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RESEARCH ARTICLE

Dietary antioxidants, but not courtship effort, affect oxidative balance in the testes and muscles of crickets

Leigh W. Simmons*, Maxine Lovegrove and Samuel J. Lymbery

ABSTRACT

Recent interest has focused on the role of reactive oxygen species (ROS) as universal constraints in life-history evolution. Empirical studies have examined the oxidative costs of reproduction for females, with little work conducted on males. The male germline is thought to be particularly susceptible to oxidative damage because the testes, and the sperm themselves, can be prolific producers of ROS. We tested the hypothesis that protection of the male germline from oxidative damage represents a cost of reproduction for males. We fed male crickets, *Teleogryllus oceanicus*, with one of two experimental diets in which we manipulated the availability of dietary antioxidants, and we induced variation in their expenditure on courtship effort by manipulating access to females. We measured the total antioxidant capacity, levels of ROS production and the amount of oxidative damage to proteins in both testis and thoracic muscle tissues. Dietary antioxidants contributed to positive oxidative balance in both tissue types. Although the testes had greater antioxidant defences than muscle tissue, they also produced considerably higher levels of ROS and sustained higher levels of oxidative damage. Courtship effort had no impact on any measure of oxidative balance. Our data confirm that the male germline is especially susceptible to oxidative stress and that dietary antioxidants can alleviate this oxidative cost of reproduction.

KEY WORDS: Oxidative stress, Fertility, Life-history trade-off, Cost of reproduction, Ejaculate quality

INTRODUCTION

A fundamental principle in life-history theory is that the evolution of fitness traits should be constrained by the existence of trade-offs between them (Stearns, 1992). Organisms are thought to have a limited pool of nutritional resources that must be allocated to competing life-history traits such as somatic maintenance and reproduction, so that allocation of resources to one comes at a cost of allocation to the other (Roff and Fairbairn, 2007; Williams, 1966). Nutrient allocation trade-offs are the foundation of the 'disposable soma' theory of ageing (Kirkwood, 1977). While a longevity cost of increased reproduction is often reported from phenotypic studies, disruptions to the signalling pathways that regulate resource allocation to reproduction do not always extend lifespan, calling into question the notion of a simple resource allocation trade-off and sparking considerable debate over the physiological mechanism(s) underlying life-history trade-offs (Harshman and Zera, 2006; Maklakov and Immler, 2016; Partridge et al., 2005).

There has been a surge of interest in the role of reactive oxygen species (ROS) as universal constraints in life-history evolution (Costantini, 2008; Dowling and Simmons, 2009; Monaghan et al., 2009). ROS are free radicals produced as byproducts of oxidative phosphorylation, the fundamental biochemical process that generates the energy to sustain life. At low levels, ROS can be important signalling molecules (Halliwell and Gutteridge, 2007), but at high levels they will inflict oxidative damage on DNA, RNA, proteins and lipids (Beckman and Ames, 1998; Harman, 1956). Animals are equipped with a variety of defence mechanisms to counter oxidative damage from excess ROS. These include endogenously produced enzyme systems, such as the enzyme superoxide dismutase, and non-enzymatic mechanisms that involve antioxidants obtained in the diet such as flavonoids, carotenoids, and vitamins E and C (Catoni et al., 2008; Nappi and Ottaviani, 2000; Noguera et al., 2015). Whether an animal experiences oxidative damage will depend largely on the balance between ROS production on the one hand and antioxidant defences on the other (Monaghan et al., 2009), a balance that may be influenced by the acquisition of dietary antioxidants and their allocation to metabolically active tissues. Reproduction is costly, requiring an elevation in metabolic rate that has been thought to increase ROS production (Blount et al., 2016). Trade-offs between reproduction and lifespan might thus be mediated via an organism's ability to protect itself against damaging ROS that are the byproducts of reproduction.

