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Identification of novel ejaculate proteins in a seed beetle and division of labour across male accessory reproductive glands

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ABSTRACT

The male ejaculate contains a multitude of seminal fluid proteins (SFPs), many of which are key reproductive molecules, as well as sperm. However, the identification of SFPs is notoriously difficult and a detailed understanding of this complex phenotype has only been achieved in a few model species. We employed a recently developed proteomic method involving whole-organism stable isotope labelling coupled with proteomic and transcriptomic analyses to characterize ejaculate proteins in the seed beetle *Callosobruchus maculatus*. We identified 317 proteins that were transferred to females at mating, and a great majority of these showed signals of secretion and were highly male-biased in expression in the abdomen. These male-derived proteins were enriched with proteins involved in general metabolic and catabolic processes but also with proteolytic enzymes and proteins involved in protection against oxidative stress. Thirty-seven proteins showed significant homology with SFPs previously identified in other insects. However, no less than 92 *C. maculatus* ejaculate proteins were entirely novel, receiving no significant blast hits and lacking homologs in extant data bases, consistent with a rapid and divergent evolution of SFPs. We used 3D micro-tomography in conjunction with proteomic methods to identify 5 distinct pairs of male accessory reproductive glands and to show that certain ejaculate proteins were only recovered in certain male glands. Finally, we provide a tentative list of 231 candidate female-derived reproductive proteins, some of which are likely important in ejaculate processing and/or sperm storage.

1. Introduction

An extraordinary complex reproductive phenotypes in animals with internal fertilization is the male ejaculate (Avila et al., 2011; Chapman, 2008; Poiani, 2006). This not only contains sperm proteins (SPs) but also a multitude of seminal fluid proteins (SFPs) and other substances, many of which affect male and female reproductive success (McGraw et al., 2015), offspring phenotype (Bromfield et al., 2014) and show rapid evolution (Avila et al., 2011; Findlay et al., 2009; Swanson and Vacquier, 2002; Walters and Harrison, 2010). As the reproductive outcome of mating is to some extent dictated by the composition of the ejaculate (Goenaga et al., 2015; Perry et al., 2013), SFPs are important potential agents of postmating sexual selection and sexual conflict (Sirot and Wolfner, 2015). Yet, unravelling the full compositional complexity of the seminal fluid has proven challenging in non-model species. The identification of SPs and SFPs has been difficult in the past and a reasonably complete understanding of this phenotype has only been achieved in a handful of species (Chapman, 2008), but whole-organism stable isotope labelling to detect male ejaculate proteins within mated females has more recently increased our understanding of seminal fluid composition (Boes et al., 2014; Dean et al., 2011; Findlay et al., 2008; Sirot et al., 2011).

The compositional complexity of the ejaculate may be matched by a similar anatomical and structural complexity of the glandular tissues that excrete SFPs. Trypsin like enzymes are common components of seminal fluid. One would predict that such enzymes and their substrates may be physically separated until the appropriate time for blending the ejaculate “cocktail”, allowing modification and other reactions to occur when required. For example, the proteins that interact to form the copulatory plug of rodents are produced in the anterior prostate and seminal vesicle glands (Schneider et al., 2016; Williams-Ashman, 1984) and only mix at ejaculation. We also note that the hypothesis that males adaptively “tailor” the composition of the seminal fluid depending on, for example, properties of their mate (Perry et al., 2013) or their competitor relies on there being a certain flexibility in the SF production line. Such flexibility would be afforded by structural complexity of the male reproductive system, but studying this in small animals is often challenging. Ascribing different proteins to distinct production

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sites is even more difficult (Meslin et al., 2017). Again, the recent development of micro-tomography, allowing 3D reconstructions of the inner anatomical details of even small organisms, has now opened new possibilities for a significantly improved understanding (Mattei et al., 2015).

The seed beetle, *Callosobruchus maculatus*, is a major agricultural pest and an emerging model system for sexual selection and sexual conflict (Berger et al., 2016; Eady, 1995; Fricke and Arnqvist, 2007; Gay et al., 2009; Hotzy and Arnqvist, 2009). Males transfer a large ejaculate, up to 8% of male body weight (Rönn et al., 2008), to females at mating and they possess injurious genital spines (Crudgington and Siva-Jothy, 2000; Hotzy and Arnqvist, 2009) that increase the transfer of seminal fluid proteins (SFPs) into the female body (Hotzy et al., 2012; Huignard, 1983). Aspects of male and female morphology and physiology evolve under sexually antagonistic coevolution (Dougherty et al., 2017; Rönn et al., 2007) and it has been suggested that reproductive proteins do as well (Brown and Eady, 2001; Fricke and Arnqvist, 2007; Yamane et al., 2008). Previous studies have shown that SFPs alter female behaviour and physiology (Huignard et al., 1977; Goenaga et al., 2015) and certain size-classes of proteins are known to modulate reproduction and sexual conflict in this species (Yamane et al., 2015). Bayram et al. (2017) recently used 2D SDS-PAGE, in conjunction with MALDI-TOF and LC-MSMS, to identify 98 putative SFPs in this species. Of the putative SFPs, 44 showed strong evidence for being SFPs, although they noted that this was likely an underestimate of the full compositional complexity of SFPs due to technical limitations of 2D SDS-PAGE analyses. Further, previous studies documenting variation in ejaculate composition across populations (Goenaga et al., 2015) and ejaculate allocation within populations (Rönn et al., 2008), have suggested that males are able to tailor their ejaculates adaptively. This not only predicts a high degree of anatomical complexity of the glandular tissues that excrete ejaculate proteins but also a certain division of labour across glands.

