ECOLOGY LETTERS

Ecology Letters, (2011) doi: 10.1111/j.1461-0248.2011.01653.x

LETTER

Effects of vitamin E and beta-carotene on sperm competitiveness

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Abstract

Sperm are particularly prone to oxidative damage because they generate reactive oxygen species (ROS), have a high polyunsaturated fat content and a reduced capacity to repair DNA damage. The dietary compounds vitamin E and beta-carotene are argued to have antioxidant properties that help to counter the damaging effects of excess ROS. Here in, we tested the post-copulatory consequences for male crickets (*Teleogryllus oceanicus*) of dietary intake of these two candidate antioxidants. During competitive fertilisation trials, vitamin E, but not beta-carotene, singularly enhanced sperm competitiveness. However, the diet combining a high vitamin E dose and beta-carotene produced males with the most competitive ejaculates, possibly due to the known ability of beta-carotene to recycle vitamin E. Our results provide support for the idea that these two common dietary compounds have interactive antioxidant properties *in vivo*, by affecting the outcomes of male reproductive success under competitive conditions.

Keywords

Antioxidants, fertility, infertility, male fertility, oxidative stress, reactive oxygen species, reproductive success, sexual selection, sperm, sperm competition.

Ecology letters (2011)

INTRODUCTION

Reactive oxygen species (ROS) are natural by-products of the metabolism of oxygen, and although important as signalling molecules and for immune responses when at low levels (Apel & Hirt 2004; Halliwell & Gutteridge 2007), excess ROS levels cause oxidative damage to cell membranes, and the RNA and DNA within the cell (Harman 1956; Beckman & Ames 1998). Because ROS react with other molecules in a non-targeting fashion, animals must employ sophisticated defence mechanisms to mitigate the production of ROS (Monaghan *et al.* 2009). Both enzymatic and non-enzymatic mechanisms are utilised to control ROS, primarily via the deployment of antioxidants (Nappi & Ottaviani 2000; Apel & Hirt 2004). In addition to endogenously produced antioxidants such as superoxide dismutase, catalase and glutathione peroxidase, a range of potentially effective antioxidants require ingestion. Dietary compounds, such as vitamin E (tocopherols), polyphenolic antioxidants (flavonoids), vitamin C (ascorbic acid) and carotenoids (carotenes and xanthophylls) are known to act in various ways to quench ROS (Catoni *et al.* 2008), at least *in vitro*. Furthermore, because dietary antioxidants are depleted once deployed and need to be replenished, it has been suggested that they might constitute a limiting factor in the expression and maintenance of traits tied to fitness and performance (von Schantz *et al.* 1999; Blount *et al.* 2001).

Male germ cells are particularly sensitive to oxidative damage. First, they are routinely exposed to ROS, given that they are themselves a site of significant ROS production via oxidative phosphorylation in the sperm mitochondria involved in motility, and because they develop within the highly metabolically active, hence ROS-leaking, testes (Taylor 2001). Second, male germ cells typically possess a high polyunsaturated fat content (making them a prime target of lipid peroxidation), they show low antioxidant capacity (tied to a reduced cytoplasm relative to other cells) and are transcriptionally silent (limiting their capacity to repair DNA damage) (Aitken & Graves 2002). Males that are unable to balance pro-oxidants and antioxidants may thus suffer infertility if sperm cells are damaged.

Several lines of evidence have emerged to support a role for dietary antioxidants, in particular vitamin E, in determining sperm quality. Much evidence comes from studies in humans (Fraga *et al.* 1991, 1996; Suleiman *et al.* 1996; Keskes-Ammar *et al.* 2003; Eskenazi *et al.* 2005). For example, oral supplementation of vitamin E in males exhibiting depressed sperm motility (asthenospermia) has been shown to have the dual effect of reducing levels of lipid peroxidation and improving sperm motility (Suleiman *et al.* 1996). And consistent with this finding, the concentration of vitamin E in the seminal plasma of males was reduced by over 30% in cigarette smokers compared to non-smokers, with smokers also exhibiting a 50% higher incidence of damage to sperm DNA (as gauged by a biomarker assessing the oxidative lesion of guanine) (Fraga *et al.* 1996). In rabbits, supplementation with vitamins C and E reduced the production of free radicals and improved semen quality (total sperm output, sperm concentration, ejaculate volume), with particularly strong effects on those rabbits supplemented exclusively with vitamin E (Yousef *et al.* 2003).