There are studies that suggest ROS as players mediating life-history trade-offs. In decorated crickets, *Gryllodes sigillatus*, protein oxidation was found to be higher in the shorter lived sex (females) and to be negatively genetically correlated with lifespan in both males and females (Archer et al., 2012). Increased reproductive effort by female fruit flies, *Drosophila melanogaster*, and zebra finches, *Taeniopygia guttata*, is associated with an increased susceptibility to oxidative stress (Alonso-Alvarez et al., 2004; Salmon et al., 2001; Wang et al., 2001). A recent meta-analysis found a general positive effect of reproductive effort on the levels of oxidative damage in the tissues of female birds and mammals (Blount et al., 2016). These studies have focused predominantly on the oxidative cost of reproduction for females. The few studies that have also considered males have focused on parental provisioning in birds, with evidence from circulating levels of oxidative markers in blood suggesting that males can suffer an oxidative cost of raising offspring (Romero-Haro et al., 2016).

The male germline is thought to be particularly prone to oxidative stress. The testes are sites of rapid germ cell proliferation, they have high metabolic rates and they are significant producers of ROS (Aitken and Roman, 2008; Boussouar and Benahmed, 2004). Moreover, sperm are among the most metabolically active of cells (Tourmente and Roldan, 2015), producing their own ROS within

Centre for Evolutionary Biology, School of Biological Sciences (M092), The University of Western Australia, Crawley, WA 6009, Australia.

*Author for correspondence (leigh.simmons@uwa.edu.au)

L.W.S., 0000-0003-0562-1474

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the mitochondria that power their motility (Aitken, 1995). At the same time, sperm are thought to be more sensitive to oxidative damage than other cells because of their relatively high content of polyunsaturated fatty acids and the fact that they are transcriptionally silent and so unable to produce their own endogenous antioxidant defences (Aitken and Baker, 2004). Oxidative stress negatively affects sperm physiology (Rojas Mora et al., 2017a,b) and fertilization capacity (de Lamirande et al., 1997), and compromises DNA integrity with severe impacts on early embryo development (O et al., 2006, 1988). As such, oxidative stress is a major factor in male infertility (Aitken and Baker, 2004).

While the testes have a battery of endogenous defences to protect the germline from oxidative stress (Aitken and Roman, 2008), dietary antioxidants have also been implicated as limiting resources involved in male reproductive trade-offs (Blount et al., 2001; Dowling and Simmons, 2009). There is good evidence that dietary antioxidants can affect sperm quality (Ahmadi et al., 2016; Liu et al., 1997; Rengaraj and Ho Hong, 2015; Showell et al., 2011; Surai et al., 1997). For example, male three-spined sticklebacks, *Gasterosteus aculeatus*, fed a diet rich in carotenoids have increased fertilization success in non-competitive fertilization assays (Pike et al., 2010). While a direct role for dietary carotenoids as antioxidants has been questioned in birds (Costantini and Møller, 2008; Koch et al., 2018), work with insects suggest that carotenoids may contribute indirectly to antioxidant capacity.

Almbro et al. (2011) fed completely artificial diets containing vitamin E and/or beta-carotene to male crickets, *Teleogryllus oceanicus*, and examined their impact on competitive fertilization success. Males fed diets rich in vitamin E had greater competitive fertilization success than those fed diets with trace amounts of vitamin E. Dietary beta-carotene had no direct effect on competitive fertilization success; however, when it was combined with vitamin E it boosted competitive fertilization success by more than 50%. Beta-carotene recycles vitamin E from its oxidized state, thereby enhancing vitamin E's role as an antioxidant (Böhm et al., 1997). Thus, while beta-carotene may not be a dietary antioxidant per se, it may contribute indirectly to oxidative balance through its effects on vitamin E. Although Almbro et al. (2011) did not measure oxidative balance in their study, they argued that the effects of dietary antioxidants on competitive fertilization success most likely arose through their protection of the male germline from oxidative damage. Here, we tested this idea explicitly by measuring levels of oxidative stress in male crickets fed the same experimental diets.

