1 **Ontogenetic changes in seminal fluid gene expression**
2 **and the protein composition of cricket seminal fluid**

3

4 **LW Simmons^{1*}, M Beveridge¹, L Li^{2,3}, Y-F Tan^{1,2,3} and AH**
5 **Millar^{2,3}**

6

7 ¹Centre for Evolutionary Biology, ²Centre for Comparative Analysis of
8 Biomolecular Networks, and ³ARC Centre of Excellence in Plant Energy
9 Biology, The University of Western Australia, Crawley, 6009, Australia.

10

11

12

13 * Author for correspondence (e mail: leigh.simmons@uwa.edu.au)

14

15

16

17 ontogeny of seminal fluid gene expression

18

19

20 Word count: 3531

21

SUMMARY The ejaculates of most internally fertilizing species consists of both sperm and seminal fluid proteins. Seminal fluid proteins have been studied largely in relation to their post-mating effects on female reproductive physiology, and predominantly in genomically well-characterised species. Seminal fluids can also play important roles in sperm maturation and performance. In the field cricket *Teleogryllus oceanicus* the viability of ejaculated sperm increases as males age, as does their competitive fertilization success. Here, using quantitative proteomics and quantitative real-time PCR, we document ontogenetic changes in seminal fluid protein abundance and in seminal fluid gene expression. We identified 11 proteins that increased in abundance in the seminal fluid of crickets as they aged. Gene expression was quantified for 5 seminal fluid protein genes, and in 4 of these gene expression increased as males aged. These ontogenetic changes were associated with a general increase in the size of the male accessory glands. Several of the seminal fluid proteins that we have identified are novel, and some have BLAST matches to proteins implicated in sperm function. Our data suggest that age related changes in competitive fertilization success may be dependent on seminal fluid chemistry.

42 INTRODUCTION

43 Sperm are typically transferred to females bathed in a complex soup of seminal fluid
44 proteins (sfps) that are manufactured by the male reproductive accessory glands
45 (Poiani 2006). Seminal fluid plays an important role in the nourishment, protection,
46 capacitation and motility of sperm within the female reproductive tract and is
47 expected to contribute to a male's reproductive success, especially when males are
48 subject to postcopulatory selection via sperm competition (Parker 1970; Simmons
49 2001), cryptic female choice (Eberhard 1996), or sexual conflict (Arnqvist and Rowe
50 2005). While there is a considerable amount of work across a diverse range of animal
51 taxa that has focused on the evolutionary consequences of postcopulatory sexual
52 selection for the evolution of sperm production and sperm form and function, much
53 less is known about sfp evolution (Simmons and Fitzpatrick 2012). What we do know
54 comes predominantly from genomically well-characterised model organisms such as
55 mice (Karn et al. 2008; Dean et al. 2009; Ramm et al. 2009), and particularly
56 *Drosophila* (Avila et al. 2011).

57 In *Drosophila*, sfps induce behavioral, physiological, and transcriptional
58 changes in females that promote male reproductive fitness, at the expense of female
59 fitness (Rice 1996; Wolfner 2009; Avila et al. 2011). The *D. melanogaster* sfp sex
60 peptide induces sexual unreceptivity and increased oviposition by the mated female at
61 a cost to female lifespan (Chapman et al. 2003; Wigby and Chapman 2005; Smith et
62 al. 2012). Other sfps are involved in the storage of sperm in the female's sperm
63 storage organs, and in promoting a male's competitive fertilization success (Chapman
64 et al. 2000; Adams and Wolfner 2007). As might be expected, there is good evidence
65 that *Drosophila* sfps have been subject to rapid and divergent evolution, driven by
66 postcopulatory sexual selection (Wagstaff and Begun 2007; Findlay et al. 2008).

67 Indeed, 138 sfps have so far been identified in *Drosophila* seminal fluid, although the
68 functional significance of many, if not most of these proteins remains largely
69 unknown.

70 Attempts to broaden our knowledge of sfps across a wider range of taxa have
71 combined expressed sequence tag (EST) analysis with proteomics to identify sfps
72 from species with little to no genomic information (Findlay and Swanson 2010). Thus
73 studies of honeybees (Baer et al. 2009), mosquitos (Sirot et al. 2011), crickets (Andrés
74 et al. 2006; Braswell et al. 2006; Marshall et al. 2011), butterflies (Walters and
75 Harrison 2010) and flour beetles (South et al. 2011) have identified novel proteins
76 transferred to females at mating. Many of these studies have revealed that these novel
77 sfps are undergoing rapid evolutionary divergence, although the functions of these
78 proteins remain largely unknown.