Here, we use whole-organism stable isotope labelling coupled with proteomic and transcriptomic analyses to provide a more comprehensive list of proteins transferred to females in *C. maculatus*. Moreover, we combine 3D micro-tomography with proteomic methods to test the explicit prediction that the ejaculate production line should be complex and should show some division of labour. We identify in total 317 male-derived proteins that are transferred to females, several of which are 'unknown' proteins that may represent rapidly evolving SFPs novel to seed beetles. We use micro-tomography to uncover the complexity of male accessory reproductive glands and show that there is some division of labour across different reproductive glands. Finally, we provide a conservative list of female-derived reproductive tract proteins. These are putative female reproductive proteins that may be important in ejaculate processing and/or sperm storage (McGraw et al., 2004; Avila et al., 2011; Sirot and Wolfner, 2015).

2. Materials and methods

2.1. 3D micro-tomography

The precise anatomy of the male accessory glands associated with reproduction is not well understood in *C. maculatus* (Singh, 1978). To provide a full description of the male reproductive glands complex we therefore conducted micro-CT analyses (Metscher, 2009). Virgin males (24–48 h post hatching) were decapitated and placed in Bouin's fixative for 4 days. Following three steps of serial rinsing in H_2O (to remove excess fixative), the samples were place in 500 µl Lugols stain (dilute potassium iodide) for 48 h. This enhances the contrast of non-mineralized tissues in micro-CT. Samples were then rinsed in 5% EtOH and left in 5% EtOH for 24 h. Over five days, samples were then serially dehydrated (20%, 40%, 60%, 70%–80% EtOH). Twenty-four hours prior to micro-CT scanning, the samples were placed in 96% EtOH.

Micro-CT was performed on a SkyScan 1172, at 22 V and 110 µA resulting in a voxel size of 0.7 µm. The resulting CT images were used to reconstruct, inspect, analyse and render 3D images of the male reproductive complex, using the software DataViewer and CT-Vox (bruker-microct.com) and SPIERS (Serial Palaeontological Image Editing and Rendering System) free software (spiers-software.org; Sutton et al., 2012). SPIERS edit was used to manually clean up the images, and mask the area for each tissue type. These images were then reconstructed and viewed in 3D using SPIERSview.

2.2. Experimental design

A major aim of the study was to identify ejaculate proteins through ^{15}N labelling and proteomic analysis. Labelling using a heavier stable isotope causes the proteins to have a greater mass. As a result, labelled proteins are not identifiable using mass spectrometry as the observed mass spectra do not match what would be predicted by the proteome database. Mating a labelled female to an unlabelled male therefore allows the identification of only male-derived proteins within a sample of reproductive tracts from mated females. Complete labelling is not required to create enough of a mass shift to render the labelled proteins unidentifiable against a standard database. Here, our aim was to create labelled beetles with approximately 50% of the nitrogen present in each protein being of the heavy isotope (^{15}N instead of ^{14}N).

2.3. Beetles

All *C. maculatus* beetles used were from the South India reference stock. Beetles were maintained under constant laboratory conditions of 29 °C, 60% RH and a 12:12 h light:dark cycle. Beetle larvae develop within the host bean and hatch as adults that are able to reproduce, without requiring further nutrition or water. Therefore, the proteins within the adult beetles derive entirely from the host bean. Eggs laid on beans containing ^{15}N will therefore result in the emergence of ^{15}N labelled sexually mature beetles.

2.4. Producing labelled beans

A protocol for producing labelled beans, where artificial beans were created from ground beans combined with labelled yeast powder, was developed based upon previously established methods for producing artificial beans (Hudaib et al., 2013) and ^{15}N labelled yeast (Findlay et al., 2008). To produce labelled yeast, 20 µl *Saccharomyces cerevisiae* cell stock was cultured in 200 µl of labelled media (98% ^{15}N, Silantes), containing 20% glucose, at 30 °C with continuous shaking at 150 rpm. After 48 h, the yeast-containing media was divided into smaller volumes (approximately 30 ml) within 50 ml falcon tubes for centrifuging at 12000g, 4 °C for 15 min. The supernatant was discarded and 10 ml of 30% EtOH added to the yeast pellet and mixed. The ethanol was added to break up the cell membranes and kill yeast cells. The ethanol – yeast mixture was centrifuged again at 12000g and 4 °C for 15 min. The supernatant was discarded and the yeast paste spread onto clean parafilm stretched over the base of a petri dish. This was left to dry, covered, on a hot plate at 40 °C over 48 h.

To produce the bean powder, black eyed peas (*Vigna unguiculata*) were briefly soaked in H_2O until the coat easily peeled off (up to 10 min). Black eyed peas were used as they are the preferred host of *C. maculatus*. The beans were then dried on a hotplate at 30 °C for 48 h. The dry beans were ground using a coffee bean grinder (Delonghi) on the finest setting. After the first grind, the powder was processed through the grinder two more times and the resulting bean flour was sieved through a fine grade mesh (300 µm/Tyler-grade 48).