Early studies of the oxidative costs of life-history traits failed to yield consistent support because they tended to focus on the measurement of antioxidant defences alone. However, what is important is the balance between antioxidant defences and pro-oxidants, and the level of oxidative damage that will result from an imbalance between these components of the system (Monaghan et al., 2009). In this study, we measured the levels of ROS, total antioxidant defences and the amount of protein damage in crickets fed antioxidant-rich and -poor diets. Our use of controlled access to chemically defined synthetic diets that varied only in the amount of dietary antioxidants allowed us to minimize the potential pitfalls associated with experimental animals having access to sufficient resources to maintain their oxidative balance (Metcalfe and Monaghan, 2013).

The costs of reproduction are often only seen when animals are forced to work harder, or under more extreme conditions, than their naturally selected optima (Beaulieu et al., 2015; Janssens and Stoks, 2018; Metcalfe and Monaghan, 2013). Moreover, recent work has begun to question the notion that increased metabolism necessarily generates increased ROS production, and studies that report more efficient mitochondrial function and reduced ROS production following increases in metabolic rate are accumulating (Speakman and Garratt, 2014; Zhang et al., 2018; Zhang and Hood, 2016). Thus, in our experimental design we also manipulated male pre-mating reproductive effort in order to determine how variation in energy expenditure affected oxidative balance.

Finally, we measured oxidative balance in multiple tissues because previous studies have found that the levels of oxidative stress can vary significantly across tissue types (Garratt et al., 2012; Speakman and Garratt, 2014). Specifically, we examined oxidative balance in the thoracic flight muscle and testes because these tissues contribute to male reproductive success through alternative mechanisms – pre-copulatory courtship and post-copulatory sperm competitiveness, respectively – and previous studies have suggested a trade-off between male courtship song and sperm quality (Simmons et al., 2010). Importantly, by measuring different tissue types, we were able to test the claim that the male germline is particularly sensitive to oxidative stress, and that its protection represents an important cost of reproduction for males (Maklakov and Immler, 2016).

MATERIALS AND METHODS

Teleogryllus oceanicus (Le Guillou 1841) used in these experiments were drawn from a large outbred laboratory stock that originated from Carnarvon, Western Australia, and is re-seeded annually with field-caught individuals. Males were isolated from the stock culture at the eighth nymphal instar, housed individually in plastic boxes (7 cm×7 cm×5 cm) and held in a constant temperature (26°C) room on a reversed 12 h:12 h light:dark cycle. Crickets were supplied with one of two completely synthetic experimental diets.

Diet manipulation

Synthetic diets were constructed in which protein, carbohydrate and mineral content were strictly controlled (for base recipe, see Almbro et al., 2011). Vitamin E (0.05 mg g^{-1}) and beta-carotene (0.1 mg g^{-1}) were added to the base recipe to produce an antioxidant-rich diet. Beta-carotene was completely excluded from the antioxidant-poor diet, which contained only trace amounts of vitamin E (0.002 mg g^{-1}). Vitamin E and beta-carotene have both been implicated as dietary antioxidants capable of quenching ROS (Catoni et al., 2008), and, as noted above, beta-carotene recycles vitamin E from its oxidized state, thereby enhancing vitamin E's role as an antioxidant (Böhm et al., 1997). Importantly, beta-carotene contributes to the role of vitamin E in promoting male competitive fertilization success (Almbro et al., 2011). Vitamin E was not completely excluded from the antioxidant-poor diet because it is essential for spermatogenesis in crickets (Meikle and McFarlane, 1965).

The quantity of food consumed by each cricket was standardized following the procedures outlined in Almbro et al. (2011); when first isolated, they received 29±1 mg day^{-1}, increasing to 35±1 mg day^{-1} when they moulted to the penultimate (ninth) nymphal instar, and to 65±1 mg day^{-1} when adult. Crickets were used in experiments 10 days after emerging as adults.

Courtship manipulation

Male crickets court females with a courtship song produced by stridulation, involving the rapid opening and closing of the forewings (Simmons et al., 2010). Studies of housefly and honeybee have found that engagement in flight has the effect of

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elevating oxidative damage and reducing lifespan (Williams et al., 2008; Yan and Sohal, 2000). In crickets, stridulation requires the use of the same thoracic flight muscles to drive forewing movement and, as with flight, requires an increase in metabolic rate (Bennet-Clark, 1970; Hack, 1998; Hoback and Wagner, 1997; Kavanagh, 1987). We therefore manipulated the expression of male courtship stridulation.