Due to their dual role as both pigments used in the expression of many sexually selected signals across animal taxa (McGraw & Ardia 2004; Blount 2004; Bertrand *et al.* 2006; Catoni *et al.* 2008) and their antioxidant potential, at least *in vitro* (Young & Lowe 2001), much empirical attention has focused on the role that carotenoids might play as antioxidants and honest signals of condition in animals (Catoni *et al.* 2008; Costantini & Møller 2008; Svensson & Wong 2011). Of these, two recent reviews have concluded that there is no, or at best only marginal, support for a general role of carotenoids as antioxidants *in vivo* (Young & Lowe 2001; Costantini & Møller 2008). Yet, several authors have identified positive associations between carotenoid rich sexual coloration and sperm quality (Locatello *et al.* 2006; Helfenstein *et al.* 2010), and have argued for a dualistic role of carotenoids in protecting sperm from oxidative damage and signalling male fertility (Blount *et al.* 2001). However, very little research has examined directly the possibility that carotenoids might serve antioxidant functions to sperm. In principle, carotenoids at least have the potential to protect the high polyunsaturated lipid content of sperm because they are fat

soluble (Catoni *et al.* 2008). Evidence for a role of carotenoids in sperm function comes from mallard ducks *Anas platyrhynchos*, where increases in plasma carotenoid concentration were associated with increases in sperm motility and velocity (Peters *et al.* 2004). Notably, the only study to date that has linked antioxidant intake directly to functional male fertility (the realised outcome of reproduction), used carotenoids as the putative antioxidant supplement (Pike *et al.* 2010). Specifically, Pike *et al.* (2010) showed that three-spined stickleback males fed higher levels of carotenoids had higher fertilisation success than males that were largely deprived of carotenoids, when provided with a clutch of unfertilised viable eggs under non-competitive mating conditions.

To date, the effects of antioxidant supplementation on male fertility have not been assessed, *in vivo*, under competitive reproductive conditions. This is striking because for most animal species, mating is an intensely competitive activity, resulting in large variance across males in realised reproductive success (Andersson 1994). Indeed, the females of many animal species typically mate with more than one male during a single reproductive bout (Simmons 2001), invoking intense post-copulatory sexual selection on the male ejaculate. In mating systems where males require high rates of spermatogenesis and highly motile sperm (with consequent higher ROS production) to maximise their reproductive fitness, Dowling & Simmons (2009) contended that differences among males in their ability to protect their sperm from oxidative damage will contribute to the variance in male fertilisation competitiveness under sperm competition.

Here in we investigate whether dietary supplementation with vitamin E alone, beta-carotene alone, or their combination, affects the competitive reproductive ability of males in the Australian cricket *Teleogryllus oceanicus*, an insect in which sperm competition is rife. Our use of vitamin E is motivated by the empirical support outlined above that implicates this compound as a likely antioxidant involved in maintaining sperm quality (Fraga *et al.* 1991, 1996; Suleiman *et al.* 1996; Keskes-Ammar *et al.* 2003; Eskenazi *et al.* 2005). Our use of beta-carotene is motivated by the findings of Pike *et al.* (2010), and by emerging evidence that antioxidants typically act in synergy in their effects (Catoni *et al.* 2008; Svensson & Wong 2011), and finally by the finding that beta-carotene is known to recycle vitamin E *in vitro* (Böhm *et al.* 1997), thereby enhancing the antioxidant potential of vitamin E.

MATERIAL AND METHODS

Cricket population and artificial diet preparation

Cricket hatchlings were sampled from a large (> 1000 individuals) outbred laboratory stock population originating from wild-type adults that were collected from a banana plantation in Carnarvon, Western Australia. Crickets in this stock population pass through *c.*four generations per year, and the population is augmented with around 50 wild caught females annually. These females will have mated with a minimum of between two to six males prior to their introduction to the laboratory population (Simmons & Beveridge 2010).

