Protein analysis of the spermatophore reveals diverse compositions in both the ampulla and the spermatophylax in a bushcricket

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Abstract. Nuptial gifts are male mating investments, which, in bushcrickets, comprise the sperm-containing ampulla and the attached spermatophylax. The functions of the spermatophylax are to deter females from premature removal of the sperm-containing ampulla, which is a nutrient resource for females, as well as a source of compounds that influence female behaviour to increase male evolutionary fitness. Placing these functions into a proteomic perspective, we analyze the protein composition of nuptial gifts from male *Poecilimon ampliatus* (Brunner von Wattenwyl, 1878) bushcrickets using large two-dimensional gel electrophoresis coupled with nano-liquid chromatography-electrospray ionization mass spectrometry and *de novo* sequencing. We separate the proteins with high resolution and detect approximately 600 protein spots in the seminal fluid (ampulla) and 300 in the spermatophylax. There is only a small fraction of overlap in protein spots, whereas the majority differ between the two compartments. As a result of the lack of a sequenced genome and protein data for this non-model insect, we are unable to identify the proteins. We discuss the diversity of proteins, as well as their size range, in light of potential protein costs and potential functions.

Key words. Bushcricket, evolutionary proteomics, mating effort, nuptial gifts, *Poecilimon*, spermatophore.

Introduction

The transfer of gametes between the sexes is essential for mating in animals. However, differential investment of the sexes into mating and reproduction leads to unbalanced costs and benefits. Males generally invest less, although some species contribute more by offering costly nuptial gifts to the female (Lewis *et al*., 2014). Nuptial gifts are provided by a large variety of animals and are particularly common in insects (Vahed, 1998). Such gifts are either of exogenous (such as captured prey) or endogenous (i.e. manufactured by the males) origin (Lewis & South, 2012; Lewis *et al*., 2014). Bushcricket males (Insecta: Orthoptera: Tettigoniidae) add endogenously derived material (called the spermatophylax) around the sperm-containing

ampulla; the spermatophylax and ampulla combined are called the spermatophore. Immediately after copulation, the females start to consume the spermatophylax (Lehmann, 2012). It is well established that the spermatophylax acts as sperm protection device, deterring the female from removing or eating the ampulla during sperm transfer (Gwynne, 1997).

There is long-lasting debate about the other functions of the bushcricket spermatophore (Vahed, 2007; Gwynne, 2008; Lehmann, 2012) and this is hindered by limited knowledge concerning spermatophore composition. The spermatophore is obviously a food source for the female. Radioactive labelling shows that traces of male-provided material are slowly incorporated into newly manufactured eggs (Bowen *et al*., 1984; Gwynne, 1988; Wedell, 1993). In some herbivorous Phaneropteridae, female bushcrickets use the spermatophylax as a fast uptake food source, replacing plant feeding in the first 12 h after mating (Lehmann & Lehmann, 2016) and male-derived carbohydrates rapidly contribute to their

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metabolism (Voigt *et al.*, 2008). When food-deprived, females of several species increase mating rates (Gwynne, 2001) and compete for gift-giving males (Gwynne *et al.*, 1998). Even females of an obligate parthenogenetic species accept heterospecific males for mating and consume the spermatophylax (Lehmann *et al.*, 2011). The spermatophore can represent an important nitrogen resource for bushcrickets (Bowen *et al.*, 1984; Wedell & Ritchie, 2004), especially for species that rely on plants poor in nitrogen (Whitman *et al.*, 1994; Awmack & Leather, 2002). In a herbivorous bushcricket (Voigt *et al.*, 2006), male-manufactured nitrogen transferred in the spermatophore is incorporated into female muscles after feeding. In female bushcrickets, female postmating responses are known that may increase the number or weight of eggs laid, delay remating for several days, lead to an earlier death (Lehmann, 2012) or alter food intake (Lehmann & Lehmann, 2016). However, relatively little is known about the proteins in the spermatophore. One proposed function is the ability of the male to affect female behaviour through chemical manipulations (Gwynne, 2008), although this still lacks experimental support.

Recent studies propose the application of proteomics to the field of evolutionary ecology (Diz *et al.*, 2012; Baer & Millar, 2016) and behavioural ecology (Valcu & Kempenaers, 2015). The main reason for this is that the proteome is closer to the phenotype than the genome and so it might be more relevant for identifying functional correlations and hence help with our understanding of the underlying natural selection and adaptation. Internal fertilizing animals are known for the transfer of seminal fluids along with the sperm cells during mating. These seminal fluids are produced in accessory glands (Gillott, 2003; Poiani, 2006) and are rich in seminal fluid proteins (SFP). Such SFPs are found in the ejaculate of several insect species (Avila *et al.*, 2011). Accordingly, in the present study, we investigate the protein composition of the complex bushcricket spermatophore.