79 Tissue-specific transcriptomics in the field cricket *Teleogryllus oceanicus*
80 have revealed uniquely expressed sequences in both testes and accessory glands that
81 show a significantly lower rate of matching to annotated *D. melanogaster* genes
82 compared with those from general body tissue, consistent with the general finding that
83 genes expressed in testes and accessory glands are rapidly evolving targets of
84 selection (Bailey et al. 2013). Proteomics analyses of *T. oceanicus* seminal fluid has
85 identified 21 sfps, many with sequence similarity to those found to play significant
86 roles in male fertility and postmating changes in female reproductive physiology in
87 other taxa (Simmons et al. 2013). In this study we examined ontogenetic changes in
88 both seminal fluid protein composition and in the expression of a sample of sfp genes,
89 in an attempt to identify proteins that may impact male competitive fertilization
90 success. Sperm viability — the proportion of live sperm in the ejaculate — is an
91 important determinant of a male's competitive fertilization success (García-González

and Simmons 2005), and seminal fluid has been shown to affect sperm viability (Simmons and Beveridge 2011) in this species. Although males will begin mating within 4 days of adult eclosion, previous studies of *T. oceanicus* have found that sperm viability increases with male age (García-González and Simmons 2005), as does a male's competitive fertilization success (Dowling et al. 2010). If sfps are responsible for changes in male competitive fertility we should expect to see ontogenetic changes in the protein composition of seminal fluid and in the expression of genes that encode sfps.

MATERIALS AND METHODS

Seminal fluid protein analysis

Male crickets were sampled from a large (>1000) outbred laboratory stock held at the University of Western Australia, and housed in a constant temperature room on a 12h:12h light:dark regime at 26°C (Simmons, Tan, and Millar 2013). Final instar nymphs were placed into individual boxes (7×7×5cm) provided with cat chow and water ad libitum and checked daily for adult emergence. Adults were sampled when they were 4(9), 8(13), 12(16), 16(10) and 20(9) days(n) post adult eclosion. At their allotted age, a spermatophore was removed from each male's subgenital plate. The ejaculate is normally transferred to the female from the spermatophore over a period of approximately 40 mins after its insertion into the female reproductive tract (Simmons et al. 2003), but will evacuate onto a microscope slide within 20 mins when the evacuating tube is cut (Simmons and Beveridge 2011). Spermatophores were placed onto individual cavity slides into 20 µl of Beadle saline solution with cocktail protease inhibitor (Roche). Once the ejaculate had discharged, the empty spermatophore capsule was discarded, and the ejaculates centrifuged at 20,000g at

117 4°C to fractionate it into sperm (pellet) and seminal fluid (supernatant). The seminal
118 fluid fractions were acetone precipitated to concentrate the proteins and to remove
119 superfluous salt.

120 Seminal fluid proteins were separated by 1D SDS-PAGE according to the
121 protocol of Laemmli (1970). We ran two Hoefer gels (14 cm x 16 cm, 1.5 mm) with
122 narrow 30-well combs (GE Healthcare), distributing half of the samples from each
123 age group across each gel. Approximately 0.6 µg molecular weight markers and 0.7
124 µg of seminal fluid proteins were loaded into the lanes. Protein samples and
125 molecular mass markers were mixed with 2-fold concentrated sample loading buffer
126 (125 mM Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 20% (v/v) β -mercaptoethanol,
127 0.004% (w/v) bromophenol blue, pH 6.8) and heated at 95°C for 5 min to denature
128 proteins. The samples were then loaded onto the gel comprising the stacking
129 component (0.1% (w/v) SDS, 4% (w/v) acrylamide:bisacrylamide (29:1), 0.05%
130 (w/v) ammonium persulphate, 0.1% TEMED, 125 mM Tris-HCl, pH 6.8) and the
131 separating component (0.1% (w/v) SDS, 12% (w/v) acrylamide:bisacrylamide (29:1),
132 0.05% (w/v) ammonium persulphate, 0.05% TEMED, 375 mM Tris-HCl, pH 8.8).

133 The anode and cathode of the electrophoresis tank was filled with running buffer (200
134 mM glycine, 1% (w/v) SDS, 25 mM Tris-HCl, pH 8.6) and electrophoresed at a
135 constant current of 20 mA per Hoefer gel until the bromophenol blue dye front was
136 near the bottom of the gel. The gel was fixed (10% (v/v) acetic acid, 40% methanol)
137 overnight and then stained with Flamingo® Fluorescent Gel Stain (Bio-rad) for 3
138 hours. To remove the background the gel was rinsed with 0.1% (w/v) Tween®-20 for
139 10 mins and then water briefly. The gel fluorescence was imaged using a Typhoon
140 scanner (Typhoon Trio, GE Healthcare) with a laser excitation at 532 nm. To control
141 for gel-gel variability, we included gel identity as a factor in our statistical analyses.

The protein bands were cut from 1D SDS-PAGE gels and in-gel digested by trypsin, peptides were extracted by extracting solution and then dried in an acid-resistant centrifuge concentrator as described previously (Taylor et al. 2005; Li et al. 2012). Dry digested peptides were reconstituted with 20 µl of 2% ACN (v/v), 0.1% (v/v) FA and filtered through a Durapore-PVDF 0.22 µm centrifugal filter before injection. An Agilent iFunnel QTOF6550 LC/MS system was used for the separation and identification of peptides with 4 µl of the filtered solution injected. Tandem mass spectrometry data were analyzed through an in-house cricket isotigs database (96,750 sequences; 29,801,538 residues; Appendix S1 in Simmons et al. 2013), using the Mascot search engine version 2.3 and utilizing error tolerances of ± 100 ppm for MS and ± 0.5 Da for MS/MS; “Max Missed Cleavages” set to 1; variable modifications of oxidation (Met) and carbamidomethyl (Cys).