To control cricket dietary antioxidant intake, a completely synthetic diet was designed, based on Meikle & McFarlane (1965), in which all nutrients, carbohydrates and minerals were strictly controlled, such that all that varied between diet treatments was the level of vitamin E and the presence or absence of beta-carotene (see supplementary material for full diet preparation procedure). To test for independent main effects of each putative antioxidant as well as the interaction between the two compounds, a total of four diets was manufactured: (1) High vitamin E (0.05 mg g^{-1} synthetic diet), plus beta-carotene (0.1 mg g^{-1} synthetic diet); (2) High vitamin E (0.05 mg g^{-1} synthetic diet), no beta-carotene; (3) Low vitamin E (0.002 mg g^{-1} synthetic diet) plus beta-carotene (0.1 mg g^{-1} synthetic diet); and (4) Low vitamin E (0.002 mg g^{-1} synthetic diet), no beta-carotene. Vitamin E was never completely excluded from the diets as small levels are necessary for spermatogenesis in gryllid crickets (Meikle & McFarlane 1965). The level of beta-carotene equates to *c.*25% of the concentration naturally found in carrots (414 μg g^{-1} dry weight; Lee 1986).

To prevent compensation for a poor quality diet (lower putative antioxidant content) by higher food consumption, the amount of food the crickets were given was strictly controlled. A pilot experiment was conducted to estimate the amount of the highest quality diet (high vitamin E, plus beta-carotene) an individual cricket of a given size ate during a 24-h period. Crickets were divided into five size groups: ∼5 mm, ∼10 mm, ∼15 mm, nymphs in their penultimate instar, and adults. Food intake was measured in 10 crickets from each of the five size groups, and repeated over three days. From the smallest size group to adult crickets, individuals consumed on average (mean ± SD) 7 ± 2.3, 9 ± 3.7, 29 ± 20.7, 35 ± 29.8, 65 ± 27.2 mg/24 h. Based on these figures, the amount of food given to each size class was limited to: 7, 9, 29, 35 and 65 mg per day per cricket. These amounts were weighed out to the nearest mg on a balance (Mettler Toledo AG245, Mettler-Toledo, Zurich, Switzerland) and distributed every two days to experimental crickets as they developed. Any unconsumed food found in boxes was removed prior to new feeding.

Experimental crickets were housed in 5 L containers provided with egg cartons as shelter, *ad libitum* access to water, and housed at a constant temperature of 25 °C with a 12 : 12 h light : dark photoperiod. Cricket hatchlings were randomly allocated to one of the four synthetic diet treatments and were fed the same food throughout their development. Each of the four treatment groups, except the penultimate and adult crickets, were spread across three 5 L containers, each housing a maximum of 30 crickets at any time. Each treatment group thus started with 90 hatchlings. As crickets grew to the designated larger size, they were randomly transferred to one of the three containers in the next size class. Thus, the crickets of each treatment experienced one of 27 possible permutations of spatial environment during the first three stages of their development (three containers per treatment and three size classes, corresponding to a 2.7% chance of any two males sharing the same combination of containers throughout these stages of development. Crickets transferred from the containers holding the smallest size class were replaced with new hatchlings. To ensure virginity as adults, males and females were separated as penultimate instar nymphs and housed in separate 5 L containers. These containers never held more than 12 (mean of 7) crickets each at any given time of the experiment. All crickets were isolated on the day of their moult to adulthood and housed individually in 7 × 7 × 5 cm containers, where they continued to receive the dietary treatment at the appropriate dose scaled for one individual for the next 10 to 11 days. The period, during which males were held and fed in isolation, is the critical period during which males mature sexually; males do not court or mate for at least four days following eclosion to adulthood, and the quality of a male's ejaculate increases over the first two weeks of adult life (García-González & Simmons 2005).