An understanding of the functional significance of the spermatophore is hindered by the limited knowledge about the content of the spermatophylax. As expected, water is the major component of the spermatophore (Bowen *et al.*, 1984; Gwynne, 1988; Wedell & Ritchie, 2004; Jarrige *et al.*, 2015). Specialized substances in the ampulla of a bushcricket are carotenoids (Heller *et al.*, 2000), which are known to act as antioxidant substances that scavenge free radicals (Costantini *et al.*, 2010), thereby potentially protecting the sperm from oxidative stress. Amino acids can also be recovered as free units, particularly glycine (Heller *et al.*, 1998). In crickets, glycine is known to affect the gustatory response of the females, keeping them occupied with feeding, leading to the formulation of the 'Candymaker hypothesis' for the spermatophylax (Warwick *et al.*, 2009). Glycine is not only the smallest, but also one of the cheapest amino acids to produce (Akashi & Gojobori, 2002; Wagner, 2005; Heizer *et al.*, 2011), making its use a cost effective way of enticing females to feed on the spermatophylax and detering them from premature removal of the sperm containing ampulla.

Little is known about the proteins involved that make up the most of the spermatophore dry mass (Heller *et al.*, 1998). Previous studies report proteins being found both in the sperm-containing ampulla and the spermatophylax (Heller *et al.*, 1998; Marchini *et al.*, 2009), although these studies employ low resolution one-dimensional gel electrophoreses and are unable to separate out individual proteins.

Systematically, the closest relatives of bushcrickets are crickets, for which we have knowledge of some protein content in the ejaculate of two species of the genus *Allonemobius* (Andrés *et al.*, 2006, 2008; Braswell *et al.*, 2006; Marshall *et al.*, 2011) and *Teleogryllus oceanicus* (Simmons *et al.*, 2013, 2014). However, crickets and bushcrickets are reported to have diverged from each other around 250 million years ago (Song *et al.*, 2015). A key difference between bushcrickets and crickets is the presence of a spermatophylax in the majority of bushcrickets (Gwynne, 1995) and the general lack of such a spermatophylax in crickets, with a few exceptions, notably *Gryllodes sigillatus*, for which the spermatophylax but not the ampulla proteome is described (Pauchet *et al.*, 2015). We still have a restricted knowledge of the protein composition in bushcricket spermatophores and especially for differences between protein patterns in the ampulla and the spermatophylax.

Based on their structure and function, protein types are very likely to differ between the ampulla and spermatophylax. Proteins transferred in the ampulla enter the female via a spermathecal duct, which leads to the spermatheca. A large proportion of these proteins may be involved in sperm transfer and consequently function to protect or maintain the male's sperm as in other insects (Avila *et al.*, 2011). By contrast, the proteins of the spermatophylax are ingested by the females, entering the gut. This may allow females the possibility of digesting and breaking down male-derived proteins (Gwynne, 2008). Males might add protective proteins that have the capacity to counter this degrading effect, such as the serine protease inhibitor found in *Gryllodes* (Pauchet *et al.*, 2015). A biochemical alternative for males would be to add prohormones, which are translated into the effector substance by female digestion.

The 'cost minimization principle' suggests that species minimize the cost of protein biosynthesis (Seligmann, 2003; Heizer *et al.*, 2011). Three aspects can be predicted to make proteins costly. First, proteins are made of amino acids, which contain a substantial amount of nitrogen as a result of the amino group. Nitrogen is a restricted source for many organisms, including the herbivore bushcricket investigated in the present study, because the nitrogen content of plants is much less than that of phytophagous insects (Awmack & Leather, 2002; Chown & Nicolson, 2004). Furthermore, nitrogen limitation can alter macromolecular elemental composition and bias the concentration of proteins (Elser *et al.*, 2011). Second, metabolic costs are higher for larger proteins simply because adding more amino acid requires more energy (Akashi & Gojobori, 2002; Bragg & Wagner, 2009; Smith & Chapman, 2010). Third, protein diversity itself can be expected to be costly to maintain. A greater number of proteins generally requires more genes, and/or post-transcriptional modifications, increasing both structural complexity and energy requirements (Lynch & Marinov, 2015; O'Brien *et al.*, 2016).