To achieve a ^{15}N content of approximately 50% in the artificial beans, and therefore in the beetles, the dry weight ratio of yeast to bean flour was 1:1.6. This was based on the supposition that yeast contains approximately 50% protein and black eyed peas approximately 30%. Water was added at a ratio of 2 ml H_2O:5 g dry ingredients, as this level

of hydration was optimal during protocol development. To obtain optimum homogenisation, water was first added to the dry yeast to rehydrate it into a paste. The bean powder was then added to the mixture, which was subsequently kneaded into dough. The dough was left at 4 °C for 5 h before shaping 0.25 g sections of dough into smooth bean shapes. The artificial beans were then left to dry at 30 °C for 1 week.

2.5. Producing labelled beetles

Two virgin females and two males were added to a petri dish containing five labelled artificial beans. The beetles were observed until mating occurred and then left overnight in the incubator under standard conditions. Males were removed after 24 h and females left to continue laying eggs for a further 24 h. After an additional 24 h, a sterile scalpel was used to scrape off excess eggs if more than four were present on each bean, to reduce larval competition for resources. The artificial **beans** were left to incubate in individual wells of a 24 well cell culture plate, to ensure beetles would remain virgins upon emergence. Beetles emerging from the artificial beans appeared and behaved normally.

2.6. Collecting ejaculates

Standard mung beans with eggs laid by the baseline population were kept in parallel to the artificial beans, to produce unlabelled control beetles. Upon emergence, both labelled and unlabelled beetles were isolated in 1.5 ml Eppendorf tubes with a ventilation hole pierced in the top and left within the incubator for 24–48 h prior to mating. From the five labelled beans, a total of 10 labelled beetles emerged. Three labelled females were each mated to an unlabelled male to allow the identification of ejaculate proteins. Additionally, two pairs where both sexes were labelled were mated as positive controls to test the labelling efficiency. A single unlabelled male was also mated to an unlabelled female to serve as a negative control. Finally, a labelled (L) male was mated to an unlabelled (UL) female, to allow the identification of female-derived reproductive tract proteins. Hence, this part of our study involved the following male-female (N) samples: UL-L (3), L-L (2), UL-UL (1), L-UL (1).

To collect reproductive tracts, the virgin focal females were first weighed to the nearest 0.00001 g (Sartorius Genius ME 235P) and introduced into a petri dish containing the male. The pair was observed until mating was completed (2–5 min). Directly upon completion, the female was weighed again and female weight gain was used to confirm ejaculate transfer from the male (Rönn et al., 2008). Immediately following this, the bursa copulatrix containing the ejaculate was dissected from the female and placed into 5 μl of lysis buffer (20 mM HEPES; 9M Urea; Complete mini EDTA-free protease inhibitor cocktail). A clean dissection needle was then used to gently rupture the ejaculate-containing bursa and to homogenise the ejaculate within it, which was briefly centrifuged before storing at −20 °C prior to LC-MSMS analysis.

2.7. Proteomic analysis

In each of the seven independent samples, the bursa containing the ejaculate was digested using trypsin according to the standard analytical pipeline within the Uppsala University SciLife proteomic facility. No protein concentration measurements were made but all material in each sample was used for digestion. Briefly, proteins were first reduced with DTT (final concentration 48 mM) and alkylated with IAA (final concentration 25 mM). Samples were diluted four times with 50 mM ammonium bicarbonate prior to tryptic enzymatic digestion (enzyme:protein ratio 1:20, Promega) at 37 °C overnight. The reaction was stopped by acidifying the sample with trifluoroacetic acid (TFA). Prior to the analysis by mass spectrometry, the peptides were purified by Pierce C18 Spin Columns (Thermo Scientific) and dried in a SpeedVac system, before resolving in 15 μL 0.1% Formic Acid (FA). Peptides from ejaculate samples were separated in reversed-phase on a C18-column, using a 90 min LC-gradient, and electrosprayed on-line to a Q Exactive Plus mass spectrometer (Thermo Finnigan). Tandem mass spectrometry was then performed applying HCD.

2.8. Proteome

A proteome was configured from the translated transcript sequences of the recently published *C. maculatus* transcriptome, based on multiple samples of larvae, pupae and adult males and females (both head/thorax and abdominal samples) (Sayadi et al., 2016). In order to ensure that no potential reproductive proteins were missed within this integrative assembled transcriptome, we have included transcript sequences obtained from different transcriptome assemblies of all abdominal samples. The obtained transcripts sequences were then translated to proteins, selecting the longest open reading frame for each transcript using TransDecoder v.2.0.1 (https://github.com/TransDecoder/), to form the final proteome database. We note that the merge of several transcriptome assemblies to one database, results in sequence redundancy. Copies of the same protein sequence from each transcript database were removed using the usearch function within the UCLUST package (Edgar, 2010). Sequences were sorted according to length prior to searching, and sequences with more than 99% identical matching were removed to reduce redundancy.