At 10 days of adult age, crickets from each diet treatment were allocated at random to one of two courtship treatments. Males were confined to one side of their container using a barrier placed across the middle of the container which had a grid of 5 mm diameter holes, spaced 5 mm apart, that allowed them to make physical contact with an individual placed on the opposite side. For courtship exposure, a single virgin female was placed in the opposite partition at the onset of the dark cycle and the pair left for 8 h. Males exposed to females interacted with them across the barrier and produced extended periods of courtship song. Because males were unable to physically mate, the treatment forced them to expend more heavily on courtship singing than they would naturally. Females were removed after 8 h and the males left alone until the following dark phase, when a second female was provided; the procedure was then repeated a third time. Males were thus exposed to females for three consecutive dark cycles. A second group of males were handled in the same way but females were not placed on the opposite side of the barrier. These males did not produce courtship song. Following the third dark cycle, crickets were frozen. A total of 114 crickets were available for our assays: 53 were fed dietary antioxidants (28 courtship exposed and 25 not exposed) and 61 were fed a diet with no antioxidants (30 courtship exposed and 31 not exposed).

Oxidative damage: protein carbonyl assay

Crickets were dissected and two tissues were removed: thoracic muscle and one testis. Each was placed in a separate 1.5 ml tube with 100 µl of lysis buffer. The tissue was homogenized using a micropestle (Interpath) and then spun for 15 min at 4°C and 15,000 rpm. The supernatant was removed into a fresh tube and stored at −80°C.

We used an assay to detect and quantify protein carbonyls, which are the products of protein oxidation and serve as markers of oxidative damage (Buss et al., 1997). First, the protein concentration of each sample was determined. Testes and muscle tissue lysates were diluted 1:10 in phosphate-buffered saline (PBS). Using the Qubit protein assay kit (Thermo Fisher Scientific) and following the manufacturer's instructions, the protein content of 2 µl of each sample was measured using the protein standards supplied in the kit and the Qubit 2.0 Fluorometer (Thermo Fisher Scientific). These samples were then diluted to 10 µg ml^{-1} and triplicate 100 µl aliquots adsorbed onto a 96-well plate (Greiner microlon 200, medium binding, flat bottom, clear polystyrene) overnight at 4°C using PBS as the blank. A protein carbonyl BSA standard curve was also prepared using 1 mg ml^{-1} oxidized and reduced BSA standards obtained from the Oxiselect protein carbonyl ELISA kit STA-310 (Jomar Life Research). These were diluted to 10 µg ml^{-1} in PBS and a series of carbonyl BSA standards were prepared by mixing the oxidized BSA and reduced BSA in the ratios specified in the manufacturer's instructions. This gave a standard curve of protein carbonyl concentrations from 0 to 7.5 nmol mg^{-1}. Triplicate 100 µl aliquots were adsorbed onto the plate alongside the samples and incubated overnight at 4°C. The wells were then washed 3 times with 250 µl PBS, emptying the wells after the last wash by tapping the plate onto a paper towel to absorb excess wash solution. A fresh working solution of dinitrophenylhydrazine (DNPH, Jomar Life Research) at 0.04 mg ml^{-1} was prepared in 2 mol l^{-1} hydrochloric acid and 100 µl was added to each well in the plate. The plate was incubated in the dark for 45 min at room temperature. The wells were washed with 250 µl PBS/ethanol (1:1, v/v) with incubation on an orbital shaker for 5 min a total of 5 times, aspirating between each wash and removing excess wash solution after the final wash as described above. The wells were washed a further 2 times with 250 µl PBS. Then, 200 µl blocking solution (5% skimmed milk powder in PBS) was added per well and incubated on an orbital shaker for 1–2 h at room temperature. Wells were washed 3 times with 250 µl 1× wash buffer (PBS, 0.1% Tween 20), removing excess wash solution after the final wash. Anti-DNP antibody produced in rabbit (Sigma) was diluted 1:10,000 in blocking solution and 100 µl was added per well and incubated on an orbital shaker for 1 h at room temperature. Wells were washed 3 times with 250 µl 1× wash buffer, removing excess wash solution after the final wash. Anti-rabbit IgG (whole molecule)-peroxidase (Sigma) was diluted 1:1000 in blocking solution and 100 µl was added per well and incubated on an orbital shaker for 1 h at room temperature. Wells were washed 5 times with 250 µl 1× wash buffer, removing excess wash solution after the final wash. After allowing the substrate to equilibrate to room temperature, 100 µl 1-step Ultra TMB-ELISA (Sigma) was added per well and incubated on an orbital shaker for 10 min at room temperature. The reaction was stopped with 100 µl per well 0.5 mol l^{-1} hydrochloric acid and the absorbance measured at 450 nm using a SpectraMax M5 plate reader (Molecular Devices). The protein carbonyl concentration of each triplicate sample was calculated from the standard curve using SoftMax Pro v5.4 software (Molecular Devices). Estimates of protein carbonyl concentration were significantly repeatable (testes: R=0.564, $F_{113,228}$=4.87, P<0.001; thoracic muscle: R=0.725, $F_{113,228}$=8.89, P<0.001), with variation among individuals being greater than variation among repeated measures of the same individual (measurement error). We took the average of our three replicates for downstream analysis.