The number of different proteins in the ampulla could be expected to be within the range found in other insects (Avila *et al.*, 2011; South *et al.*, 2011; Boes *et al.*, 2014; Scolari *et al.*, 2016). By contrast, based on the cost assumptions and our

knowledge of the bushcricket spermatophore (Lehmann, 2012), we provide two alternative hypotheses for protein numbers and protein diversity in the bushcricket spermatophylax:

The first hypothesis comprises 'the cheap spermatophylax hypothesis'. If the bushcricket spermatophylax exists to protect the ampulla and deter females from removing it until successful sperm transfer, then males may be selected to produce the cheapest spermatophylax possible. Under such a cost reduction scenario, we might expect the spermatophylax to have low protein diversity, as well as more proteins with smaller mass.

The second hypothesis comprises 'the expensive spermato-phylax hypothesis'. Here, if the spermatophylax also helps to chemically influence female behaviour to favour the male's fit-ness, we can expect many different proteins, including those of high molecular mass, in the spermatophylax.

The present study aims to test these two hypotheses by exam-ining protein composition and diversity of the spermatophore in a bushcricket.

Materials and methods

Bushcrickets

For the experiments, the herbivorous bushcricket species *Poecilimon ampliatus* (BRUNNER VON WATTENWYL, 1878) was used. The genus *Poecilimon* has several species that are well studied for evolutionary and ecological questions. Our study species has a moderately large spermatophore (G. U. C. Lehmann & A. W. Lehmann, unpublished data) and is well studied with respect to acoustic ecology (Lehmann *et al.*, 2007; Strauß *et al.*, 2012, 2014) and spermatophore investment (Lehmann & Lehmann, 2016).

In June 2014, 200 individuals were caught as nymphs in Slovenia (Gabrče 45°32′ N, 13°58′ E). All animals were trans-ferred to the laboratory at the Humboldt University Berlin, Germany and individually housed in 175-mL transparent plas-tic vials (*Drosophila* rearing boxes; Greiner Bio-one GmbH, Germany; www.GreinerBioone.com). We maintained their natural light/dark photocycle LD 14 : 10 h and normal habitat temperature (22 °C). They were provided with an *ad libitum* food mixture of *Taraxacum* leaves and flowers and bee pollen from a healthfood store.

Mating experiments to obtain probes

After reaching sexual maturity, 10 males and 10 females were paired for mating in gauze cages. Because relative sper-matophore mass varies with male age (Lehmann & Lehmann, 2009; G. U. C. Lehmann & A. W. Lehmann, unpublished data for *P. ampliatus*), we used males of either 5, 7, 11 or 13 days after the final moult. Matings were observed and the spermatophore from each of the 10 matings was removed from females using forceps immediately after attachment. Both components of the spermatophore were separated with forceps, stored individually in cryotubes and immediately frozen using dry ice (at −80 °C).

Sample preparation and two-dimensional gel electrophoresis (2-DE) of spermatophylax and ampulla proteins

From the 10 matings, we selected four spermatophylaces and ampullae for 2-DE, representing each one male of either 5, 7, 11 or 13 days of age. Six volumes of sample preparation buffer (6 M urea, 3 M thiourea, 70 mM dithiothreitol) were added to the four individual spermatophylaces or ampullae, followed by five freeze/thaw cycles. After incubation for 30 min at room temperature, samples were centrifuged for 45 min at 15 000 *g* and the supernatant was used for determination of the protein concentration (Bradford, 1976).

The Proteome Factory (Berlin, Germany) performed 2-DE based on the technique of Klose & Kobalz (1995). For the analysis, 100 μg of protein was mixed with 2% ampholytes (pH 2–11) and applied to vertical rod gels (9 M urea, 4% acry-lamide, 0.3% piperazine diacrylamide, 5% glycerol, 0.06% *N,N,N',N'*-tetramethylethane-1,2-diamine (TEMED) and 4% carrier ampholytes (pH 2–11), 0.02% ammonium persulphate) for isoelectric focusing at 8820 Vh in the first dimension. After focusing, the isoelectric focused (IEF) gels were incu-bated in equilibration buffer, containing 125 mM Tris/phosphate (pH 6.8), 40% glycerol, 65 mM dithiothreitol and 3% sodium dodecyl sulphate (SDS) for 10 min and subsequently frozen at −80 °C. The second dimension SDS-polyacrylamide gel electrophoresis (PAGE) (23 × 30 × 0.1 cm) comprised 375 mM Tris/HCl buffer (pH 8.8), 15% acrylamide, 0.2% bisacrylamide, 0.1% SDS and 0.03% TEMED. After thawing, the equilibrated IEF gels were immediately applied to SDS-PAGE gels. Elec-trophoresis was performed using 140 mA for 5.5 h until the front reached the end of the gel. After 2-DE separation, the gels were stained with FireSilver (PS2001; Proteome Factory, Germany) or with Periodic acid-Schiff staining (Zacharius *et al.*, 1969; Kapitany & Zebrowski, 1973). The fixed and 3 × distilled water washed gel was treated with oxidizing reagent (1% periodic acid in 3% acetic acid for 50 min) and subsequently washed six times with water. The gel was then stained with Schiff's reagent for 50 min and the staining was stabilized with 0.75% metasulphite in 10 mM HCl for 1 h, followed by three washes in water.