2.9. Protein identification

Peak lists obtained from MS/MS spectra were identified using MS-GF + version Beta (v10282) (Kim and Pevzner, 2014). The search was conducted using SearchGUI (v3.1.1) (Vaudel et al., 2011). Protein identification was conducted against the protein database described above, which contained 82397 target sequences. The decoy sequences were created by reversing the target sequences in SearchGUI. The identification settings were as follows: trypsin, with a maximum of 2 missed cleavages, 10.0 ppm as MS1 and 0.5 Da as MS2 tolerances. Modifications were defined as, a fixed carbamidomethylation of C, and variable oxidation of M. Peptides and proteins were inferred from the spectrum identification results using PeptideShaker version 1.16.4 (Vaudel et al., 2015). Peptide Spectrum Matches (PSMs), peptides and proteins (≥2 unique peptides) were validated at a 1.0% False Discovery Rate (FDR) estimated using the decoy hit distribution. Only proteins deemed confident by PeptideShaker (Vaudel et al., 2015) were included. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2015) partner repository with the dataset identifier PXD009967.

2.10. Transcriptomic data analysis

Identified protein sequences were matched back to the transcriptome data of Sayadi et al. (2016) and Immonen et al. (2017), to identify sequences and allow differential expression analyses. Multiple transcript isoforms often translate into the same protein, so expression data for all matching sequences were tested. Differential expression in males and females were analysed using DESeq2 package in R (Love et al., 2014). The analyses reported here focussed on 12 independent RNA samples of the abdomen of virgin and mated individuals of both sexes (3 replicates; total N = 12). All transcripts were included in the normalisation and analysis steps to ensure the transformation was not skewed. The count data for all transcripts was regularised log transformed (rld: $y = \log 2 (n + 1)$), blind to the experimental design. Mating status (mated or virgin) was included in the model whilst testing for differences based on sex. Preliminary plots revealed samples to be much more different according to sex than mating status. Overrepresentation of Gene Ontology terms were tested with the GOstats package v.2.46.0 (Falcon and Gentleman, 2007). The P - values associated with each term were adjusted for multiple testing using FDR (Benjamini and Hochberg,

1995).

2.11. Collecting male accessory glands

To collect samples of male reproductive tissue, drops of lysis buffer (20 mM HEPES, 9M Urea, Complete mini EDTA-free protease inhibitor cocktail) diluted 1:10 in milliQ water were placed on a clean glass slide under a dissecting microscope. Individual virgin males aged between 24 and 72 h post hatching were killed and the contents of their abdomen extracted into a drop of the diluted lysis buffer. The alimentary tract and excess tracheal tissue were removed, and the reproductive organ complex rinsed in lysis buffer and moved to a clean drop of lysis buffer to reduce the risk of contamination. Individual glands were then gently isolated and pinched off of the mass of reproductive tissues and pooled into 1.5 ml eppendorfs containing 30 µl of lysis buffer. We prepared two biological replicates for the testes and two for each of the five different types of male accessory reproductive glands identified by our micro-CT analyses ($2 \times 6 = 12$ samples). The total number of males dissected per sample differed between 26 and 46, as differences in the size of the different glands meant that a different absolute number of glands were needed to generate a sufficient quantity of proteins in all sample types. Samples were then Vortexed (13000 g for 30s), briefly centrifuged and stored at −20 °C prior to LC-MSMS analysis.

The protein concentration in the samples was measured using the Bradford Protein Assay with bovine serum albumin (BSA) as standard. Aliquots corresponding to 20 µg protein were taken out for digestion. The proteins were reduced, alkylated and in-solution digested by trypsin according to the standard operating procedure described above. Thereafter the samples were purified by Pierce C18 Spin Columns (Thermo Scientific), dried and resolved in 0.1% FA. Proteomic analyses and protein identification were then carried out as described above. As for the labelled samples, the mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2015) partner repository with the dataset identifier PXD009967.

3. Results

3.1. Male reproductive glands

The accessory reproductive glands in male seed beetles are not fully understood and there is striking variation across species in anatomy and structure. For example, the number of distinct glands varies between 2 and 5 pairs across species (Singh, 1978). Glands are of both mesodermal and ectodermal origin, but ascribing homology has proven difficult (Singh, 1978; Suzuki, 1988). Our 3D micro-CT analysis revealed that the complex of accessory glands and testes is tightly wrapped-up in the male abdomen *in vivo* (Fig. 1) and it allowed us to unambiguously distinguish 5 distinct pairs of accessory reproductive glands. Two pairs of large mesadenial glands (M1 and M2) dominate, together with the testes (T) and the ejaculatory bulb, the outer layer of the complex. The two mesadenial glands are sometimes considered to constitute a single large bilobed gland (Khaled et al., 2015), but are here considered to be distinct. Three pairs of ectadenial glands (E1-E3) are more centrally located. All glandular and testicular ducts eventually feed into the seminal vesicles in the centre of the reproductive complex, where the ejaculatory duct also originates (see Fig. 1).

3.2. Ejaculate proteins

The proteomic analyses of the two samples of the ejaculate-containing bursa copulatrix where both the male and the female were ^{15}N labelled yielded no identified proteins, and only three doubtful peptide matches. The unlabelled control sample, where neither of the sexes were labelled, yielded 1002 identified proteins. Therefore, the ^{15}N labelling experiment of male and female beetles was successful and resulted in a near complete nondetection of proteins from labelled individuals.

