Apis mellifera* homologue |
|---|---|---|---|---|---|---|---|---|
| TC001930 | 20.75 | 10, 12 | EC, ME | No homology | | | | |
| TC003522 | 11.64 | 10,12 | EC, ME, MB | No homology | | | | |
| TC005744 | 41.86 | 4,6,7,9 | EC, ME | Serine protease inhibitor | | | CG8137<br>Serine-type endopeptidase inhibitor<br>Known SFP | GB16472-RA<br>Serine protease inhibitor |
| TC008976 | 31.89 | 10,12 | EC, ME | Serine protease inhibitor | AAEL009795<br>Papilin glycoprotein | AGAP009766<br>Known SFP | CG33103<br>Papilin glycoprotein | GB16153-RA<br>Papilin glycoprotein |
| TC009459 | 11.28 | 10,12 | EC, ME | Pheromone/odorant binding protein | | | | |
| TC010065 | 12.10 | 10,12 | EC, ME | No homology | | | | |
| TC010064 | 12.15 | 10,12 | EC, ME | Pheromone/odorant binding protein | | | | |
| TC011481 | 22.60 | 1,4,6,7,12 | EC, ME | No homology | | | | |
| TC012560 | 10.66 | 10,12 | EC, ME | Inosine triphosphate pyrophosphatase | AAEL000200 | | | |
| TC013049 | 33.81 | 4,6,9 | EC, ME, MB | Senescence marker protein | AAEL000757<br>Fat body protein | AGAP007794 | CG7390<br>Senescence marker protein | GB18633-RA<br>Senescence marker protein |
| TC015056 | 13.74 | 10,12 | EC, ME, MB | No homology | AAEL006131 | AGAP001708 | CG31326<br>Serine-type endopeptidase | |
| TC015849 | 41.89 | 1,3 | EC | Prophenoloxidase | AAEL014544<br>Prophenoloxidase | AGAP002825<br>Prophenoloxidase | CG8193<br>Monophenol monooxygenase activity | GB18313-RA<br>Prophenoloxidase |
| TC006088 | 11.18 | 10,12 | EC, ME, MB | Nucleoside triphosphate hydrolase | | | | |
| TC010066 | 13.05 | 10,12 | EC, ME, MB, FB | Pheromone/odorant binding protein | AAEL002617<br>Odorant binding protein | | | GB18363-RA<br>Odorant binding protein |

Tissue types: EC, ectadenia gland; ME, mesadenia gland; MB, male body; FB, female body.

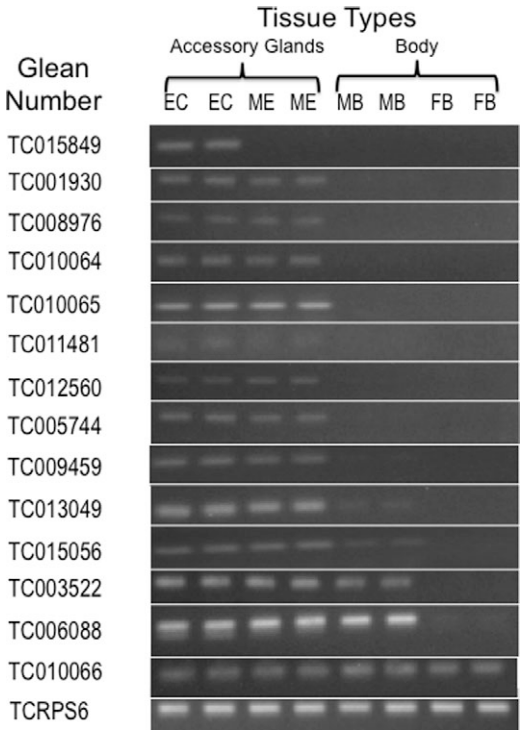


**Figure 3.** Qualitative reverse transcription PCR to determine the presence of 14 putative seminal fluid protein gene transcripts in different tissue types of *Tribolium castaneum*. EC, ectadenia gland; ME, mesadenia gland; MB, male body; FB, female body. The bottom row shows expression of TCRPS6, a protein that is part of the small ribosomal subunit. This was used to standardize the amounts of template cDNA used. This figure represents a composite of gels, as the products of the reverse transcription PCR from each gene were visualized on separate gels and then assembled for this figure.

1990; Mueller *et al*., 2004, Wolfner, 2009). In mammals, proteolysis-regulating SFPs influence the breakdown of semenogelin and affect sperm motility (Kise *et al*., 1996; Robert *et al*., 1997; Malm *et al*., 2000). Two of the putative *T. castaneum* SFPs reported here, TC005744 and TC008976, are predicted to be proteolysis regulators (Table 1). Interestingly, TC005744 is a predicted protease inhibitor with serpin domains that has a homologue amongst the known *D. melanogaster* SFPs (Ravi Ram *et al*., 2005; Findlay *et al*., 2008). That *Drosophila* SFP, CG8137, has predicted hydrophobic domains similar to those found in serpin class members that constitute a large component of mammalian seminal fluid. These serpins are capable of binding hormones and are crucial for male fertility in mammals (Uhrin *et al*., 2000). Given the known conservation of the general roles of reproductive proteins, it is possible that the *T. castaneum* putative proteolysis-regulating SFPs identified here share functions with those observed in other taxa.