Gene expression assays

A further 15 crickets were sampled for each age class for gene expression assays. Each cricket was frozen, the accessory gland removed, weighed, placed in *RNAlater* (Life Technologies), stored overnight at 4°C and then placed at -20°C. RNA was extracted from the entire accessory gland using the PureLink RNA mini kit (Life Technologies) following the manufacturer’s instructions. The tissue was disrupted using a homogenizer (Life Technologies) and DNA was removed using an on-column PureLink DNase treatment (Life Technologies). RNA yield was quantified using the Qubit® 2.0 fluorometer (Life Technologies) and assessed for integrity by denaturing RNA electrophoresis in TAE buffer (Masek et al. 2005). RNA was eluted into 100µl and yields ranged from 254 to 790µg/ml per sample.

Using the high capacity RNA-to-cDNA kit (Life Technologies), 2µg of each RNA sample was converted to cDNA in a 20µl reaction volume following the manufacturer's instructions and stored at -20°C.

Five seminal fluid proteins were selected for gene expression assay based on their observed changes in seminal fluid abundance and our ability to identify the isotig associated with that protein (Table S1 in the online supplementary material). We selected *actin* as a reference gene (Isotig01761 from the *T. oceanicus* sequence data base lodged with Genbank under the project number SRA044883.2; Bailey et al. 2013). Isotig01761 was found to have 100% homology with mRNA for the *actin* gene of *Gryllus bimaculatus*. TaqMan gene expression custom assays (Life Technologies) were designed for these 6 genes using the online Custom TaqMan assay design tool. Forward, reverse and reporter sequences can be found in Table S1. In order to determine a suitable starting concentration of cDNA for the gene expression assays, a standard curve of cDNA was prepared from one of the 4 day samples, to include 100ng/µl, 10ng/µl, 1ng/µl, 0.1ng/µl and 0.001ng/µl. Gene expression assays were then set up in triplicate for each of the standards and for each of the assays as follows: 1µl cDNA, 5µl 2x TaqMan Gene expression master mix (Life Technologies) and 0.5µl 20x TaqMan custom assay mix in a 10µl reaction volume. The assays were run in compatible 96-well plates on a StepOne Plus Real-Time PCR system (Life Technologies) using the following cycling conditions: 50°C for 2 minutes, 95°C for 10 minutes and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The efficiency of each assay was assessed with all R^2 values >0.998 and slopes from -3.636 to -3.34 corresponding to efficiencies varying from 88% to 99%. For all 6 candidate genes, 10ng cDNA gave average C_T values varying from 19.97 to 25.40 so this was the input amount for all further assays.

191 Preliminary gene expression assays were set up for the reference gene, *actin*.
192 Five samples from each age class were assayed by qPCR as described above and
193 analysed for variation in gene expression between treatments. As there was no
194 change in gene expression between treatments we determined that this was an
195 appropriate reference gene.

196 Gene expression assays were then carried out on all samples using 10ng
197 cDNA, each assay run in triplicate for each candidate gene and reference gene with
198 negative controls (without cDNA) on each plate. Results were analysed by
199 StepOne™ software v2.1 (Life Technologies) and the results exported into
200 DataAssist™ v3.0 software (Life Technologies) for sample comparison using the
201 comparative C_T ($2^{-\Delta\Delta C_T}$) method (Schmittgen and Livak 2008).

202

203 **RESULTS**

204 **Testes and accessory gland weight**

205 After controlling for the effect of male body mass ($F_{1,54}=7.11$, $P=0.010$), accessory
206 gland mass increases with age post-adult eclosion ($F_{1,54}=166.91$, $P<0.001$) (Figure
207 1a). Conversely, after controlling for body mass ($F_{1,54}=3.91$, $P=0.053$), testes mass
208 declines with age ($F_{1,54}=21.41$, $P<0.001$) (Figure 1b).

209

210 **Seminal fluid protein composition**

211 We were able to quantify variation in 35 distinct protein bands on our 1D SDS-PAGE
212 gels (Figure 2, Table 2, Table S2). In 66% of cases we were able to match these
213 proteins via peptide mass spectrometry to their 454 sequencing derived transcripts,
214 and find their closest BLAST matches (Table S2).