Sperm competition assay and paternity assignment

The effect of diet on sperm competitiveness was tested by mating one focal experimental male and one standard male competitor with a single

standard female. Experimental males were wild type and had black eyes whereas all standard females and competitor males were homozygous autosomal recessive for the white-eye phenotype, a mutation which does not affect the fertility of males (Simmons *et al.* 2003). Paternity could thus be assigned to each of the two males that the female had mated with by determining the eye colour (black or white) of the offspring. Female and competitor males were sampled from a white-eye stock population (> 1000 individuals maintained under the same conditions as the wild-type stock population) as hatchlings and reared under the same general conditions as focal males, but were fed a standard diet of cat chow provided *ad libitum*. All focal and competitor males were 10 to 11-days-old at the time of mating whereas females ranged from 10–19 days of age (13 ± 2.3 days, mean ± SD). There was no significant difference in age between females across the four treatment groups (ANOVA, $F_{3,60} = 0.151$, $P = 0.929$). The second male mated with the female within 24 h after the first, and male mating order was randomised (focal male mated first and then the competitor male, and *vice versa*), allowing measures of both defensive (P1) and offensive (P2) sperm competition. To ensure complete transfer of the ejaculate by each male, each female was observed for 40 min to ensure that she did not remove the spermatophore. Females were then isolated with food and water, and allowed to oviposit for seven days in moist cotton pads. Cotton pads were incubated at 25 °C and one week after the onset of hatching, or when no more hatchlings emerged, all offspring were euthanised by freezing (−18 °C), counted and scored for eye colour under a stereoscope. Only the female identity was known at the time of paternity scoring (not the identity or treatment classifications of the males).

Statistical analyses

Our final sample size was 64 females that were successfully mated to both a focal male and a competitor male. The total number of hatchlings per clutch ranged between 24 and 237 with an average of 125 ± 46 (mean ± SD). To investigate the effect of the two putative antioxidants on reproductive success, we used a generalised linear model with a binomial error distribution and logit link function. Vitamin E, beta-carotene and mating order (first or second male to mate) were entered as main effects, the number of black eyed offspring (sired by the focal male) as the response variable, and the total number of offspring produced by the female as the binomial denominator. The data were overdispersed, so we used *F*-ratios to test for significance, as recommended by Crawley (1993). To better appreciate the biological effects we observed, we also calculated effect sizes (Pearson's r) and their 95% confidence intervals following the procedures outlined by Nakagawa & Cuthill (2007).

RESULTS

The mean proportion of offspring sired by focal males was 0.37 ± 0.39 (mean ± SD) and ranged from 0 to 1. Vitamin E significantly increased sperm competitiveness but beta-carotene did not (Table 1, Fig. 1 a,b). However, there was a significant interaction effect between vitamin E and beta-carotene (Table 1). Mating order affected paternity by skewing reproductive success towards the first male (P1) to mate with a female (Table 1, Fig. 1 a,b). The three-way interaction between vitamin E, beta-carotene and mating order was not significant ($F_{1,56} = 0.64$, $P = 0.426$), and was removed from the model. There was also no significant interaction effect between vitamin E and mating order ($F_{1,56} = 2.62$, $P = 0.111$) or between beta-carotene and mating order ($F_{1,56} = 1.65$, $P = 0.205$), so these interaction terms were also removed from the final model.

Table 1 Summary statistics from a generalised linear model examining the effects of putative dietary antioxidants on the competitive fertilisation success of male crickets

Factor	Deviance	d.f.	F	*P*	Effect size {95% CI}
Vitamin E	96 200	1	9.07	0.004	0.36 {0.12, 0.55}
Beta-carotene	6062	1	0.57	0.453	0.09 {−0.16, 0.33}
Mating order	85 720	1	8.08	0.006	0.35 {0.10, 0.53}
Vitamin E*beta-carotene	68 390	1	6.45	0.014	0.31 {0.07, 0.51}
Residual	579 200	59			