Protein identification by liquid chromatography (LC)/mass spectrometry (MS)

Protein identification using nano-LC-electrospray ionization (ESI)-tandem MS (MS/MS) was performed by the Proteome Factory. To prepare the samples for the MS experiments, protein spots (from the four 2-DE gels) or protein preparation in urea from the remaining six spermatophores were utilized. Spots were shrunk/swollen in 60% acetonitrile (ACN) w/50 mM triethylammonium bicarbonate (TEAB)/100 mM TEAB buffer in three cycles for 20 min each. During swelling of the spots, reduction of disulphide was performed by the addition of 5 mM tris(2-carboxyethyl)phosphine (TCEP) and subsequent alkylation by the addition of 10 mM iodoacetamide (IAA) in the dark. Proteins in urea were reduced by addition of 5 mM TCEP for 20 min and then 10 mM IAA was added to alkylate the liberated cysteine residues in a dark environment. For the spots,

200 ng of trypsin in 20 µL of 10% ACN in 50 mm TEAB was used for in-gel proteolysis overnight at 35 °C. After proteolysis, peptides were acidified by the addition of 0.5 volumes of 2% formic acid (FA), resulting in 6.6% ACN and 0.6% FA.

The MS system consisted of an Agilent 1100 nanoLC system (Agilent, Germany), PicoTip electrospray emitter (New Objective, Woburn, Massachusetts) and an Orbitrap XL or LTQ-FT Ultra mass spectrometer (ThermoFisher, Germany). Acidified peptides from a single spot were applied to nano-LC-ESI-MS/MS. Peptides were trapped and desalted on an enrichment column (Zorbax SB C18, 0.3 × 5 mm; Agilent) for 5 min using 2.5% ACN/0.5% formic acid as eluent, and then peptides were separated on a Zorbax 300 SB C18, 75 µm × 150 mm column (Agilent) using an ACN/0.1% formic acid gradient from 5% to 35% ACN within 40 min. MS/MS spectra were recorded data-dependently by the mass spectrometer in accordance with the manufacturer's instructions. Identification of proteins was performed by an MS/MS ion search with the Mascot search engine (Matrix Science, U.K.). The ion charge in the search parameters for ions from ESI-MS/MS data acquisition was set to 1+, 2+ or 3+.

Further search parameters were: carbamidomethylation (fixed at C), deamidation (variable at N or Q) and oxidation (variable at M). These parameters were chosen with respect to sample preparation and common modification events. Different types of databases were used in the experiments: the NCBInr database (https://www.ncbi.nlm.nih.gov/refseq) for undirected protein identification in various species and an NCBInr-derived Orthoptera database containing 21 247 protein entries (January 2016) from various species. The Mascot search results were filtered by selecting $P < 0.05$ and an ion score > 20. Proteomic data of the tandem MS/MS search (raw data, peak lists and search files) are stored in accordance with the requirements of the ProteomeXchange Consortium (Vizcaíno *et al.*, 2014) and documented in the jPOSTrepo (https://repository.jpostdb.org; Okuda *et al.*, 2017) with the accession numbers: Ampulla spots 17, 18, 19: JPOSTID: JPST000190 PXID: PXD005024; Spermatophylax spots 1–16: JPOSTID: JPST000189 PXID: PXD005023. Ampulla Spots 9, 10, 11: JPOSTID: JPST000194 PXID: PXD005030; Ampulla liquid sample: JPOSTID: JPST000193 PXID: PXD005029: Spermatophylax liquid sample: JPOSTID: JPST000191 PXID: PXD005027.