Antioxidant capacity: ABTS decolourization assay

Antioxidant activity was measured using an ABTS radical cation decolourization assay described in Re et al. (1999). The assay measures the amount of pre-formed radical ABTS$^{\cdot+}$ that is quenched by a given tissue sample. A 7 mmol l^{-1} solution of ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt; Sigma] was combined with 2.45 mmol l^{-1} potassium peroxodisulphate (Sigma) in equal volumes to produce a stock solution of ABTS radical cation (ABTS$^{\cdot+}$). This was left overnight in the dark at room temperature to obtain maximum absorbance. An ABTS$^{\cdot+}$ working solution was prepared by diluting the stock solution with 20 mmol l^{-1} sodium acetate pH 6.5 (Biostrategy) to give an absorbance of 0.8–1.0 at 734 nm when 100 µl was placed in a microtitre plate. A 1.5 mmol l^{-1} stock solution of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Sigma) was prepared in PBS pH 7.4 and used to produce a standard curve from 1 to 0.1 mmol l^{-1} in 20 mmol l^{-1} sodium acetate. Samples were prepared by diluting the tissue lysates 1:5 in 20 mmol l^{-1} sodium acetate. Then, 10 µl diluted sample, 10 µl standard and 10 µl sodium acetate as a blank were placed in duplicate in a polystyrene flat-bottom 96-well plate (Interpath) followed by 100 µl diluted ABTS$^{\cdot+}$ and incubated at 30°C for 5 min in the dark. The absorbance was then read at 734 nm using a SpectraMax M5 plate reader (Molecular Devices). The antioxidant activity of each sample was calculated from the Trolox standard curve using SoftMax Pro v5.4 software (Molecular Devices). Measures of antioxidant activity

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were significantly repeatable (testes: R=0.977, $F_{113,114}$=84.78, P<0.001; thoracic muscle: R=0.944, $F_{113,114}$=76.32, P<0.001). We took the average of our two replicates for downstream analysis.

ROS assay

ROS were assayed using a commercial kit (Amplex Red hydrogen peroxide/peroxidase assay kit A22188, Thermo Fisher Scientific). This kit contains a sensitive, one-step assay that uses the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) to detect hydrogen peroxide. All reagents were prepared as per the manufacturer's instructions including a H_2O_2 standard curve with final concentrations between 0 and 5 µmol l^{-1}. Tissue samples were prepared by diluting 1:10 in insect Ringer solution. Samples were placed in duplicate in a Greiner clear-bottom black plate (Interpath) alongside the standard curve and the assay was carried out according to the manufacturer's instructions. Fluorescence was measured using excitation at 530 nm and detection at 590 nm using a SpectraMax M5 plate reader (Molecular Devices). The ROS value for each sample was calculated from the standard curve using SoftMax Pro v5.4 software (Molecular Devices). Because tissues were lysed, the assay provided a measure of the total amount of intracellular and extracellular H_2O_2 in the tissue of interest. Measures of ROS were significantly repeatable (testes: R=0.991, $F_{113,114}$=216.42, P<0.001; thoracic muscle: R=0.988, $F_{113,114}$=167.83, P<0.001). We took the average of our two replicates for downstream analysis.