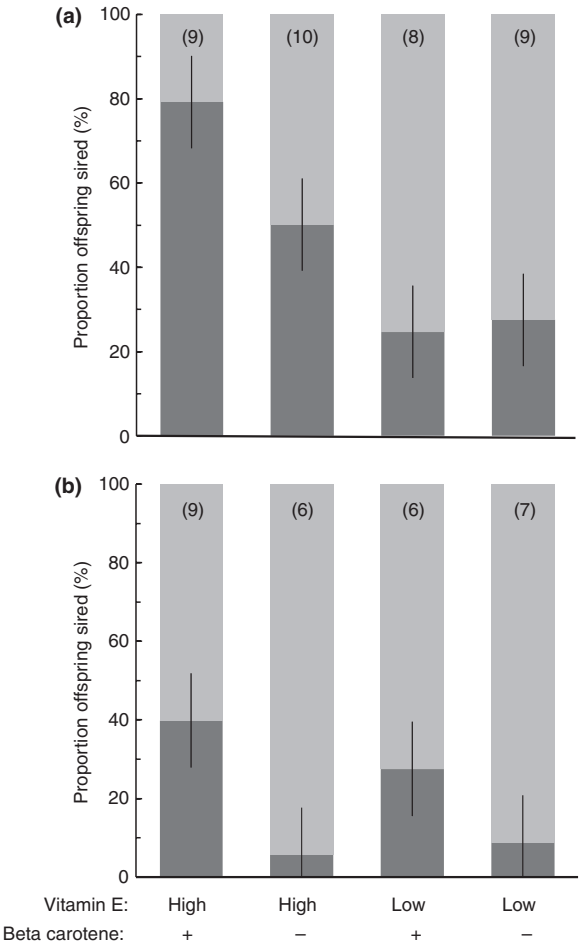

Figure 1 Proportion of offspring sired by focal (dark grey bars) and competitor males (light grey bars) in each of the four diet treatments in (a) the defensive P1 (focal male mated with female first) and (b) offensive P2 (competitor male mated with female first) position respectively. Black bars indicate standard deviations.

DISCUSSION

Here in we report that the availability of the putative dietary antioxidants vitamin E and beta-carotene make a major contribution to the reproductive outcomes for males under sperm competition in an invertebrate model. Male crickets reared on larger amounts of

vitamin E sired significantly more offspring than males receiving only trace amounts of vitamin E. Beta-carotene alone did not significantly influence sperm competitiveness, with the effect size being 75% lower than that of vitamin E. Notably, however, beta-carotene availability did interact significantly with vitamin E dosage to increase sperm competitiveness. Thus, our results directly link the dietary intake of both putative antioxidants to functional fertility under post-copulatory competitive conditions that are typical across animal species.

Functional fertility in males has been hypothesised to be influenced by the state of the antioxidant defence system because germ cells are exceptionally sensitive to oxidative damage (Blount *et al.* 2001). The protective properties of various putative antioxidants, however, are presumably not equal (Catoni *et al.* 2008). Vitamin E has been implicated as a vital antioxidant linked to components of fertility in humans and fowl (reviewed by Catoni *et al.* 2008) and our results demonstrate a significant positive effect of supplementary vitamin E on competitive reproductive success in this invertebrate model. The link between vitamin E and male fertility presumably stems from vitamin E's established capacity to protect the spermatozoa from oxidative damage, thereby ensuring their motility and fertilisation capacity (Fraga *et al.* 1996; Suleiman *et al.* 1996; Velando *et al.* 2008; Golzar Adabi *et al.* 2011). Carotenoids, on the other hand, have been suggested to be less powerful mitigators of oxidative stress in the absence of other antioxidants (Catoni *et al.* 2008). In accordance, our study shows that beta-carotene *per se* does not significantly influence sperm competitiveness in crickets. Neither could it substitute for vitamin E in spermatogenesis in the cricket *Acheta domesticus* (McFarlane 1992). However, we did reveal a significant interaction effect of vitamin E and beta-carotene; the particularly high proportion of offspring (~80%) sired by focal males when in the defensive (first to mate) position, and that were fed both high levels of vitamin E and beta-carotene, indicates that such a combination is far better than either vitamin E or beta-carotene alone. We acknowledge the possibility that vitamin E and beta-carotene may have exerted their effects in our study through physiological processes that were not linked to their antioxidant capacity. For instance, vitamin E is known to have effects on cellular functions that are unrelated to antioxidants, including inhibiting cell proliferation, and in modulating the expression of certain genes (Halliwell & Gutteridge 2007). While this alternative interpretation is possible, we believe that it is inconsistent with the accumulating evidence that vitamin E improves sperm quality by reducing oxidative damage to sperm. Thus, we believe that our results are more likely attributable to the antioxidant potential of dietary vitamin E, and the interaction effects of beta-carotene on vitamin E (Böhm *et al.* 1997).

