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Sclerotised spines in the female bursa associated with male's spermatophore production in cantharidin-producing false blister beetles

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ABSTRACT

Cantharidin is a defence chemical synthesised in only two beetle families Meloidae and Oedemeridae. In Meloidae, cantharidin is used as a defence chemical in eggs. However, in Oedemeridae the function of cantharidin remains unclear. Based on morphological comparison of female internal reproductive organs in 39 species of Oedemeridae, we found that some species have sclerotised spines in the bursa copulatrix (bursal spines), while others have no such spines. Molecular phylogenetic trees inferred from mitochondrial 16S and nuclear 28S rRNA gene sequences suggested multiple evolutionary origins of bursal spines from an ancestor without spines. In the species which lacked spines, males transferred small amounts of ejaculates to females; however, in species with spines, males transferred large spermatophores. Deposited spermatophores gradually disappeared in the bursa, probably owing to absorption. To compare the amounts of cantharidin in eggs laid by species with and without bursal spines, we constructed a new bioassay system using the small beetle *Mecynotarsus tenuipes* from the family Anthicidae. *M. tenuipes* individuals were attracted to droplets of cantharidin/acetone solution, and the level of attraction increased with cantharidin concentration. This bioassay demonstrated that the eggs of *Nacerdes caudata* and *N. katoi*, both of which species have conspicuous bursal spines, contain more cantharidin than the eggs of *N. waterhousei*, which lacks spines. In the former species, males transfer large spermatophores to the female, and spermatophores are eventually broken down and digested within the female's spiny bursa. Thus, females with bursal spines may be able to provide more cantharidin to their eggs.

1. Introduction

Nuptial gifts are food or items that are transferred between mates in several groups of animals, generally from males to females. Nuptial gifts are used by recipients for reproduction and to maintain physical condition (Boggs, 1995; Vahed, 1998; Gwynne, 2008). Lewis et al. (2014) redefined nuptial gifts as materials (beyond the obligatory gametes) provided by a donor to a recipient during courtship or copulation in order to improve donor fitness, and divided them into four types: exogenous oral gifts, endogenous oral gifts, endogenous genital gifts, and endogenous transdermal gifts. Exogenous oral gifts are materials, such as nuptial prey (e.g., Thornhill, 1976; Cumming, 1994) or plant seeds (e.g., Albo and Costa, 2010), collected by donors. Such gifts may improve mating success, copulation duration, and the quantity of sperm transferred by donors. Endogenous oral gifts comprise orally ingestible gifts derived from the donor's physiological systems, such as spermatophores that attach externally to the female genitalia (often called the spermatophylax) (Gwynne, 1984), haemolymph, or other body parts (Fedorka and Mousseau, 2004). Endogenous genital gifts are also manufactured by the donor (particularly by the donor's reproductive glands) and absorbed within the recipient's genital tract; they consist of spermatophores containing nutrients (e.g., Rooney and Lewis, 1999) or non-nutritive substances, such as immunostimulants or antibiotics (Poiani, 2006), water (Arnqvist et al., 2005), ions or minerals (Engebretson and Mason, 1980), or defensive compounds (Eisner and Meinwald, 1995). In some insects, leeches, squid, polychaetes, turbellarians, and acochlidan sea slugs, males traumatically inject their ejaculates and accessory gland fluids into their mates (Lange et al., 2013); such fluids are considered endogenous transdermal gifts.

Males of some insect species that use defence chemicals against predators store defensive compounds as nuptial gifts with their spermatophores; for example, pyrrolizidine alkaloids (Dussourd et al., 1988; Eisner and Meinwald, 1995) and cyanogenic glycosides (Cardoso and Gilbert, 2007). These chemicals are transferred from

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males to females through copulation, and allow females to protect their eggs from predation. Cantharidin, a toxic terpenoid compound, may also serve as a nuptial gift in some beetles in the same context as above. This defensive compound is produced by true blister beetles (Family Meloidae) and false blister beetles (Oedemeridae) (Carrel and Eisner, 1974; Carrel et al., 1986), and stored in their haemolymph and various other tissues at the larval and adult stages (Dixon et al., 1963; Carrel et al., 1986, 1993; Frenzel and Dettner, 1994; Holz et al., 1994). In true blister beetles, only males continue to synthesise cantharidin after adult eclosion, and newly synthesised cantharidin is moved to their reproductive accessory glands, then transported to the epididymis and vas deferens, and finally deposited and accumulated in the testes (Nikbakhtzadeh et al., 2007). Adult females synthesise cantharidin only during the larval period (Sierra et al., 1975; Carrel et al., 1993). Thus, adult females require supplemental cantharidin from male-derived spermatophores to defend their eggs successfully. After mating, cantharidin accumulates in the female spermatophoral receptacle (bursa copulatrix) and is allocated to eggs (Carrel et al., 1993; Nikbakhtzadeh et al., 2007, 2012).

In contrast, false blister beetle adult females, as well as adult males, can synthesise cantharidin (Carrel et al., 1986; Frenzel and Dettner, 1994). In most cases, field-caught females contain more cantharidin than males (Frenzel and Dettner, 1994; Abtahi et al., 2012). Holz et al. (1994) reported that no cantharidin, or only a very small amount of cantharidin, is transferred from males to females at mating, and thus that its contribution as a nuptial gift may be negligible in *Oedemera femorata*. Thus, it is still unclear whether male false blister beetles use cantharidin as a nuptial gift.