Mating has the potential to introduce a variety of pathogens into the female reproductive tract that could negatively affect the reproductive success of both sexes. In *D. melanogaster*, several SFPs have been found to have either direct antimicrobial activity and may protect gametes and zygotes or may modulate a female's ability to fight infection (Samakovlis *et al*., 1991; Lung *et al*., 2001; Mueller *et al*., 2007, Wolfner, 2009). Putative SFPs in both *Ae. aegypti* and *An. gambiae* fall into protein classes suggesting that these may also play a role in immune response (Rogers *et al*., 2008; Sirot *et al*., 2008). Here we identified a putative SFP in *Tribolium*, TC015849, which is possibly involved in an immune response. This putative SFP is a predicted prophenoloxidase, a class of proteins that have been demonstrated to be important components of innate immune response within arthropods. The activation of prophenoloxidase leads to melanin synthesis and the stimulation of the Toll signalling pathway, which in turn induces the assembly of antimicrobial proteins (Ferrandon *et al*., 2007; El Chamy *et al*., 2008; Kan *et al*., 2008; Kanost & Gorman, 2008; Buchon *et al*., 2009; Roh *et al*., 2009;). The homologues of TC015849 in *Ae. aegypti*, *An. gambiae* and *Ap. mellifera* are also predicted to have prophenoloxidase domains (Baer *et al*., 2009; Lawson *et al*., 2009), whereas the *D. melanogaster* homologue, CG8193, is predicted to have monophenol monoxygenase activity and be involved in defence response (Asano & Takebuchi, 2009). However, none of those homologues are putative or known SFPs. Prophenoloxidase activity is typically associated with haemocytes (Cerenius & Soderhall, 2004), and given that haemocytes would be likely to be found in male and female body samples, it is surprising that TC015849 transcripts were not detected in those tissues if expression is indeed limited to haemocytes. Furthermore, we did not detect TC015849 proteins in virgin female reproductive tracts using mass spectrometry. Given that mating represents an opportunity for the introduction of foreign materials into the female reproductive tract and that prophenoloxidase is responsible for initiating melanin deposition around such foreign objects, it is conceivable that prophenoloxidase could be transferred within the male ejaculate to serve a defensive function within the female. The lack of expression at levels detectable in our study of TC015849 in any other tissue besides male AGs lends credence to this prediction, but certainly requires further investigation before any definitive conclusions can be drawn. Additionally, it is worth noting that such defensive strategies are likely to be coupled with other, more immediate forms of protections, such as lysis of bacterial cells (Otti *et al*., 2009).

Several of the possible *T. castaneum* SFPs fall into the classification of being predicted pheromone/odorant binding proteins (Table 1). These are small, soluble proteins that bind semiochemicals such as pheromones and odour molecules and deliver those molecules to olfactory receptors (see Pelosi *et al*., 2006 for review), supporting a role in insect molecular recognition. Odorant binding

proteins are a class of putative SFPs identified in *D. melanogaster* and *Ae. aegypti* (Findlay *et al*., 2008; Sirot *et al*., 2008). The functional significance of these proteins in reproduction has yet to be identified, but recent work suggests that odorant molecules play a chemoattractant role for sperm (Fukuda *et al*., 2004). Others (Takemori & Yamamoto, 2009) have suggested that the odorant binding proteins in *D. melanogaster* have organ-specific signalling roles in reproduction.

Three of the other classes of proteins identified here represent novel types of putative SFPs, but little or nothing is known about how these classes of proteins might function in reproduction. TC013049 is predicted to be a senescence marker protein, and its homologue in *D. melanogaster*, CG7390, and *Ap. mellifera*, GB18633, are known to be senescence marker proteins (Tweedie *et al*., 2009). This protein has been implicated in mammalian cells to have a role in $Ca^{2+}$ ion homeostasis and signalling (Inoue *et al*., 1999; Son *et al*., 2008). $Ca^{2+}$ has been demonstrated to play a crucial role in mammalian sperm function where it regulates such activities such as capacitation, chemotaxis and acrosome reaction (Publicover *et al*., 2007; Costello *et al*., 2009). TC012560 is predicted to be an inosine triphosphate pyrophosphatase, a protein that hydrolyses nucleoside triphosphates into monophosphates and is typically referred to as a house-cleaning enzyme (Galperin *et al*., 2006). Finally, TC006088 is predicted to be a nucleoside triphosphate hydrolase, an enzyme that can degrade nucleotides into simpler forms. The potential role of these proteins in reproductive processes remains to be investigated.