215 For inference testing we conducted a Discriminant Analysis (DA) of the
 216 relative abundances of each of the 35 proteins in the seminal fluid as covariates, and
 217 male age as the categorical variable. The DA returned 3 canonical axes (CA) that each
 218 explained >10% of the variance in protein abundance (Eigenvalue[% variance
 219 explained]: CA1: 11.7[63.0]; CA2: 2.9[15.6]; CA3: 2.4[12.7]) (Table S3 in the online
 220 supplementary material). The analysis correctly classified 100% of our samples into
 221 their correct age categories, which differed significantly in the relative abundance of
 222 sfps (Wilk's Lambda = 0.002, $F_{140,74}=1.91$, $P=0.001$; Figure 2). The unknown 26 kD
 223 protein and *ToSfp007* contributed most strongly to CA1 (see Table S3). Tabachnick &
 224 Fidell (2007) recommend that the number of variables in a DA should be less than the
 225 sample size available in each treatment group. We had an average of 11 ejaculates per
 226 age category, and a range of 9 — 16. Therefore, we repeated our DA using the 8 sfps
 227 with the lowest P-values in univariate tests (Table 1). The reduced DA returned 3
 228 canonical axes (Eigenvalues[% variance explained]: CA1: 1.33[61.8]; CA2:
 229 0.43[20.2]; CA3: 0.30[13.9]) (Table S4). The analysis correctly classified 65% of our
 230 samples into their age categories, which differed significantly in the relative
 231 abundance of sfps (Wilk's Lambda=0.211, $F_{32, 168}=2.74$, $P<0.001$) (see Figure S1).
 232 Classifications were generally better for the youngest and oldest age categories
 233 (correct classifications 89%, 69%, 56%, 40% and 78% for age categories 4, 8, 12, 16
 234 and 20 respectively). Seminal fluid proteins *ToSfp014* and *ToSfp024* contributed
 235 particularly strongly to the first canonical axis in this analysis, while *ToSfp007*
 236 contributed particularly strongly to the second canonical axis.

237 We used scores on the first canonical axis from our full DA as a measure of
 238 the amount of sfps, and asked whether males with larger accessory glands ejaculated
 239 greater amounts of sfps. As would be expected from our DA, the seminal fluid of

older males contained a greater abundance of sfps ($F_{1,54}=58.26$, $P<0.001$) and after controlling for age effects, males with larger accessory glands had a greater abundance of sfps in their ejaculates ($F_{1,54}=5.63$, $P=0.021$). Neither accessory gland weight nor male age predicted variation in CA2 or CA3 (analyses not shown).

Sfp gene expression

Univariate tests indicated that four of the sfp genes analysed showed changes in gene expression with male age post adult eclosion (Table 2). Discriminant analysis returned two canonical axes that independently explained >10% of the variation in sfp gene expression (Table 3). The analysis correctly classified 61.3% of samples into their age categories which differed significantly in sfp gene expression (Wilk's Lambda = 0.178, $F_{20,220}=7.49$, $P<0.001$; Figure 3). Classifications were generally better for the younger age categories (correct classification 87%, 73%, 47%, 40%, 60% for age categories 4, 8, 12, 16 and 20 respectively). Based on the magnitude of the eigenvectors, expression of isotig01610 (*ToSfp014*) and isotig02293 (*ToSfp025*) contributed most to CA1, while isotig00263 (*ToSfp007*) contributed most to CA2 (Table 2).

DISCUSSION

Our proteomic analyses revealed significant changes in the abundance of proteins within the seminal fluid of male *Teleogryllus oceanicus* as they aged. Gene expression analyses of some representative sfps confirmed that the increased abundance of these proteins was due to an age dependent change in gene expression. Previous studies of this species have shown how sperm viability, the proportion of live sperm in an ejaculate, increases with male age (García-González and Simmons

2005), as does a male's competitive fertilization success (Dowling et al. 2010). Seminal fluid affects sperm viability in this species (Simmons and Beveridge 2011), so it seems likely that the ontogenetic changes in seminal fluid composition we have observed contribute to age dependent sperm competitive ability.

Interestingly age dependent increases in the abundance of sfps in the ejaculate were associated with a general increase in the size of the male reproductive accessory gland, and a concomittent regression in testes size. In crickets, spermeogenesis occurs during the last two nymphal stages of juvenile development (Suzuki and Nishimura 1997). Our data suggest that male crickets may have produced much of the sperm they will require for reproduction by the time of adult eclosion, and that after eclosion resources are directed away from the testes to the production of accessory gland secretions that make up the seminal fluid, and the structural components of the spermatophore that are required for the transfer of sperm and seminal fluid to the female. The maturation of male accessory glands and increased expression of accessory gland protein genes following adult eclosion has also been reported in *Tenebrio molitor*, where maturation appears to be under the control of juvenile hormone (Parthasarathy et al. 2009).

There were a number of specific sfps that appeared to contribute particularly strongly to the ontogenetic changes in seminal fluid composition, and some of these sfps have been identified previously. In particular *ToSfp007* contributed strongly to the discriminant axes that described changes in sfp composition and changes in gene expression. This protein is a trypsin-like serine protease (Simmons, Tan, and Millar 2013) similar to those reported from the accessory glands of the crickets *Gryllus pennsylvannicus* (Andrés et al. 2006), *Allenemobius socius* and *A. fasciatus* (Marshall et al. 2011). In *A. socius* RNA interference knockdown has shown that ejaculate

serine protease mediates the induction of egg laying in mated females (Marshall et al. 2009). Serine proteases are also involved in sperm function and fertility in invertebrates and vertebrates alike (Green and Summers 1980; Inaba et al. 1993; Kodama et al. 2002). A second protein that increased in abundance in the seminal fluid with male age was *ToSp027*. Although this protein was originally identified from the *T. oceanicus* sperm proteome, it has also been found in the seminal fluid proteome (*ToSfp001*; Simmons et al. 2013). The protein is an apyrase. Apyrases are often found to be tightly associated with sperm and, like serine proteases, have been implicated as important proteins for sperm function (Simmons et al. 2013).