After neutralising ROS, vitamin E is itself turned into a radical and other compounds (carotenoids and vitamin C) reduce the radicalised vitamin E (Vertuani *et al.* 2004), creating an effective and important recycling chain that improves the overall antioxidant system (Surai 2002). Such recycling is entirely consistent with the interaction effect we observed. It is noteworthy that in some species beta-carotene can be converted to vitamin A (von Lintig & Vogt 2004), which in mammals plays an important role in sperm production (Thompson *et al.* 1964). Thus beta-carotene could influence sperm competitiveness indirectly after conversion to vitamin A. We believe that this latter alternative is unlikely to hold true in insects, however, where vitamin A is important for vision, but plays no role in growth or reproduction (Bowers & McCay 1940; Kerkut & Gilbert 1985). Moreover, while there is evidence that beta-carotene can interact with vitamin E to enhance its beneficial properties, as observed here, the same is not expected for vitamin A. The enhanced post-copulatory competitiveness of males fed both vitamin E and beta-carotene is thus in line with our theoretical predictions that both compounds will work in synergy.

What is ultimately clear from our work is that the deprivation of two putative dietary antioxidants reduced the post-copulatory reproductive competitiveness of individual males, in a species where post-copulatory selection makes a pivotal contribution to the outcome of total male fitness. The observed differences in sperm competitiveness across treatments could be driven by three factors; differences in the proportion of dead sperm per ejaculate, differences in the hatching success of embryos that were the product of sperm with compromised DNA integrity, or differences in the sperm production capacity of males (Velando *et al.* 2008). We are unable to distinguish among these possibilities, nor do we know whether the resulting diet-mediated fertility outcomes are the result of direct sperm competition between males (Dowling & Simmons 2009) or cryptic choice processes exerted by females (Velando *et al.* 2008). Promiscuity may help protect females against the risk of mating with a male whose sperm has succumbed to oxidative stress, because males with undamaged sperm will secure the main share of paternity. Moreover, if the capacity to acquire, assimilate and utilise dietary antioxidants to combat oxidative stress varies between individuals (Surai 2002), and is heritable, females could increase the quality of their offspring by adopting not only pre-copulatory, but also post-copulatory mechanisms to avoid breeding with poor quality males. Such a process would be facilitated if ROS constituted a general constraint in the expression of life-history traits such as sexual signalling and fertility (Dowling & Simmons 2009).

Dowling & Simmons (2009) suggested that ROS may indeed provide a universal constraint in life history evolution, and empirical work highlighted in their review indicates that this may be the case. Some evidence exists for a link between sperm quality and carotenoid-based sexual ornaments (Pike *et al.* 2010), and it has also been suggested that scope exists for females to discriminate between males via other types of condition-dependant displays that may signal a male's ability to combat oxidative stress, such as courtship song (von Schantz *et al.* 1999). Consistent with this suggestion, Simmons *et al.* (2010) recently provided quantitative genetic evidence for a trade-off between sperm viability and courtship song in *T. oceanicus*, which in turn suggests that high energetic functional costs are associated with the expression of each trait. It is possible that this trade-off could be mediated by ROS production. These previous results, together with the evidence in our study that sperm competitiveness is linked to the intake of two putative antioxidants, suggest that research into the role that antioxidants play in mediating trade-offs in males, particularly over episodes of pre- and post-copulatory reproductive investment, will be a fruitful avenue for further research.

To summarise, we demonstrate the effect of two putative dietary antioxidants in promoting sperm competitiveness in an insect model. Our results show that males supplemented with vitamin E sired significantly more offspring, when under sperm competition, than did males with limited access to vitamin E. Our data are consistent with the suggestion that beta-carotene is also an important compound for male fitness, but indirectly, possibly through its ability to recycle spent vitamin E.

ACKNOWLEDGEMENT

This study was supported by funding from the Australian Research Council and the West Australian Centres of Excellence in Science and Innovation programme (to LWS), and a University of Western Australia Research Development Award (to DKD).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 Synthetic diet base recipe.

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Editor, Greg Grether
Manuscript received 28 February 2011
First decision made 30 March 2011
Manuscript accepted 21 May 2011