RESULTS

We entered measures of antioxidant capacity, ROS or oxidative damage into separate repeated measures ANOVA. Diet had a significant effect on the antioxidant capacity of the sampled tissues (between subjects $F_{1,111}$=8.73, P=0.004) but courtship treatment did not (between subjects $F_{1,111}$=0.10, P=0.752) (Fig. 1, Table 1). The interaction effect was not significant and was removed from the model (between subject $F_{1,110}$=0.96, P=0.330). There was a significant within-subject effect of tissue type (Table 2). Testis tissue had greater antioxidant capacity than thoracic muscle (Fig. 1).

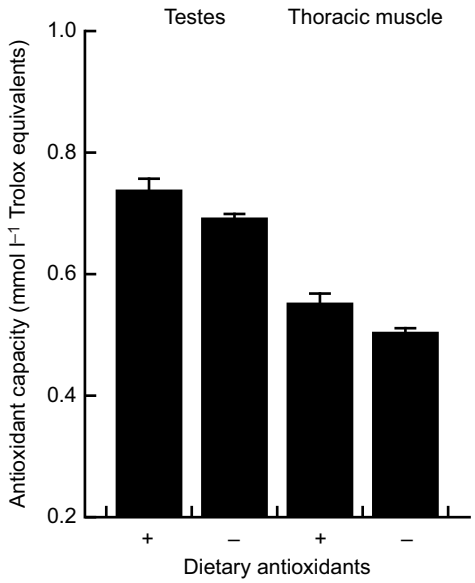

Fig. 1. Total antioxidant capacity of tissue lysates of testis and thoracic muscle of male crickets, *Teleogryllus oceanicus*, fed an antioxidant-rich (+) or -poor (–) diet. Data are means±s.e.m.

Table 1. Biological markers of oxidative stress (mean±s.e.m.) in the testis and thoracic muscle of non-courting male crickets, *Teleogryllus oceanicus*, and those induced to produce courtship song by exposure to females

	Tissue	Courtship	No courtship
Antioxidant capacity (mmol l^{-1} Trolox equivalents)	Testis	0.71±0.01	0.72±0.02
	Muscle	0.53±0.02	0.52±0.01
ROS (µmol l^{-1} H_2O_2)	Testis	4.27±0.24	4.27±0.19
	Muscle	1.33±0.10	1.41±0.08
Protein carbonyl (nmol l^{-1})	Testis	0.39±0.06	0.44±0.06
	Muscle	0.16±0.01	0.17±0.01

ROS, reactive oxygen species.

Diet did not influence the levels of ROS in tissue lysates (between subject $F_{1,110}$=2.78, P=0.099) and there was no effect of courtship treatment (between subject $F_{1,110}$=0.04, P=0.852). The interaction was again not significant (between subject $F_{1,110}$=0.25, P=0.622) and was removed from the model (Fig. 2, Table 1). However, within subjects there was a significant effect of tissue type on the levels of ROS (Table 2). Testis tissue had higher levels of ROS than thoracic muscle (Fig. 2).

The concentration of oxidized proteins in tissue lysates did not vary significantly between diet (between subject $F_{1,111}$=3.69, P=0.057) or courtship exposure treatments (between subject $F_{1,111}$=0.49, P=0.488). The interaction was not significant and was removed from the model (between subject $F_{1,110}$=0.02, P=0.877). Within subjects there was a significant effect of tissue type and an interaction between tissue type and diet (Table 2). Males fed an antioxidant-rich diet had a lower concentration of oxidative damage in their testes but not in their thoracic muscle (Fig. 3).