Our analysis identified 6 novel sfps not previously described from the *T. oceanicus* seminal fluid proteome. Three of these are of unknown function. However, the closest BLAST match to *ToSfp024* was carbonic anhydrase. Carbonic anhydrases are involved in maintaining pH balance and are important for the capacitation of insect sperm in the vas deferens (Bebas et al. 2002; Kotwica et al. 2006). The carbonate system has also been shown to be important in controlling sperm motility in squids (Hirohashi et al. 2013). *ToSfp024* contributed strongly to the first discriminant axis describing changes in sfp abundance, and the increased abundance of carbonic anhydrase may contribute to the improved viability and competitive fertilization ability of sperm as males age.

A significant proportion of the variation in protein abundance in the seminal fluids was attributable to the size of the accessory glands, even after accounting for age differences. For a given age class, males that invested more in their accessory glands produced ejaculates with greater amounts of sfps. Previous studies of *T. oceanicus* have shown that accessory gland size holds important fitness consequences for the development of embryos; the viability of embryos depends strongly on the

accessory gland size of a female's mating partner (García-González and Simmons 2005, 2007, 2010). These findings strongly implicated seminal fluid proteins as imparting important paternal effects on reproduction. Our finding that indeed, males with larger accessory glands transfer ejaculates with a greater abundance of sfps supports this view. Identifying the sfps that affect embryo viability will require targeted RNA interference knockdown of the sfps identified from the *T. oceanicus* ejaculate.

In conclusion, using quantitative proteomics and realtime quantitative PCR, we have uncovered ontogenetic changes in the abundance of seminal fluid proteins in the ejaculate of *T. oceanicus* that are determined by changes in sfp gene expression. A number of the sfps that were found to increase in abundance are implicated in sperm capacitation or motility. These findings suggest that changes in sperm viability and competitive fertilization reported previously in this species may be due to changes in seminal fluid composition.

ACKNOWLEDGEMENTS

LWS was supported by a Professorial Fellowship from the ARC (DP110104594), and AHM was supported by a Future Fellowship from the ARC (FT110100242) and ARC grant (CE0561495).

REFERENCES

Adams, E. M. and Wolfner, M. F. 2007. Seminal proteins but not sperm induce morphological changes in the *Drosophila melanogaster* female reproductive tract during sperm storage. *Journal of Insect Physiology* 53 (4):319-331.

339 Andrés, J. A., Maroja, L. S., Bogdanowicz, S. M., Swanson, W. J., and Harrison, R.
 340 G. 2006. Molecular evolution of seminal proteins in field crickets. *Molecular*
 341 *Biology and Evolution* 23:1574-1584.
 342 Arnqvist, G. and Rowe, L. 2005. *Sexual Conflict*. New Jersey: Princeton University
 343 Press.
 344 Avila, F. W., Sirot, L. K., LaFlamme, B. A., Rubinstein, C. D., and Wolfner, M. F.
 345 2011. Insect seminal fluid proteins: identification and function. *Annual Review*
 346 *of Entomology* 56:21-40.
 347 Baer, B., Heazlewood, J. L., Taylor, N. L., Eubel, H., and Millar, A. H. 2009. The
 348 seminal fluid proteome of the honeybee *Apis mellifera*. *Proteomics* 9:2085-
 349 2097.
 350 Bailey, N. W., Veltsos, P., Tan, Y.-F., Millar, A. H., Ritchie, M. G., and Simmons, L.
 351 W. 2013. Tissue-specific transcriptomics in the field cricket *Teleogryllus*
 352 *oceanicus*. *G3: Genes|Genomes|Genetics* 3 (2):225-230.
 353 Bebas, P., Cymborowski, B., and Giebultowicz, J. M. 2002. Circadian rhythm of
 354 acidification in insect vas deferens regulated by rhythmic expression of
 355 vacuolar H⁺-ATPase. *Journal of Experimental biology* 205 (1):37-44.
 356 Braswell, W. E., Andres, J. A., Maroja, L. S., Harrison, R. G., Howard, D. J., and
 357 Swanson, W. J. 2006. Identification and comparative analysis of accessory
 358 gland proteins in Orthoptera. *Genome* 49 (9):1069-1080.
 359 Chapman, T., Bangham, J., Vinti, G., Seifried, B., Lung, O., Wolfner, M. F., Smith,
 360 H. K., and Partridge, L. 2003. The sex peptide of *Drosophila melanogaster*:
 361 Female post-mating responses analyzed by using RNA interference.
 362 *Proceedings of the National Academy of Sciences USA* 100:9923-9928.