Five of the putative *T. castaneum* SFPs did not fall into any predicted protein class. Four of these proteins had no homologues in any of the four species (*Ae. aegypti*, *An. gambiae*, *D. melanogaster* and *Ap. mellifera*) investigated; one of the five proteins had homologues in all three dipteran species, but functional information is available for only one of those homologues, CG31326 in *D. melanogaster*, which is a serine-type endopeptidase that is upregulated in response to bacterial infection (Maia *et al*., 2007). We have no functional information on the remaining four proteins in this group. However, all of these proteins remain of interest because they show male-biased expression, with TC010065 expression detected exclusively in the male accessory glands.

## Conclusions

The primary goal of this research was to identify a subset of the SFPs that are transferred to female *T. castaneum* in the male ejaculate. We have successfully identified several novel putative SFPs in *T. castaneum* by utilizing a combination of proteomics and gene expression approaches. This represents the first proteomic-based method of identifying SFPs in any coleopteran species, and is the first to identify putative SFPs that are likely transferred to the female in the male-derived spermatophore. Fourteen putative SFPs were identified by mass spectrometry and 13 of those 14 were shown to be male-specific, nine showing expression limited to the two pairs of male reproductive AGs. Two of the 14 identified genes were demonstrated to have homologues amongst the respective known and putative SFPs of *D. melanogaster* and *An. gambiae*. The predicted protein classes of many of these putative SFPs are similar to those SFPs reported from a variety of other insects. However, several of the identified putative SFPs did not have conserved protein domains, or identifiable homologues in BLAST searches, indicating that these proteins may be rapidly evolving.

*T. castaneum* represents an extremely amenable model system for addressing questions about sexual selection and sexual conflict because of the successful implementation of a systematic, integrative approach towards uniting episodes of sexual selection (Fedina & Lewis, 2008). By knocking down individual SFPs (eg using RNA interference) in such a system, researchers can assess the role and importance of ejaculate proteins in mediating mating success, fertilization success, sperm competition and potentially even reproductive isolation (Walters & Harrison, 2008; Findlay & Swanson, 2010). Therefore, exploring the male reproductive proteins identified in this and another recent investigation (Parthasarathy *et al*., 2009) may not only provide insight into the possible mechanisms of sexual selection, but also help control the reproductive output of this economically important agricultural pest.

## Experimental procedures

### *Identification of seminal fluid proteins using mass spectrometry*

The reproductive tract of *T. castaneum* males is dominated by a pair of lobed testes and two pairs of male accessory glands (Fig. 1A). The testes are connected to the ejaculatory duct through the vas deferentia, which dilate into the slightly wider seminal vesicles as they approach the ejaculatory duct. The two pairs of male AGs are distinguishable by their morphology; the EC are smaller and rod-shaped, whereas the ME are longer and more tubular. The seminal vesicles and two pairs of AGs terminate in the ejaculatory duct. The two types of AGs together produce a spermatophore, which is transferred to the female bursa copulatrix during mating (Fig. 1B).

Putative *T. castaneum* SFPs were directly identified using mass spectrometry. For this analysis, proteins were collected and pooled from several different tissue types: virgin male reproductive AGs (EC and ME separately), the bursa copulatrix from mated females containing a newly transferred spermatophore (Fig. 1B), and the bursa copulatrix of virgin females. Additionally, male body tissue lacking AGs was collected. For each tissue type, at least two independent biological replicates representing tissue

pooled from 30 individuals were collected. Mated females were obtained by placing a virgin male and female into a mating arena. Within 30 s of the cessation of copulation, females were removed and the entire female reproductive tract was dissected in autoclaved, Dulbecco's phosphate buffered saline (DPBS; Sigma, St Louis, MO, USA) with protease inhibitors (Roche, Indianapolis, IN, USA). This rapid removal of the female reproductive tract following mating reduced the possibility of our sample containing proteins that the female produces in response to the presence of male-derived proteins. The bursa copulatrix containing the spermatophore was then separated from the remainder of the tissue. Similar dissections were carried out to remove the bursa from virgin females and AGs from virgin males. By including both the mated and virgin female bursa copulatrices in our analysis, we were able to distinguish probable male-derived proteins from proteins normally present in the female bursa copulatrix. For the samples of the male and female bodies without the above tissue types, as much of the remainder of the tissue was removed from the abdomen, thorax and the head as possible. Exoskeleton fragments were removed from the samples. Following dissection, tissues were placed in 40 μl 10% Dulbecco's PBS with protease inhibitors. Samples were centrifuged at 11 000 *g* for 30 min at 4 °C, and supernatant was removed and placed in new tubes. Pellets were re-suspended in 20 μl 10% DPBS with protease inhibitors and 20 μl 2 × sodium dodecyl sulfate (SDS) sample buffer (125 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.001% bromophenol blue) was added to the supernatant and pellet samples. Proteins were separated on a one-dimensional 5–15% gradient SDS-polyacrylamide gel and stained with SimplyBlue SafeStain (Invitrogen, Carlsbad, CA, USA) for visualization.