The three samples of labelled females mated to unlabelled males yielded 195, 286 and 240 identified male-derived proteins, of which 98 were present in all three samples (Fig. 2). In total, our analyses identified 317 male-derived proteins within the bursa copulatrix of mated females. This forms our comprehensive set of ejaculate proteins in *C. maculatus* (SI Table 1). We note that a previous effort to identify SFPs in this species (Bayram et al., 2017) using 2D SDS-PAGE identified 98 putative SFPs, of which 29 are also part of our comprehensive set. Of the 98 putative SFPs from the previous analyses, 44 had strong evidence of being probable SFPs based on secretion signals, identification as SFPs in other species, and male-biased transcript expression (Bayram et al., 2017). All 29 of the proteins found in both the previous analyses and identified here within the labelled male samples were part of this more confident set of 44 putative SFPs of Bayram et al. (2017).

Many of the 317 ejaculate proteins detected here are likely not SFPs, but represent sperm proteins and other groups of proteins such as products of constitutively expressed genes. We used three hierarchical criteria to identify proteins that are most likely to represent true SFPs. First, we assessed signals of secretion within the identified protein sequences, using SignalP (Petersen et al., 2011) and SecretomeP (Bendtsen et al., 2004), while noting that not all secretory proteins show signals of secretion. Of the 317 male-derived proteins, 196 showed signals of secretion (Table S1). Second, transcript expression data for the 317 protein sequences identified here as ejaculate proteins were analysed from male and female abdominal samples (N = 12). The abdomen of both sexes of *C. maculatus* is to a large extent composed of reproductive tissue. Genes that are highly expressed in males, but not expressed in females, are likely male-specific reproductive proteins. Statistical analysis using a negative binomial generalized linear model (GLM) was performed to determine differential expression. As the mated and virgin data was highly similar, we are only presenting the differential expression analyses between male and female abdominal samples. Mating status was included in the model as a factor. As the 317 proteins derive from different assembled transcriptomes, and as we only have differential expression data from the integrative assembled transcriptome, we used BLAST to match the 317 protein sequences back to the transcriptome. This resulted in 411 transcript sequences with identical matching. There are more transcript sequences than protein sequences here because there were multiple isoforms for 52 proteins, each of which matched to multiple RNA transcripts. We analysed the data for all identically matching sequences. Of the 411 transcript sequences, 340 showed significant differential expression. A great majority of these, i.e. 315, had significantly greater expression in male abdominal samples than in females (FDR adjusted p-value < 0.05) (SI Fig. 1). When selecting transcripts with greatest total abundance (i.e., one isoform per protein), 234 out of the 317 ejaculated proteins showed more than four-fold, 220 more than 10-fold and 194 more than 100-fold expression in males relative to females (Table S1). Out of the 196 proteins showing signs of secretion, 122 also showed more than 100-fold expression in males relative to females.

Third, although SFPs can be produced in testes and associated ducts, we conservatively inspected those ejaculate proteins that were recovered from one or more of the accessory glands but were not found in testes (see below). There were 69 such proteins. Out of these 69, 41 also showed a signal of secretion and, simultaneously, a more than four-fold expression in males relative to females. These 41 ejaculate proteins form our most confident set of SFPs (Table S1).

Remarkably, no less than 92 ejaculate proteins (i.e., 29%) received no significant blast hits and lacked homologs in the non-redundant (nr) protein database (SI Table 1). These thus represent novel proteins. A functional enrichment analysis of the 317 ejaculate proteins, against a reference consisting of the total male-specific transcriptome of Sayadi et al. (2016) provided some insights (SI Table 5). In terms of biological processes, the set was enriched with many proteins involved in general

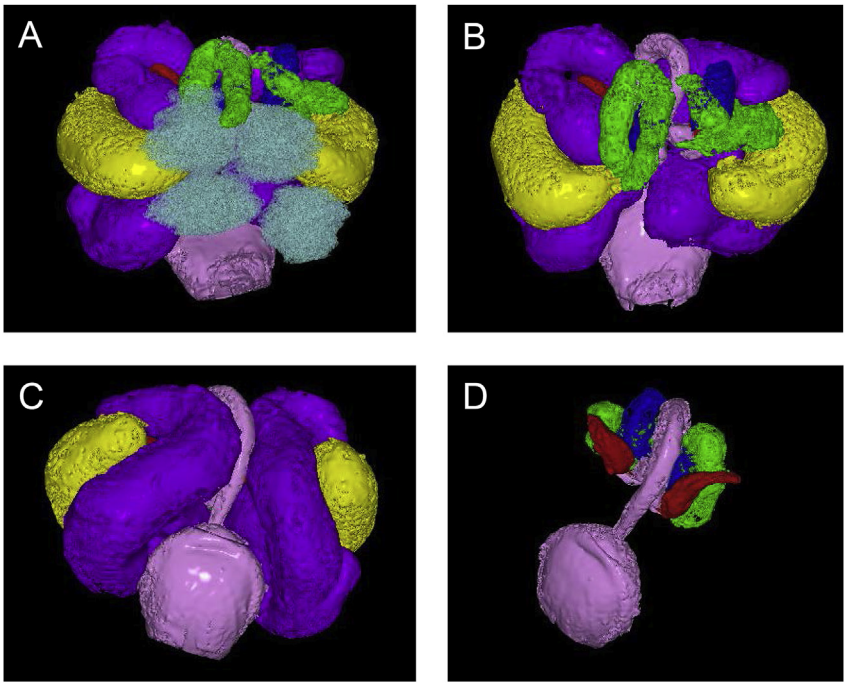

Fig. 1. Reconstructions of the internal reproductive complex of male *C. maculatus* from micro-CT scans. (A) The two characteristic bilobed testes (light blue) are located in the outer layer of the complex. (B) Removing the testes uncovers the underlying five pairs of accessory reproductive glands of which (C) the larger ones are the mesadenial glands (M1 – purple; M2 yellow). (D) Embraced by the mesadenial glands are the three pairs of ectadenial glands (E1 – green; E2 – blue; E3 – red). Shown is also the ejaculatory bulb and duct (pink), which originates from the seminal vesicle region in the centre of the complex. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