363 Chapman, T., Neubaum, D. M., Wolfner, M. F., and Partridge, L. 2000. The role of
 364 male accessory gland protein Acp36DE in sperm competition in *Drosophila*
 365 *melanogaster*. *Proceedings of the Royal Society of London B* 267:1097-1105.
 366 Dean, M. D., Clark, N. L., Findlay, G. D., Karn, R. C., Yi, X., Swanson, W. J.,
 367 MacCoss, M. J., and Nachman, M. W. 2009. Proteomics and comparative
 368 genomic investigations reveal heterogeneity in evolutionary rate of male
 369 reproductive proteins in mice (*Mus domesticus*). *Mol. Biol. Evol.* 26:1733-
 370 1743.
 371 Dowling, D. K., Nystrand, M., and Simmons, L. W. 2010. Maternal effects, but no
 372 good or compatible genes for sperm competitiveness in Australian crickets.
 373 *Evolution* 64:1257-1266.
 374 Eberhard, W. G. 1996. *Female Control: Sexual Selection by Cryptic Female Choice*.
 375 Princeton: Princeton University Press.
 376 Findlay, G. D. and Swanson, W. J. 2010. Proteomics enhances evolutionary and
 377 functional analysis of reproductive proteins. *BioEssays* 32:26-36.
 378 Findlay, G. D., Yi, X., MacCoss, M. J., and Swanson, W. J. 2008. Proteomics reveals
 379 novel *Drosophila* seminal fluid proteins transferred at mating. *PLoS Biol.*
 380 6:e178.
 381 García-González, F. and Simmons, L. W. 2005. The evolution of polyandry: intrinsic
 382 sire effects contribute to embryo viability. *Journal of Evolutionary Biology*
 383 18:1097-1103.
 384 ———. 2005. Sperm viability matters in insect sperm competition. *Current Biology*
 385 15:271-275.
 386 ———. 2007. Paternal indirect genetic effects on offspring viability and the benefits
 387 of polyandry. *Current Biology* 17:32-36.

388 García-González, F. and Simmons, L. W. 2010. Male-induced costs of mating for
389 females compensated by offspring viability benefits in an insect. *Journal of*
390 *Evolutionary Biology* 23:2066-2075.

391 Green, J. and Summers, R. 1980. Ultrastructural demonstration of trypsin-like
392 protease in acrosomes of sea urchin sperm. *Science* 209 (4454):398-400.

393 Hirohashi, N., Alvarez, L., Shiba, K., Fujiwara, E., Iwata, Y., Mohri, T., Inaba, K.,
394 Chiba, K., Ochi, H., Supuran, C. T., Kotzur, N., Kakiuchi, Y., Kaupp, U. B.,
395 and Baba, S. A. 2013. Sperm from sneaker male squids exhibit chemotactic
396 swarming to CO₂. *Current Biology* 23:775-781.

397 Inaba, K., Akazome, Y., and Morisawa, M. 1993. Purification of proteasomes from
398 salmonid fish sperm and their localization along sperm flagella. *Journal of*
399 *Cell Science* 104 (3):907-915.

400 Karn, R. C., Clark, N. L., Nguyen, E. D., and Swanson, W. J. 2008. Adaptive
401 evolution in rodent seminal vesicle secretion proteins. *Molecular Biology and*
402 *Evolution* 25:2301-2310.

403 Kodama, E., Baba, T., Kohno, N., Satoh, S., Yokosawa, H., and Sawada, H. 2002.
404 Spermosin, a trypsin-like protease from ascidian sperm. *European Journal of*
405 *Biochemistry* 269 (2):657-663.

406 Kotwica, J., Ciuk, M. A., Joachimiak, E., Rowinski, S., Cymborowski, B., and Bebas,
407 P. 2006. Carbonic anhydrase activity in the vas deferens of the cotton
408 leafworm — *Spodoptera littoralis* (Lepidoptera: Noctuidae) controlled by
409 circadian clock. *Journal of Physiology and Pharmacology* 57, Supp 8:107-
410 123.

411 Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head
412 of bacteriophage. *Nature* 227:680-685.

413 Li, L., Nelson, C. J., Solheim, C., Whelan, J., and Millar, A. H. 2012. Determining
 414 degradation and synthesis rates of *Arabidopsis* proteins using the kinetics of
 415 progressive ¹⁵N labeling of two-dimensional gel-separated protein spots.
 416 *Molecular and Cellular Proteomics* 11:M111.010025.

417 Marshall, J. L., Huestis, D. L., Garcia, C., Hiromasa, Y., Wheeler, S., Noh, S.,
 418 Tomich, J. M., and Howard, D. J. 2011. Comparative proteomics uncovers the
 419 signature of natural selection acting on the ejaculate proteomes of two cricket
 420 species isolated by postmating, prezygotic phenotypes. *Molecular Biology and*
 421 *Evolution* 28 (1):423-435.

422 Marshall, J. L., Huestis, D. L., Hiromasa, Y., Wheeler, S., Oppert, S., Oppert, C.,
 423 Marshall, S. A., Tomich, J. M., and Oppert, B. 2009. Identification, RNAi
 424 knockdown, and functional analysis of an ejaculate protein that mediates a
 425 postmating, prezygotic phenotype in a cricket. *Plos One* 4 (10):e7537.

426 Masek, T., Vopalensky, V., Suchomelova, P., and Pospisek, M. 2005. Denaturing
 427 RNA electrophoresis in TAE agarose gels. *Analytical Biochemistry* 336:46-50.

428 Parker, G. A. 1970. Sperm competition and its evolutionary consequences in the
 429 insects. *Biological Reviews* 45:525-567.

