Carotenoids in the spermatophores of bushcrickets (Orthoptera: Ephippigerinae)

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During mating male bushcrickets transfer large spermatophores, which have been demonstrated to play an important role in female nutrition and egg production. Until now only relatively unspecific substances such as water and proteins were known to be present within these spermatophores. We found that in the bushcricket *Ephippiger zelleri* the spermatophores contain substantial amounts of carotenoids (mainly lutein and zeaxanthin) that are also found in the eggs of this species. Carotenoids are well known for their positive effects on survival and reproduction in animals. This is the first example, to our knowledge, where such specific vitamin-like substances were found to be transferred from male to female during mating.

Keywords: nuptial gift; lutein; zeaxanthin; *Ephippiger*; Tettigonioidea

1. INTRODUCTION

In many insect species, males transfer not only sperm but also other materials to females during courtship and copulation. These materials are classified as nuptial gifts when they exert positive effects on females or offspring. Nuptial gifts often consist of materials produced by male glands (for examples see Thornhill & Alcock 1983; Vahed 1998) and are absorbed within the reproductive or gastrointestinal tract of the females. Positive effects produced by nuptial gifts include protection of females or eggs by transferred chemicals such as cantharidin (e.g. Dussourd *et al.* 1988; Schütz & Dettner 1992; Eisner *et al.* 1996), increased egg number (e.g. Simmons 1990), increased offspring fitness (e.g. Reinhold 1999) or increased female life span (e.g. Brown 1997).

In some of the latter cases these nutritional effects may be based on the large amount of material transferred, in others, however, obviously small amounts of very specialized substances seem to be involved (Brown 1997). The kinds of substances responsible for these effects, however, are largely unknown. Generally, the knowledge of the composition of the glandular nuptial gifts is relatively poor. The few analyses have concentrated mainly on elemental, amino-acid and protein composition (Fleck *et al.* 1996; Heller *et al.* 1998). Specialized substances have not been detected except for urates, which have been found to be a significant source of male-derived nitrogen (Mullins *et al.* 1992; Schal & Bell 1982) in cockroaches, and some inorganic substances such as Zn²⁺ (Engebretson & Mason 1980) and Na⁺ (e.g. Smedley & Eisner 1996) in Lepidoptera.

However, there are many examples indicating that small amounts of highly specialized and effective substances are transferred during mating for purposes other than nutrition. The most prominent substances are anti-aphrodisiacs and oviposition stimulants, which are

found in nuptial gifts of many insect species (for a review, see Eberhard 1996; Vahed 1998).

In bushcrickets and some species of crickets the males transfer a large spermatophore consisting of the sperm-containing ampulla and the sperm-free proteinaceous spermatophylax, which is consumed by the female after copulation. The gland products in the spermatophylax have been demonstrated to play an important role in female nutrition and egg production (for a review, see Gwynne 1997). The positive effects can often be mimicked by additional food but in some cases special substances may be involved (e.g. Gwynne 1988).

In the following we present the first example, to our knowledge, of highly specific substances, carotenoids, that are enriched and modified by the male and transferred via his spermatophore. The first clue that carotenoids are involved in this species was given by the yellow colour of the ampulla (figure 1). To study whether the carotenoids are also important for egg production we examined eggs of this species for the presence of carotenoids.

2. MATERIAL AND METHODS

First and second instar larvae of *Ephippiger zelleri*, ssp. *melisi* Baccetti, 1958 were collected in the Gran Sasso mountains in Italy, near the type locality of that subspecies (42°27' N, 13°18' E). The animals were kept singly and fed exclusively on leaves of *Taraxacum officinale* under laboratory conditions. About 20 days after their imaginal moult, males and females readily copulated when given the opportunity. Immediately after mating, the spermatophores were removed and frozen at -20 °C. Three virgin females were killed at an age of ca. 35 days; their eggs were removed and frozen at -20 °C.

Spermatophores and eggs were homogenized in a solution of chloroform and methanol (5 ml; 3:1) for 3 min (Ultra-Turrax homogenizer; Jahnke+Kunkel, Staufen, Breisgau, Germany). The homogenates were centrifuged at 3000 g for 10 min in order to separate the chloroform phase, containing the lipophilic compounds and therefore the carotenoids, from the aqueous phase. The aqueous phase was discarded and the chloroform phase was completely dried with nitrogen and rediluted in 100 µl ethanol. This solution was directly charged to a

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Figure 1. Female bushcricket, *Ephippiger zelleri*, with spermatophore. Note the yellow content of the ampulla containing the carotenoids.

high-pressure liquid chromatography (HPLC). After saponification, the carotenoids of *T. officinale* leaves were extracted using the method described by Britton (1995).

The carotenoid contents of spermatophores, eggs and leaves were analysed by an HPLC with attached diode-array detector (Jasco, Gro-Umstadt, Germany; RP-C30 column). The fluid phase was a linear gradient of solution A (81% methanol (MeOH), 15% methyl-*tert*-butyl ether (TBME), 4% H₂O) to solution B (6% MeOH, 90% TBME, 4% H₂O) within 90 min. For each measurement we injected 20 µl of the ethanolic carotenoid extracts. The absolute amounts of the different carotenoids were calculated from the peak areas of the chromatograms. The standard substances for the calibration of the HPLC were obtained from Sigma (Deisenhofen, Germany) (β-carotene, β-cryptoxanthin, lutein) and Merck (Darmstadt, Germany) (zeaxanthin).