De novo *sequencing with peaks*

Because neither the NCBInr database, nor the NCBInr-derived database contain actual bushcricket protein sequences, the identification had to rely on homologous sequences from other species, which could result in a failure to identify bushcricket proteins. To overcome this limitation, the MS datasets were used for *de novo* sequencing at a peptide level. The LC–MS/MS data files acquired in high resolution mode were used for analysis using PEAKS, version 7.5 (Bioinformatics Solutions Inc., Canada). The data refined parameters were: precursor correction, no additional filtering or merging of data. *De novo* parameters: parent mass error tolerance 3 ppm, fragment mass error tolerance 0.02 Da. The modifications selected were identical to

the Mascot database searches. The NCBInr-derived Orthoptera database was used for error-tolerant *de novo* peptide matching. Peptide candidates with a calculated false discovery rate < 0.1% were taken into account.

Results

Protein content

A spermatophore of *P. ampliatus* contains (mean ± SD) 5.17 ± 1.34 mg of protein ($n = 4$). Within the spermatophore, the nutritious spermatophylax has more than double the amount of protein (3.57 ± 0.67 mg, $n = 4$) than the ampulla (1.60 ± 0.91 mg). The fresh spermatophore mass was 93.33 ± 23.50 mg ($n = 4$). From other probes of this species, we know that 85% of the spermatophore is water. The remaining 15% of the spermatophore comprises 14 mg dry mass. Using this simple approximation, the 5.17 mg of protein in a spermatophore can be calculated as comprising 36% of the dry mass. As such, the protein fraction forms a major part of the spermatophore dry mass. We did not detect an increase in protein content with male age for the spermatophylax. By contrast, the protein mass in the ampulla was higher in the 13-day-old male (2.93 mg) than in the remaining three ampulla of younger males (ranging from 0.93 to 1.45 mg).

Two-dimensional electrophoresis to determine the protein composition of ampulla and spermatophylax

The 2-DE analysis that we applied has a capacity to separate up to 5000 spots (Klose & Kobalz, 1995). Both the ampulla and spermatophylax had complex protein compositions, with approximately 850 different protein spots in total. The protein composition differed remarkably between the ampulla and spermatophylax. The spot number was twice as high in the ampulla (number of spots: range 573–602 for four different males) compared with the spermatophylax (number of spots: range 285–312 for four different males) (Fig. 1).

Testing the four different males across the adult age range of 5–13 days revealed limited variation between individuals (Fig. 2). Different individuals produced similar results for both the ampulla and the spermatophylax in terms of spot number, location and size (Fig. 2). The size distributions of proteins show clear differences between the ampulla and the spermatophylax (Fig. 1). Approximately 50 spots had the same position within the ampulla and the spermatophylax 2-DE pattern, which may be indicative of the same protein. These common protein spots are restricted to the middle protein mass of 30–60 kDa, especially prominent around 40 kDa. The range of protein size in the ampulla is smaller, with mass varying between 20 and 120 kDa, compared with a range from under 14.5 to 130 kDa in the spermatophylax. The distribution in the ampulla is relatively even, with a majority of spots found above 20 and below 65 kDa. By contrast, within the spermatophylax, several large spots are
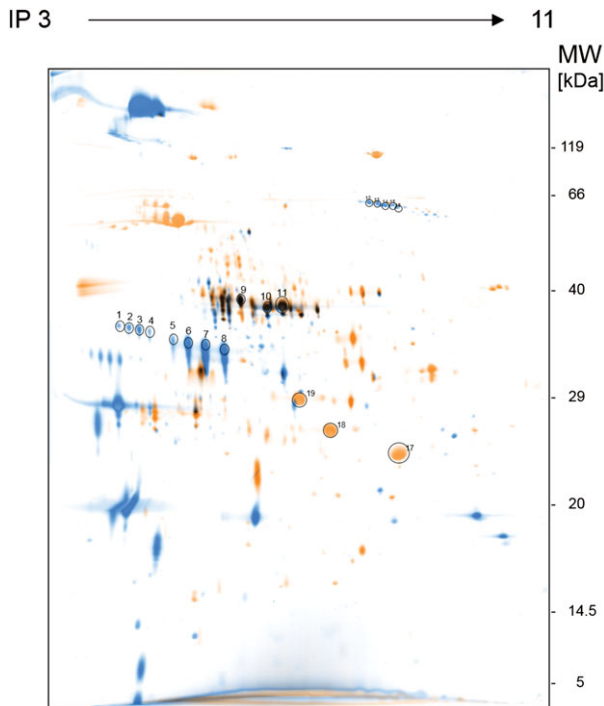
Fig. 1. Two-dimensional gel electrophoresis (2-DE) to separate the proteins of the spermatophore of the bushcricket *Poecilimon ampliatus*. The probes of the ampulla and the spermatophylax were analyzed independently. The probes were separated by isoelectric focusing in the first dimension on a pH gradient of 3–11 and in the second dimension by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (23 cm × 30 cm). After 2-DE separation, the gels were stained by Fire-Silver (PS2001; Proteome Factory). Molecular weight (kDa) is indicated at the right of the gel. The example shown represents an overlay of the ampulla and spermatophylax patterns of spermatophore number 8. Protein spots found in the ampulla are orange, those in the spermatophylax are blue spots, and those recovered in both the ampulla and the spermatophylax are black spots. Circled and numbered spots were used in the attempts to identify the proteins. [Colour figure can be viewed at wileyonlinelibrary.com].