430 Parthasarathy, R., Tan, A., Sun, Z., Chen, Z., Rankin, M., and Palli, S. R. 2009.
 431 Juvenile hormone regulation of male accessory gland activity in the red flour
 432 beetle, *Tribolium castaneum*. *Mechanisms of Development* 126 (7):563-579.

433 Poiani, A. 2006. Complexity of seminal fluid: a review. *Behavioral Ecology and*
 434 *Sociobiology* 60 (3):289-310.

435 Ramm, S. A., McDonald, L., Hurst, J. L., Beynon, R. J., and Stockley, P. 2009.
 436 Comparative proteomics reveals evidence for evolutionary diversification of

437 rodent seminal fluid and its functional significance in sperm competition. *Mol.*
438 *Biol. Evol.* 26:189-198.

439 Rice, W. R. 1996. Sexually antagonistic male adaptation triggered by experimental
440 arrest of female evolution. *Nature* 381:232-234.

441 Schmittgen, T. D. and Livak, K. J. 2008. Analysing real-time PCR data by the
442 comparative CT method. *Nature Protocols* 3:1101-1108.

443 Simmons, L. W. 2001. *Sperm Competition and its Evolutionary Consequences in the*
444 *Insects*. Princeton: Princeton University Press.

445 Simmons, L. W. and Beveridge, M. 2011. Seminal fluid affects sperm viability in a
446 cricket. *Plos One* 6:e17975. doi:10.1371/journal.pone.0017975.

447 Simmons, L. W. and Fitzpatrick, J. L. 2012. Sperm wars and the evolution of male
448 fertility. *Reproduction*:doi:10.1530/REP-12-0285

449 Simmons, L. W., Tan, Y.-F., and Millar, A. H. 2013. Sperm and seminal fluid
450 proteomes of the field cricket *Teleogryllus oceanicus*: identification of novel
451 proteins transferred to females at mating. *Insect Molecular Biology*
452 Online:doi: 10.1111/imb.12007.

453 Simmons, L. W., Wernham, J., García-González, F., and Kamien, D. 2003. Variation
454 in paternity in the field cricket *Teleogryllus oceanicus*: no detectable influence
455 of sperm numbers or sperm length. *Behavioral Ecology* 14:539-545.

456 Sirot, L. K., Hardstone, M. C., Helinski, M. E. H., Ribeiro, J. M. C., Kimura, M.,
457 Deewatthanawong, P., Wolfner, M. F., and Harrington, L. C. 2011. Towards a
458 semen proteome of the Dengue vector mosquito: protein identification and
459 potential functions. *PLoS Negl Trop Dis* 5 (3):e989.

460 Smith, D. T., Sirot, L. K., Wolfner, M. F., Hosken, D. J., and Wedell, N. 2012. The
 461 consequences of genetic variation in sex peptide expression levels for egg
 462 laying and retention in females. *Heredity* 109 (4):222-225.
 463 South, A., Sirot, L. K., and Lewis, S. M. 2011. Identification of predicted seminal
 464 fluid proteins in *Tribolium castaneum*. *Insect Molecular Biology* 20 (4):447-
 465 456.
 466 Suzuki, A. C. and Nishimura, K. 1997. The testes development in 3rd- to 6th-instar
 467 nymphs of the cricket, *Gryllus bimaculatus*. *Zoological Science* 14:637-639.
 468 Tabachnick, B. G. and Fidell, L. S. 2007. *Using Multivariate Statistics 5th ed.* Boston,
 469 MA: Pearson International.
 470 Taylor, N. L., Heazlewood, J. L., Day, D. A., and Millar, A. H. 2005. Differential
 471 impact of environmental stresses on the pea mitochondrial proteome.
 472 *Molecular and Cellular Proteomics* 4:1122-1133.
 473 Wagstaff, B. J. and Begun, D. J. 2007. Adaptive evolution of recently duplicated
 474 accessory gland protein genes in desert drosophila. *Genetics* 177 (2):1023-
 475 1030.
 476 Walters, J. R. and Harrison, R. G. 2010. Combined EST and proteomic analysis
 477 identifies rapidly evolving seminal fluid proteins in *Heliconius* butterflies.
 478 *Molecular Biology and Evolution* 27 (9):2000-2013.
 479 Wigby, S. and Chapman, T. 2005. Sex peptide causes mating costs in female
 480 *Drosophila melanogaster*. *Current Biology* 15:316-321.
 481 Wolfner, M. F. 2009. Battle and ballet: molecular interactions between the sexes in
 482 *Drosophila*. *J. Hered.* 100:399-410.

483

484

485

486 Figure legends

487

488 **Fig. 1.** The effect of male age on reproductive accessory gland weight (top) and
489 combined testes weight (bottom) in *Teleogryllus oceanicus*.

490

491 **Fig. 2.** 1D SDS-PAGE of the seminal fluid of *Teleogryllus oceanicus* from 4
492 different age classes ranging from 4 to 20 days post-adult eclosion. Protein bands are
493 labeled by their molecular weight, with the four lanes shown in isolation on the left in
494 order to indicate the relative positions of the 35 different protein bands.