In our preliminary observations of male and female false blister beetle internal reproductive organs, we found that some species have conspicuous sclerotised spines within the female bursa copulatrix (bursal spines), while other species have no such spines. These bursal spines have not previously been described in these beetles. The females of most moths and butterflies (Lepidoptera) also have spines in the bursa, and these spines are thought to break and digest spermatophore envelopes (Cordero and Baixeras, 2015). In this study, therefore, we examined the possible functions of false blister beetle bursal spines in relation to cantharidin donation from males to females via spermatophores. First, we mapped the evolutionary patterns of bursal spines using phylogenetic trees inferred from mitochondrial 16S and nuclear 28S ribosomal DNA sequences from 11 genera and 39 species collected in Japan. Second, we observed the size and transfer process of spermatophores in the laboratory, and compared them with the characteristics of female bursal spines. Finally, using a new bioassay system with the small beetle *Mecynotarsus tenuipes* from the family Anthicidae, we compared the relative quantity of cantharidin contained in eggs laid by spiny and spineless species. *M. tenuipes* was attracted to a droplet of cantharidin/acetone solution to feed upon, and the number of individuals attracted increased with cantharidin concentration. Our working hypothesis is that females from species with bursal spines can digest larger spermatophores and provide more cantharidin to their eggs than females from species without spines. Spermatophores thereby serve as endogenous genital gifts according to the definition of Lewis et al. (2014).

2. Materials and methods

2.1. Morphological measurements and mating experiments

A total of 554 false blister beetle individuals, consisting of 11 genera and 39 species, were collected across Japan from March to June, 2012 to 2015, and reared at 25 ± 1 °C with a 14 h light and 10 h dark cycle. Within 3 days after collection, they were cooled at −20 °C for 10 min. After their maximum elytral lengths were measured to the nearest 0.1 mm using a binocular eyepiece (Leica MZFL3), internal reproductive organs were dissected in insect saline consisting of 0.9 g NaCl, 0.02 g $CaCl_2$, 0.02 g KCl, and 0.02 g $NaHCO_3$ in 100 mL water. A Nikon H550S stereomicroscope with a Nikon DS-L3 camera system was used to examine bursal spines, count the number of spines, and measure the length of spines. If the female carried a spermatophore, the major and minor axes of the spermatophore were measured under a Nikon H550S stereomicroscope with a Nikon DS-L3 camera system, and its volume was calculated as a spheroid.

Mating was observed in plastic vials (27 mm in diameter, 110 mm in depth) that contained a male and female. If mating occurred, copulation duration was recorded. Females were removed and dissected about 30 min after copulation to determine the presence or absence of the spermatophore. If present, its volume was calculated as mentioned above.

2.2. Molecular phylogenetic analysis

139 individuals from 11 genera and 39 species of false blister beetles were used for molecular phylogenetic analysis (Table S1). These beetles were collected across Japan from January to November, 2007 to 2015, and comprised all four tribes of the subfamily Oedemerinae distributed in Japan: Asclerini, Ditylini, Nacerdini, and Oedemerini. Collected beetles were preserved in 99.5% ethanol or as dried specimens. *Meloe coarctatus* of the family Meloidae was used as an outgroup species (Table S1).

Total genomic DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). For all 140 samples, fragments of mitochondrial 16S rRNA and nuclear 28S rRNA genes were amplified using Ex Taq® (TaKaRa, Tokyo, Japan) with three primer sets: 5′-GGGAGGAAAAGAAACTAAC-3′ (Ober, 2002) and 5′-CACGTACTCTTGAACTCTCTCT-3′ (Snäll et al., 2007) for the 28S D1 region, 5′-AGAGAGAGTTCAAGAGTACGTG-3′ (Snäll et al., 2007) and 5′-TTGGTCCGTGTTTCAAGACGGG-3′ (Snäll et al., 2007) for the 28S D2 region, and 5′-CGCCTGTTTAWCAAAAACAT-3′ (Hundsdoerfer et al., 2009) and 5′-CTCCGGTYTGAACTCAGAT CAAGT-3′ (Hundsdoerfer et al., 2009) for the 16S region. The PCR reaction mix (total volume 10 μL) contained 1.0 μL 10 × Ex Taq Buffer, 0.8 μL 25 mM dNTP mix, 0.5 μL each of the forward and reverse primers (10 pM), 0.05 μL Taq polymerase, 6.15 μL distilled deionized water, and 1.0 μL template DNA. PCR was carried out using a T100™ thermal cycler (Bio-Rad, Hercules, CA, USA). PCR protocols were as follows: for the 28S rRNA gene, an initial 3-min denaturing step at 93 °C, 35 cycles of 15 s at 98 °C, 30 s at 57 °C, and 40 s at 72 °C, with a final 3-min extension at 72 °C (Lopez-Vaamonde et al., 2001); and for the 16S rRNA gene, an initial 2-min denaturing step at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 53 °C, and 80 s at 72 °C, with a final 5-min extension at 72 °C. PCR products were purified with illustra™ ExoProStar™ 1-Step (GE Healthcare, Buckinghamshire, UK) and sequenced using BigDye® Terminator ver. 3.1 (Applied Biosystems, Foster City, CA, USA) on an ABI 3130xl Genetic Analyzer (Applied Biosystems). Direct sequencing data were aligned to 448 bp 16S and 805 bp 28S rRNA sequences using MEGA5 (Tamura et al., 2011) (For accession numbers in GenBank, see Table S1). Phylogenetic analyses were performed using MEGA5 with the neighbour-joining (NJ) method based on p-distance and maximum likelihood (ML) estimation based on Kimura's 2-parameter model with gamma-distributed rates (28S), the Tamura-Nei model with gamma-distributed rates and invariant sites (16S), and the General Time Reversible model with gamma-distributed rates and invariant sites (28S+16S) (Tamura et al., 2011). The best-fit nucleotide substitution model was estimated based on the Bayesian Information Criterion (BIC: Schwarz, 1978) using MEGA5 (Tamura et al., 2011).