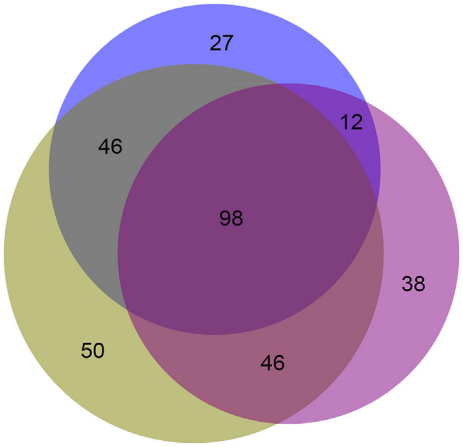

Fig. 2. Venn diagram illustrating the number of identified male-derived proteins in three female reproductive tract samples (each given a unique colour) from just-mated females. In total, 317 such proteins were identified. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

metabolic and catabolic processes but also with proteolytic enzymes and proteins involved in protection against oxidative stress. The pattern of enrichment for molecular function matched this overall pattern well. In terms of cellular components, the set was enriched for mitochondrial and extracellular location.

We also compared the 317 ejaculate proteins against sequences previously identified as SFPs in proteomic analyses of *Tribolium castaneum*, *Apis mellifera* and *Drosophila melanogaster*, using the method detailed in Bayram et al. (2017). This yielded 37 significant hits (SI Table 6). Notable among those shared with *Drosophila* are several serpins (including Acp76A), which function as protease inhibitors.

3.3. Division of labour across male glands

Our proteomic analyses showed that the testicular and accessory reproductive gland proteomes were rich (see Table S2). When including only proteins that occurred in both samples of a given gland, we identified 1161 distinct proteins in the testes and each of the five different pairs of accessory glands contained > 400 proteins. There was considerable overlap in protein composition across glands (Table SI 3 and 4), although the number of proteins unique to specific glands was sizeable; T:336, E1:97, E2:34, E3:58, M1:8 and M2:14. Most of these proteins are no doubt proteins that build, and function within, the cellular structures and tissues that make up the testes and the glands. Below, thus, we focus exclusively on the subset of these proteins that also occurred in our comprehensive set of 317 ejaculate proteins since these male-derived proteins are demonstrably transferred to females.

We recovered 316 out of all 317 proteins identified in our labelling experiment in at least one of the 12 samples from the reproductive glands. We here restrict this to include only proteins that occurred in both samples of at least one given gland, totalling 283 of the 317 proteins. Out of these 283, we found 214 in testes, 219 in E1, 243 in E2, 199 in E3, 150 in M1 and 147 in M2. Overall, most proteins were found in more than one gland (Fig. 3). The testes showed most unique proteins (N = 14), of which several were general metabolic enzymes but also structural proteins (i.e., tubulin) and myosin, known to be involved in sperm production and motility (Schliwa, 2003; Gupta, 2006; Li and Yang, 2016). We found substantial differentiation across male accessory glands. The two large mesadenial glands were distinct in that they contained fewest and a low number of unique proteins (N = 12) (Fig. 3). Among these were a few catalytic enzymes (e.g., glutathione peroxidase, transketolase). The three ectadenial glands contained more unique proteins: 30 were recovered only in one or more of the ectadenial glands (Fig. 3). Among these are several digestive enzymes, such as proteases and lipases, but also proteinase inhibitors. Thus, it is clear that the difference seen in ultrastructure between the mesadenial and ectadenial glands (Khaled et al., 2015) is reflected by the fact that they, to some extent, also produce distinct proteins. We note that 79 of the 283 proteins belonged to the set of novel proteins, and out of these 70 occurred in more than one type of gland, 3 only in ectadenial glands, 4 in mesadenial glands and 2 only in testes.

3.4. Female reproductive proteins

From a sample of an unlabelled female mated to a labelled male, we identified 676 female-derived proteins within the bursa copulatrix.