Discussion

Protein diversity in the ampulla and the spermatophylax
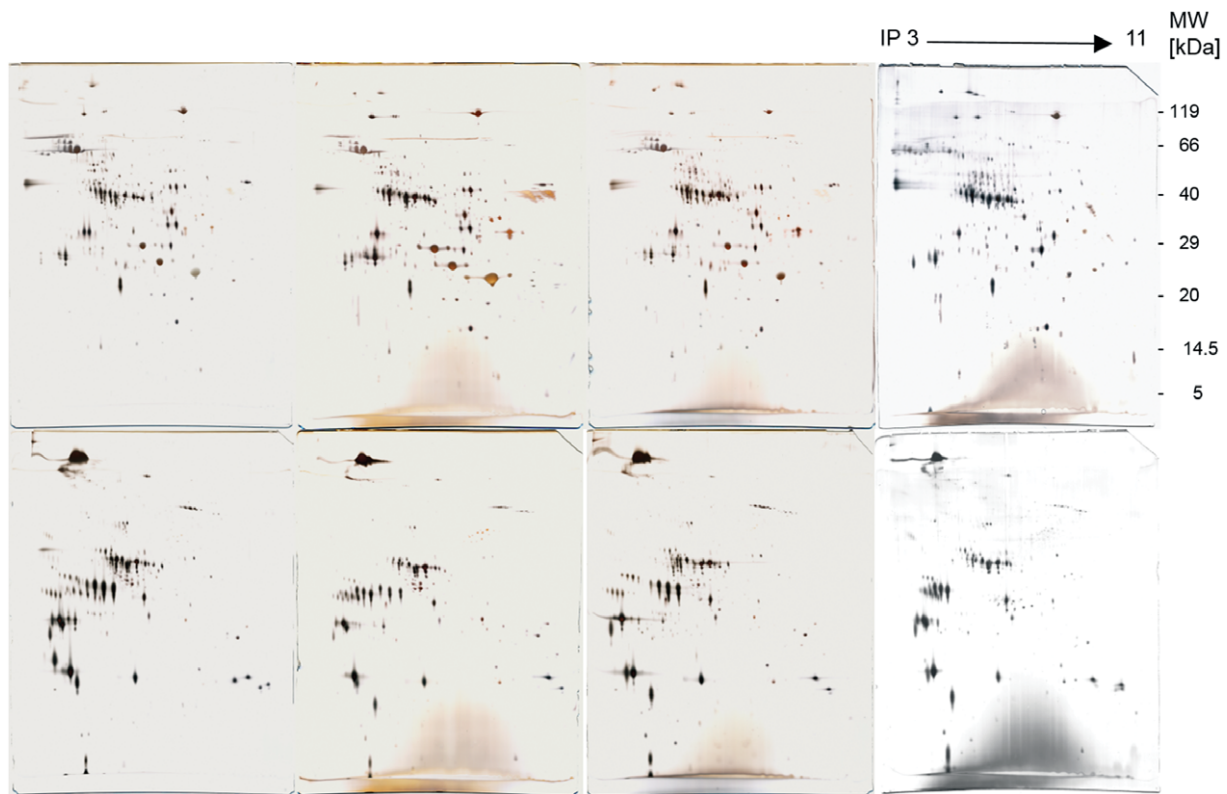
We find a large number of putative proteins in both the ampulla and the spermatophylax, many more than reported for other species of insects. The number of approximately 600 protein spots in the ampulla is higher than the 38–280 proteins detected in the ejaculate of other insect species (Avila *et al.*, 2011; South *et al.*, 2011; Boes *et al.*, 2014; Scolari *et al.*, 2016). Because methods vary in accuracy, the results might be not fully comparable with respect to the number of detected SFPs. Nonetheless, even with identical methods, the number of SFPs varies between 116 and 146 in three species of the genus *Drosophila* (Findlay *et al.*, 2008, 2009), indicating that protein number might be a rapidly evolving character. The approximately 300 proteins in the spermatophylax is an order of magnitude larger than the 30 proteins detected in the spermatophylax of the cricket *Gryllodes sigillatus* (Pauchet *et al.*, 2015).