3. RESULTS

The spermatophores ($n = 4$; mass of spermatophore 0.39 ± 0.025 g (mean $\pm$ s.d.), about 40% of male body weight) contained substantial amounts of two different carotenoids and small amounts of a third one: lutein (0.90 ± 0.61 µg), zeaxanthin (0.69 ± 0.41 µg) and β-cryptoxanthin (0.02 ± 0.0001 µg per spermatophore). The different carotenoids were identified by their characteristic retention time in the HPLC and their absorption pattern (figure 2). All eggs (about 90) found in females ready to start oviposition contained the same substances, but in a different ratio. Here, lutein (1.54 ± 0.69 µg per all eggs of a female) was dominant, followed by zeaxanthin (0.17 ± 0.06 µg) and β-cryptoxanthin (0.04 ± 0.01 µg) (figure 2). Extractions from *T. officinale* leaves contained only lutein (0.55 µg per g leaves (fresh mass); $n = 2$ extractions from 20 g leaves each) and β-carotene (0.28 µg per g

leaves). No zeaxanthin or β-cryptoxanthin was found (figure 2).

4. DISCUSSION

The discovery of carotenoids in the spermatophores of some bushcricket species is interesting for two reasons. First, it is the first example of complex organic molecules important for female survival and egg production transferred via a spermatophore. Carotenoids are thought to be very important in the diet of nearly all insects since the visual pigment chromophore retinal is derived from them and insects are unable to synthesize it (e.g. Bennett & White 1989). They may be an example of small amounts of important substances that are transferred during nuptial feeding.

Surprisingly, the carotenoids are not transferred within the large spermatophylax but within the much smaller ampulla. Possibly these fat-soluble molecules cannot be transported efficiently within the aqueous protein matrix of the spermatophylax. In bushcrickets the spermatophylax typically contains more than 85% water (Heller *et al.* 1998). In Lepidoptera and Coleoptera the highly specific substances are also assumed to be transported in the seminal fluid (Eisner *et al.* 1996). Moreover, the form of the transport could indicate a special function of the carotenoids. As well-known antioxidants (Felton & Summers 1995) they could protect the sperm in the ampulla. Some of the carotenoids may be transferred to the spermatheca and continue their protective function there.

Carotenoids in the spermatophore are certainly not restricted to *E. zelleri*. In some other species of the subfamily Ephippigerinae (e.g. *E. ephippiger* (photography in Tauscher 1986), *Ephippigerida taeniata* (K.-G. Heller,

Figure 2. Chromatograms and absorbance spectra (in milli-absorption units (mAU)). The chromatograms for (a) the food plant *Taraxacum officinale*, (b) a spermatophore and (c) eggs are presented. The extractions from *T. officinale* contained only lutein (A) and β -carotene (D). The chromatogram of the spermatophore shows lutein (A), zeaxanthin (B), β -cryptoxanthin (C) and β -carotene (D) (an asterisk marks the injection artefact). The eggs contained lutein (A), zeaxanthin (B), β -cryptoxanthin (C) and β -carotene (D). The absorbance spectra of the carotenoids in (d) *T. officinale*, (e) the spermatophore and (f) the eggs are also presented (absorbance spectra of β -cryptoxanthin (C) and β -carotene (D) of the spermatophore not shown).

unpublished observation)) a yellow colour within the ampulla has also been observed, indicating the presence of carotenoids.

A general antioxidant function may be the explanation for the high levels of carotenoids in the eggs of *Ephippiger* as well as in those of other animal species (Blount *et al.* 2000). In the locust *Schistocerca gregaria* (Dadd 1961) the carotenoid reserves in the egg seem to be sufficient to permit growth of the hatchlings even under a carotene-free diet. However, rearing the larvae without carotene results in smaller, lighter and less active animals. In addition, carotenoids are known to have other functions. However, for none of them is an exceptionally high use in *Ephippiger* obvious. *E. zelleri* has no bright coloration, which could be improved by high levels of carotenoids as found in guppies (Kodric-Brown 1989). The bushcrickets are not known to feed on highly toxic plants, which could require them to have high levels of antioxidant substances. They also do not store highly reactive substances as do butterflies (Rothschild *et al.* 1986). Carotenoids are also known to be necessary for the regulation of the biological clock (e.g. Claret & Volkoff 1992), however, there are no indications that *Ephippiger* needs more carotenoids for this purpose than other insects.

The second point of interest is the chemical transformation of carotenoids by the insects. The leaves of *Taraxacum* contain only lutein and β -carotene. Results of the present study show that males and females of *Ephippiger* can oxidize β -carotene to β -cryptoxanthin and zeaxanthin. This kind of chemical conversion has not been demonstrated in any other insects. Why different carotenoids are found in the spermatophore and in the eggs is still completely unknown. Comparing the amounts of the different carotenoids suggests that the zeaxanthin content of the eggs could be improved by feeding on the spermatophores. To obtain a similar amount of lutein as from the spermatophore a female would have to eat nearly 2 g of *Taraxacum* leaves. Under favourable food conditions this would not be difficult, since bushcrickets are able to eat the equivalent of their body mass per day (K.-G. Heller, unpublished observation). Thus the spermatophore seems to be an important source of some but not all of the carotenoid provitamins used for egg production.

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