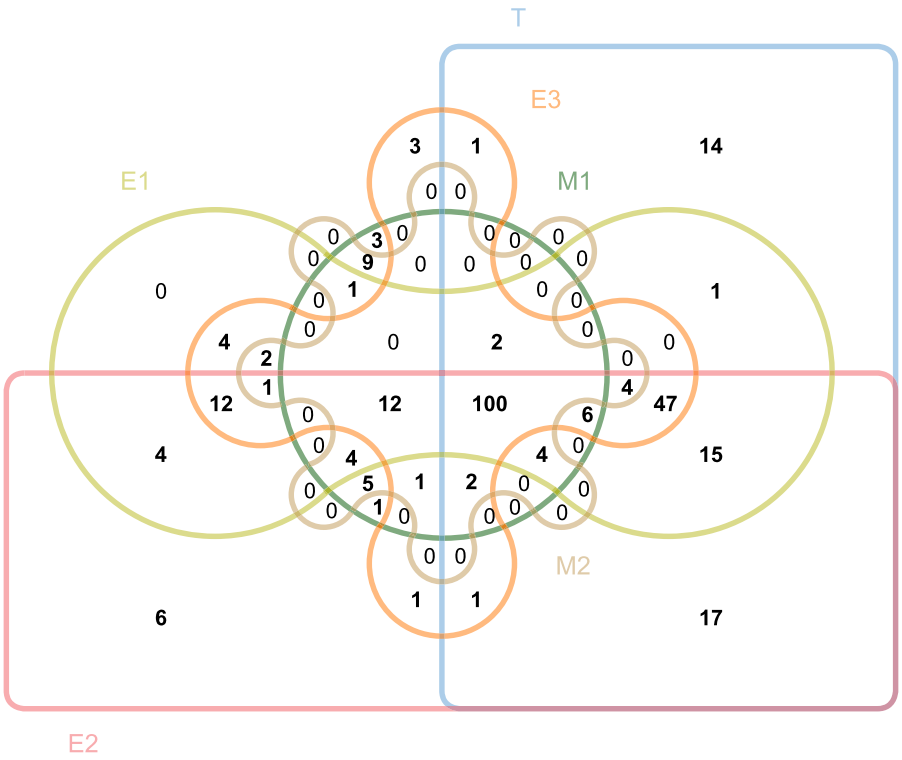

Fig. 3. Edwards–Venn diagram illustrating the localization of the 283 male-derived ejaculate proteins that were recovered in both replicate samples of the testes (T), the three ectadenial glands (E1-E3) or the two mesadenial glands (M1-M2). About one third (N = 100) were found in all six types of glands, while testes (N = 14) and E2 (N = 6) showed most unique proteins.

Many of these proteins are unlikely to qualify as female reproductive proteins, although most were female-biased in expression (SI Fig. 2). To gain a list of candidate female reproductive proteins, we scanned this set of proteins for transcripts that are upregulated in the abdomen of just mated, compared to virgin, females (Immonen et al., 2017). In total 231 of the 676 female-derived protein transcripts were significantly upregulated in the female bursa copulatrix after mating (SI Table 7). Although this is no doubt an incomplete list of female-derived proteins in the reproductive tract, the combined fact that they (1) are present in the bursa copulatrix and (2) are upregulated after mating makes them putative candidate female reproductive proteins. A functional enrichment analysis of the 231 proteins, using BLAST2GO against a reference consisting of the total female transcriptome of Sayadi et al. (2016) (SI Table 7) showed enrichment for proteins involved in translation, proteolysis, protein metabolism and ATP metabolism.

4. Discussion

Our efforts show that the seed beetle *C. maculatus* aligns well with a handful of other insect species where comprehensive studies of male ejaculates have been made (e.g., Boes et al., 2014; Dean et al., 2011; Findlay et al., 2008; Sirot et al., 2011): male seed beetles transfer hundreds of proteins to females at mating. It is clear that the use of whole-organism isotopic labelling, in conjunction with a rich set of transcriptomic data, offers an efficient methodological route to the identification of male-derived proteins in non-model taxa. We stress that the method yielded excellent results, despite the fact our protocol relied on incomplete labelling (ca 50% ^{15}N). This aspect of our work offers considerable hope for systems where complete labelling is difficult to achieve.

Previous studies of *C. maculatus* have identified a more limited set of putative SFPs (Bayram et al., 2017) and have revealed functional significance of variation in ejaculate composition (Goenaga et al., 2015). The current study adds quantitative and qualitative weight to this previous research, and identified almost one hundred ejaculate proteins novel to science. In terms of the general composition of the ejaculate of *C. maculatus*, it shares many general features with the seminal fluid of other animals. The fact that metabolic and catabolic proteins were abundant, as were proteolytic enzymes and proteins involved in protection against oxidative stress, is characteristic for male seminal fluid generally and is consistent with our view of a high biological activity of the seminal fluid inside the female reproductive tract (Avila et al., 2011; Chapman, 2008; Poiani, 2006). There are also a large number of more specific similarities. For example, the seminal fluid of *C. maculatus* contained a large number of serine protease inhibitors (i.e., serpins), of which several were homologous to serpins found in seminal fluid of *Drosophila* and other insects. Serpins are thought to protect sperm from proteolytic attack in the female reproductive tract (Dean et al., 2009), suggesting complex interactions between female-derived proteases and male-derived protease inhibitors (Sirot et al., 2015). The fact that our list of candidate female reproductive proteins included many proteases is consistent with this suggestion.