An obvious feature from the 2-DE gels is that many of the putative proteins form a series of horizontal oriented spots. This might be indicative of protein speciation, caused by post-translational modifications at the same translated protein (Jungblut *et al.*, 1996). It is worth noting that such protein speciation cannot be detected in the genome, transcriptome or the bottom-up analyzed proteome. Taking protein speciation into account and tentatively assigning spots lined horizontally on the 2-DE gels to one canonical protein, the number of genes coding for proteins in the *P. ampliatus* spermatophore compartments can be estimated to be one-quarter of the spot numbers: approximately 100 in the ampulla and more than 50 in the spermatophylax. With lower detection accuracy, these modifications might go unnoticed. Protein composition profiles are consistent between individuals (Fig. 2), regardless of the age of the adult, which varies from 5 to 13 days.

Protein analysis

Even though we are unable to identify the kind of proteins in the bushcricket spermatophylax, it is reasonable to postulate that many diverse protein functions will be presented by the high number of different proteins. Our stored data will allow the identification of the proteomes after a complete genome sequence and annotation becomes available for *P. ampliatus* or a related species. This will also allow the investigation of potential manipulative proteins in the ejaculate, as shown for some proteins in *Drosophila* (Avila *et al.*, 2011).

Different protein species of the same protein may have different functions (Okkels *et al.*, 2004; Lange *et al.*, 2014) and different regulatory controls (Holland *et al.*, 2011; Thiede *et al.*, 2013). We provide clear evidence for glycosylation for many of the protein spots in the spermatophylax. Staining of glycoprotein is especially strong for the most intense spots on the 2-DE pattern, containing the largest amount of protein (Fig. 3). Glycosylation, comprising the binding of one or more sugar residues to the protein, is a common post-translational

exclusively found below 14.5 kDa and a very large protein is prominent at 130 kDa.

Attempts to identify proteins with mass spectrometry in 19 isolated spots from a 2-DE gel (for spot location, see Fig. 1) and in the total protein fraction were not successful. The Mascot searches and Peaks investigations were dominated by the identification of keratins, which are not known to be present in insects.

However, the reproducibility of the protein spot patterns from independent preparations from four different males (Fig. 2) reduces the probability of contamination as a cause for the spots. One explanation is that the digestion of the proteins was hindered by glycosylation and other post-translational modifications. Indeed, we confirmed post-translational modifications using glycostaining by identifying a great overlap between original protein spots and glycosylated protein spots within a bushcricket spermatophylax (Fig. 3).

Fig. 2. Two-dimensional gel electrophoresis of ampulla (A1−A4, top) and spermatophylax (P1−P4, bottom) from the four different individuals varying in age (male age: 13, 11, 7 and 5 days respectively). Gel size 23 × 30 cm. Proteins were stained with FireSilver (PS2001; Proteome Factory). [Colour figure can be viewed at wileyonlinelibrary.com].

modification, prominent in eukaryotes (Mann & Jensen, 2003; Walsh *et al.*, 2005), including insects (Vandenborre *et al.*, 2011). Protein glycosylation is reported in almost every protein class. Common groups in which glycosylation occurs include the membrane and transmembrane proteins; however, this might not apply to the spermatophylax, which contains no cells. Other major classes of glycosylated proteins are ribosomal proteins and metabolic enzymes, such as dehydrogenases, proteases and amylases (Walsh *et al.*, 2005). Proteases and amylases are of special interest: the first includes vitellogenin, an important glycoprotein for reproduction that is detected in most major insect lineages (Vandenborre *et al.*, 2011; Tufail *et al.*, 2014).

Another functionally highly significant class of proteins, often found to be glycosylated, is the class 'structural proteins'. Their inclusion would be important to stabilize the spermatophylax because it has a water content of approximately 80% (Heller *et al.*, 1998; G. U. C. Lehmann & A. W. Lehmann, unpublished data). This stabilizing function could also be accomplished by solutions of high-molecular weight glycoproteins that tend to be viscous and serve as lubricants, as in mucus (Li *et al.*, 2008).