495

496 **Fig. 3.** Separation of five *Teleogryllus oceanicus* male age classes (4 - 20 days post
497 adult eclosion) on the first 2 canonical axes of a discriminant function analysis of the
498 relative abundances of 35 protein bands found by 1D SDS-PAGE of the seminal fluid.

499

500 **Fig. 4.** Separation of five *Teleogryllus oceanicus* male age classes (4 - 20 days post
501 adult eclosion) on the first 2 canonical axes of a discriminant function analysis of the
502 expression of 5 seminal fluid protein genes.

Table 1. Age dependent abundance of protein bands quantified from 1D SDS-PAGE gels of *Teleogryllus oceanicus* seminal fluid. Protein names, reference number of 454 sequence-derived transcript and the tissue of origin of transcript sequence are provided, along with the predicted protein function based on annotation of closest BLAST match.

Band MW	Protein name	Transcript reference number ¹	Tissue origin of transcript	Summary of Predicted function ²	Univariate effect of age class on protein abundance	
					<i>F</i> 4,51	<i>P</i>
70	<i>ToSfp019</i>	isotig01393/isotig01111	testis/body	protease	1.62	0.182
63	<i>ToSfp004</i>	isotig00790	accessory gland	protease	1.47	0.226
59	<i>ToSp027</i>	isotig01262	accessory gland	apyrase	0.29	0.881
55*	<i>ToSp027/ToSfp022</i>	isotig01262/isotig00444	accessory gland	apyrase	3.92	0.008
51					3.37	0.016
48	<i>ToSp027</i>	isotig01262	accessory gland	apyrase	1.08	0.377
45	<i>ToSp027</i>	isotig01262	accessory gland	apyrase	0.82	0.516
43	<i>ToSp009</i>	isotig00560	head	enolase	6.69	<0.001
41.5	<i>ToSfp005</i>	isotig01832	accessory gland	dipeptidase	3.70	0.010
39.5					1.14	0.346
38					1.43	0.237
36					0.26	0.906
35					0.18	0.946
33*	<i>ToSfp007/ToSfp023</i>	isotig00263/isotig00811	accessory gland	unknown/ trypsin like protease	3.69	0.010
32					0.87	0.49
31	<i>ToSfp010</i>	isotig00307	accessory gland	unknown	0.95	0.442
30*	<i>ToSfp024</i>	isotig02293	accessory gland	carbonic anhydrase	4.09	0.006

29.5					1.98	0.112
28					0.34	0.847
27.5*	<i>ToSfp014/ToSfp003</i>	isotig01610/isotig01821	accessory gland	unknown	2.29	0.070
27					0.78	0.543
26					0.39	0.815
25.7	<i>ToSfp003</i>	isotig01821	accessory gland	lipocalin	0.28	0.897
25.3	<i>ToSfp003</i>	isotig01821	accessory gland	lipocalin	0.23	0.918
25	<i>ToSfp003</i>	isotig01821	accessory gland	lipocalin	0.40	0.805
24.5	<i>ToSfp025</i>	isotig01042	accessory gland	unknown	2.22	0.080
20			testis		1.57	0.196
19*	<i>ToSfp017</i>	isotig05129	testis	unknown	3.14	0.022
18	<i>ToSfp026</i>	isotig06304	testis	unknown	3.66	0.011
17					1.85	0.100
16	<i>ToSfp026</i>	isotig06304	testis	unknown	3.55	0.013
15	<i>ToSfp026</i>	isotig06304	testis	unknown	3.06	0.025
14.5	<i>ToSfp027</i>	isotig00169	accessory gland	unknown	3.97	0.007
13.5	<i>ToSfp011</i>	isotig01709	body	unknown	4.78	0.002
13	<i>ToSfp009</i>	isotig00102	accessory gland	unknown	0.80	0.532

¹full sequence data can be found in Appendix S1 of Simmons et al. (2013); ²peptide mass spectrometry matching data and closest BLAST matches can be found in Table S2 of the online supplementary material.

Table 2. Seminal fluid protein gene expression, and gene expression changes with age in *Teleogryllus oceanicus*

Protein name or Molecular weight	Transcript reference	$\Delta\Delta C_T \pm SE$	Univariate effect of age class on gene expression	
			$F_{4,70}$	P
<i>ToSfp014</i>	isotig01610	0.16 $\pm$ 0.01	10.41	<0.001
<i>ToSfp025</i>	isotig02293	0.26 $\pm$ 0.01	7.92	<0.001
<i>ToSfp017</i>	isotig05129	5.42 $\pm$ 0.12	8.50	<0.001
<i>ToSfp007</i>	isotig00263	0.05 $\pm$ 0.00	0.50	0.737
<i>Tosfp001</i>	isotig01262	0.20 $\pm$ 0.01	4.02	0.006

Table 3. Canonical structure from discriminant analysis of *Sfp* gene expression among age categories of male *Teleogryllus oceanicus*.

Canonical Axis	CA1	CA2
Eigenvalue	2.76	0.38
% variance	85.6	11.8
isotig01610	-29.49	6.24
isotig02293	14.14	-3.26
isotig05129	0.45	0.74
isotig00263	-7.28	-23.10
isotig01262	1.87	9.32