Bands were selected for mass spectrometry-based identification if they were present in the AGs (we only selected proteins from the ME sample, as they more closely matched the mated female sample) and mated female bursa samples but light or absent in the virgin female bursa (indicated with boxes in Fig. 2). This increased the likelihood that our samples contained male-derived proteins that were transferred to females. In-gel digestion, tryptic peptide extractions and Nano liquid chromatography with tandem mass spectrometry (LC-MS/MS) were conducted by the Cornell University Life Sciences Core Proteomics and Mass Spectrometry facility following previously published methodology (Morris *et al.*, 2007). The data emerging from the mass spectrometry were submitted for database comparison using ProteinPilot software (Applied Biosystems, Foster City, CA, USA) against the *T. castaneum* predicted peptide fasta database (http://www.beetlebase.org), with identifications being based on a ProtScore >1.3 and representing >95% statistical significance. We also tested for predicted secretion signal sequences using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) and for predicted protein domains with SMART (http://smart.embl-heidelberg.de/) and InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/).

Predicted protein sizes were calculated with the protein molecular weight calculator at the Sequence Manipulation Suite (http://www.bioinformatics.org/sms/prot_mw.html). We compared the amino acid sequence of the identified putative SFPs to translated *D. melanogaster*, *Ae. aegypti*, *An. gambiae*, *Ap. mellifera* transcripts using tblastn against predicted *D. melanogaster* (http://flybase.bio.indiana.edu/), *Ae. aegypti* (http://aaegypti.vectorbase.org/index.php), *An. gambiae* (http://agambiae.vectorbase.org/index.php) and *Ap. mellifera* (http://hymenopteragenome.org/beebase/) gene databases. The amino acid sequences of the top hits were then compared back to the Beetlebase3_NCBI_DB database on the Beetlebase website (http://beetlebase.org/) using tblastn to determine whether the original *D. melanogaster*, *Ae. aegypti*, *An. gambiae* or *Ap. mellifera* transcript was the top hit. *T. castaneum* genes were considered homologues if the two were reciprocal best BLAST hits with *e*-values $<1 \times 10^{-3}$ and identities ≥30%.

### *Patterns of gene expression*

The expression patterns of the putative SFP genes identified through MS were investigated using qualitative RT-PCR. Total RNA was extracted separately from male EC and ME (pooled from 40 males), male whole bodies without the accessory glands, and female bodies (pooled from 30 individuals of each sex). All *T. castaneum* individuals were virgin and approximately 2 weeks old. Dissections were carried out as described above. Pooled tissue samples were kept on ice in Trizol (Invitrogen) until dissections for each tissue type were completed. Tissues were then ground in Trizol, and RNA was extracted following the manufacturer's instructions. Extracted RNA was treated with RNAse free DNase (Promega, Madison, WI, USA) to remove any DNA contamination. For each tissue type, 1 μg RNA was used to synthesize the first strand cDNA with Superscript II Reverse Transcriptase (Invitrogen). TCRPS6, a protein that is part of the small ribosomal subunit, was used to standardize the amount of cDNA used to determine the presence/absence of seminal fluid protein transcripts in each tissue type and also act as a control. PCR was conducted with first strand cDNA using Platinum PCR Supermix (Invitrogen) and gene-specific primers (Table S1). PCR cycles were as follows: one cycle at 94° C for 5 min; 33 cycles of 94° C for 30 s, 62 °C for 30 s and 72 °C for 30 s; followed by 5 min at 72 °C. For two genes, TC006088 and TC010066, primers failed to amplify gene products using this PCR programme. Therefore, the PCR programme used for those genes were: one cycle at 94 °C for 5 min; 16 cycles of 94 °C for 30 s, 66 °C for 30 s, 72 °C for 1 min; 20 cycles of 94 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min; followed by 5 min at 72 °C. TC006088 and TC010066 PCR product was sequenced to ensure proper product amplification. PCR was conducted on at least two independent biological replicates of each tissue type. The results from each gene-specific RT-PCR from all tissues tested were visualized on the same gel to determine the presence of a transcript, but relative expression was not quantified. Gel imaging was carried out using Quantity One (Bio-Rad, Hercules, CA, USA) software.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/j.1365-2583.2011.01083.x

**Table S1.** Primer sequences used for PCR amplification of genes encoding putative *Tribolium castaneum* seminal fluid proteins.

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