The ejaculate proteins found in *C. maculatus* include several well-known structural sperm proteins, such as actin, myosin and tubulin, but also proteins known to be involved in sperm motility and sperm protection in other species. For example, lactate dehydrogenase is known to catalyse basic oxidative metabolic processes and is a key enzyme for sperm mobility in mice (Baccetti et al., 1975; Odet et al., 2011). Similarly, oxidative stress can damage sperm and reduce male fertility (e.g. Almbro et al., 2011) and proteins with anti-oxidative properties, such as glutathione and superoxide dismutase, were among those discovered here. Indeed, removal of superoxide radicals and response to superoxide were among those biological processes that showed significant enrichment in ejaculate proteins. Yet other proteins are likely to be involved in sperm-egg interactions. Lectin is one of these, being important in gamete recognition in several taxa (e.g., Nicolson et al., 1975; Glabe et al., 1982). The presence of a zinc metalloproteinase is also interesting in this context, considering that it is involved in sperm-egg fusion in other taxa (Wolfsberg et al., 1993; Beek et al., 2012).

The presence of several prostaglandin dehydrogenases in the male seminal fluid is interesting for several reasons. Prostaglandins and other eicosanoids are important mediators of insect immune function (Stanley, 2006), and these dehydrogenases are likely to metabolize prostaglandins into biologically inactive derivatives (Duffy et al.,

2005). They may thus represent male SFPs that modulate female immune response to mating (McGraw et al., 2004) that might otherwise harm sperm. However, prostaglandins also play several more direct roles in insect reproduction, such as regulating egg-laying behaviour (Stanley-Samuelson and Loher, 1986) and inhibiting ovarian uptake of yolk proteins (Medeiros et al., 2002), and prostaglandin dehydrogenases may also affect such processes.

Despite the many general similarities with SFPs of other insects, only 12% of all *C. maculatus* ejaculate proteins showed significant homologies with SFPs in other insects. More strikingly, 29% lacked homologs in extant databases and are in this sense novel proteins. We suggest that these facts combined provide a striking illustration of the common observation that SFPs show rapid and divergent evolution (Avila et al., 2011; Findlay et al., 2009; Swanson and Vacquier, 2002; Walters and Harrison, 2010). An important mission in future comparative genomic work in seed beetles will now be to compare the rate of evolution across different functional groups of ejaculate proteins and to probe the genome for signals of divergent selection.

We found evidence for division of labour across the multiple secondary sex-glands in males, although overlap across glands was significant as was overlap with testes. Overlap could in part be due constitutively expressed house-keeping genes producing proteins that are transferred to females or to cross-contamination during dissections. Although great care was made to prevent the latter, it is difficult to avoid completely given that the testes and glands are immediately adjacent in the male abdomen (Fig. 1). This would render our assessment of division of labour conservative. Yet, it is clear that the differences in ultrastructure between different types of glands noted previously (Singh, 1978; Suzuki, 1988; Khaled et al., 2015) do correspond to actual functional differentiation. The fact that the mesadenial glands produced unique catalytic enzymes and the ectadenial glands produced unique digestive enzymes is consistent with functional specialization of different types of glands as well as with the suggestion that amalgamating products from different glands may trigger posttranslational modification and other forms of activation of specific compounds upon mixing. It is difficult to assess how our observed degree of division of labour relates to that in other taxa, as there are very few proteomic studies of male ejaculate proteins across multiple male glands. In butterflies, different parts of the male reproductive tract are known to contribute to different structural components of the ejaculate (Meslin et al., 2017), suggesting that more careful studies of the biochemical complexity of male ejaculates are needed. At a general level, the fact that different proteins are produced in distinct glands provides novel evidence for a potential proximate explanation for short-term and plastic tailoring of ejaculate composition (Perry et al., 2013; Meslin et al., 2017): by mixing products from different glands into the seminal vesicle, male beetles would in theory be able to rapidly modulate ejaculate composition. The composition of male ejaculates is known to differ dramatically across populations in *C. maculatus* (Goenaga et al., 2015), and this must at least in part be the result of variation in activity across different male reproductive glands and the mixing of their products.

Many SFPs are known to have direct effects on female physiology and behaviour (Avila et al., 2011; Sirot et al., 2015) and > 2000 genes significantly change their expression in female *C. maculatus* as a result of mating (Immonen et al., 2017). Following mating, male SFPs and sperm interact with female-derived molecules within the female reproductive tract but relatively little effort has been devoted to their identification (Sirot et al., 2015). Yet, in order to fully understand changes triggered in females by male-derived molecules, a crucial first step is the identification of female-derived molecules. Although this was not the main aim of the current study, our results do provide two insights. First, we demonstrate that whole-organism isotopic labelling and proteomic analyses of the female reproductive tract after mating provides a promising method also for the identification of female-derived reproductive proteins. Second, we provide a tentative list of 231 candidate female reproductive proteins. These are female-derived proteins present in the female reproductive tract which are also known to be upregulated in females after mating. These female-derived molecules were enriched for many biological processes in *C. maculatus*, including metabolism and protein modification which are also enriched in female reproductive proteins in *D. melanogaster* (McGraw et al., 2004, 2008). In this sense, our results can serve as the basis for more exhaustive and detailed work on female reproductive proteins in *C. maculatus* and of interactions between male- and female-derived reproductive molecules.

Author contributions

Conceived and designed the experiments: GA and HB. Conducted bioinformatic analyses: HB, AS and EI. Performed the experiments: HB (proteomic) and EI (transcriptomic). Analysed the data: HB, GA, AS and EI. Drafted the first manuscript: GA and HB. Revised and edited the manuscript: GA. Approved the final manuscript: HB, AS, EI and GA.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2018.12.002.

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