It is not surprising that the protein compositions vary greatly between spermatophylax and ampulla in the bushcricket *P. ampliatus*. The sperm-protecting spermatophylax has a very different function from the sperm-containing ampulla in bushcrickets (Lehmann, 2012). The spermatophylax contains twice the protein mass as the ampulla, whereas the number of

types of proteins detected in the spermatophylax is around half of the number found in the ampulla. Protein sizes in the ampulla appears to be smaller than for the spermatophylax. This greater diversity of ampulla proteins is in line with the many different functions that it has to serve during sexual reproduction. Protein functions in ejaculates are known to be complex, supporting the transport and storage of sperm, protection from rival sperm, facilitation of egg−sperm interactions and changing female reproductive physiology (Pitnick *et al.*, 2009; Avila *et al.*, 2011). The spermatophylax has just two known main functions, protecting the ampulla from premature removal and influencing female postmating behaviour for the male's benefit. Therefore, the lower number of proteins in the spermatophylax may be reasonable given the more limited functions.

Hypotheses for spermatophylax protein diversity

Although the smaller number of protein spots in the spermatophylax compared with the ampulla in bushcrickets could be consistent with a 'cheap meal' function for the spermatophylax (Vahed, 2007; Gwynne, 2008), comparison with other insect data makes this less likely. The bushcricket spermatophylax appears to contain more types of proteins than the cricket spermatophylax (Pauchet *et al.*, 2015). It also has as many or more types of proteins as are found in the ejaculates/ampulla of
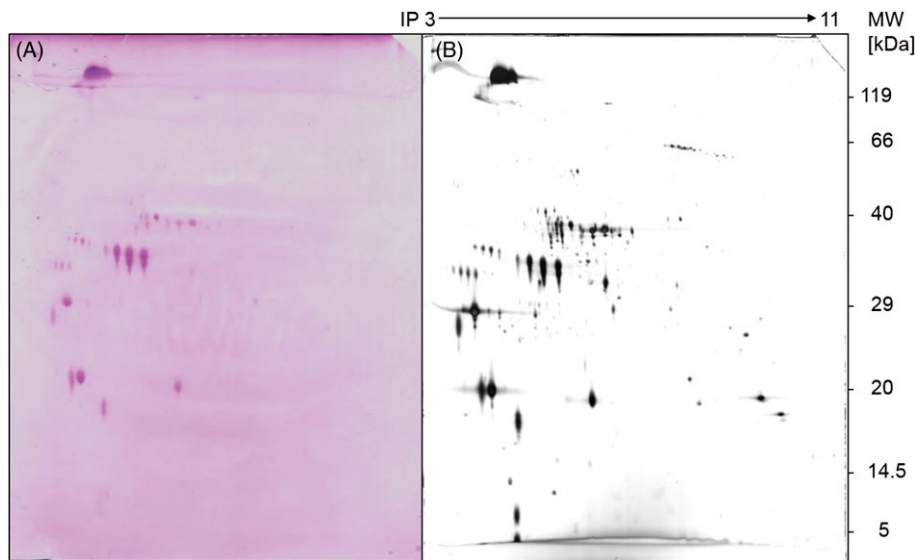
Fig. 3. Two-dimensional gel electrophoresis (2-DE) gel images of spermatophylax-derived proteins. (A) Glycoprotein-directed Periodic acid-Schiff staining of 2-DE gel. (B) The same sample after silver staining. [Colour figure can be viewed at wileyonlinelibrary.com].

other insect species (Avila *et al.*, 2011). Furthermore, the diversification and size distribution of proteins in the spermatophylax is higher than expected, indicating that functions other than just structural integrity and the provision of a 'cheap meal' are involved. Even if protein diversity in the bushcricket spermatophylax is more a consequence of post-translational modifications rather than genomic diversity, this weakens support for the 'cheap spermatophylax hypothesis' because post-translational modifications also require significant metabolic energy investment (Seligmann, 2003; Heizer *et al.*, 2011).

Currently, there is insufficient evidence to determine whether the expensive spermatophylax hypothesis fits the results. We have to wait until we are able to identify the detected proteins and especially understand their function. The pending sequencing of the complete genome of a bushcricket will help to improve protein identification of male-derived proteins. Nonetheless, the results of the present study are a promising addition to our understanding of the functional nature of male–female postmating interaction in bushcrickets, and demonstrate that their spermatophylax is neither a simple, nor a cheap 'gift'.

Acknowledgements

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