DISCUSSION

An oxidative cost of reproduction offers an intuitively appealing mechanism for the evolution of life-history trade-offs and ageing (Costantini, 2008; Dowling and Simmons, 2009; Metcalfe and Alonso-Alvarez, 2010; Monaghan et al., 2009). Nevertheless, for a variety of reasons, empirical tests for oxidative costs of reproduction have yielded mixed results (Speakman and Garratt, 2014; Zhang and Hood, 2016). The vast majority of studies have focused on the oxidative costs of reproduction for females (Blount et al., 2016). We adopted an experimental approach to explore the oxidative costs of reproduction for males. We manipulated the availability of dietary antioxidants and the amount of pre-mating courtship delivered by males, and measured oxidative balance in the testes and thoracic muscle. Dietary antioxidants contributed to positive oxidative balance in both tissue types, protecting the testes and muscle from oxidative damage. Although the testes had greater antioxidant defences than muscle tissue, they also produced considerably higher levels of ROS and sustained higher levels of oxidative damage. Accordingly, the antioxidant defences of the testes were affected to a greater extent by dietary antioxidants than were the defences of muscle tissue. Elevated courtship effort had no impact on any measure of oxidative balance.

Our findings support the notion that the male germline is particularly prone to oxidative stress (Blount et al., 2001; Dowling and Simmons, 2009), and that its maintenance is likely to represent a significant cost of reproduction (Maklakov and Immler, 2016). Studies of the oxidative cost of reproduction have mostly measured oxidative stress in blood and liver, and to a lesser extent in muscle and kidney (Speakman and Garratt, 2014). Surprisingly,

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Table 2. Within-subject effects from repeated measures ANOVA exploring the effects of dietary antioxidants and courtship exposure on biological markers of oxidative stress in the testis and thoracic muscle of male crickets, *Teleogryllus oceanicus*

	Tissue ($F_{1,111}$)	*P*	Tissue×diet ($F_{1,111}$)	*P*	Tissue×courtship ($F_{1,111}$)	*P*
Antioxidant capacity	200.87	<0.001	0.01	0.940	0.29	0.592
ROS	349.96	<0.001	2.60	0.109	0.11	0.738
Protein carbonyl	36.98	<0.001	4.09	0.046	0.16	0.690

reproductive tissues have rarely been examined. Regardless of diet, testis tissue was found to contain greater quantities of ROS than muscle (Fig. 2), consistent with findings from vertebrates that the testes are sites of elevated ROS production (Aitken and Roman, 2008). However, males fed a diet that included beta-carotene and vitamin E had testes with greater antioxidant capacity (Fig. 1) and reduced levels of protein oxidation (Fig. 3) compared with those of males fed a diet with only trace amounts of vitamin E. This finding supports the view that, regardless of endogenous antioxidant defences present in the testes (Aitken and Roman, 2008), dietary antioxidants are important components of germline protection (Ahmadi et al., 2016; Liu et al., 1997; Rengaraj and Ho Hong, 2015; Showell et al., 2011; Surai et al., 1997). Our findings cannot be explained in terms of general nutritional resource acquisition and allocation because our completely synthetic diets differed only in the specific micronutrients beta-carotene and vitamin E, and not in macronutrients such as proteins and carbohydrates, the availability of which was standardized across diet treatments. Previous studies using these same diet treatments found that male *T. oceanicus* fed the diet rich in beta-carotene and vitamin E had greater competitive fertilization success than males fed the antioxidant-poor diet (Almbro et al., 2011). Almbro et al. (2011) argued that dietary antioxidants protected the germline from oxidative stress, thereby improving the quality and fertilization capacity of sperm. Our data support this conclusion, and together these studies demonstrate a link between oxidative stress to the germline and functional male fertility.

We found no effect of elevated courtship activity on markers of oxidative stress (Table 1). Generally, thoracic muscle had relatively low antioxidant defences, contained lower amounts of ROS and had less oxidative damage. Elevated courtship activity did not result in elevated oxidative stress, suggesting that males may not face an oxidative cost of courtship. Stridulation is an energetically expensive activity (Bennet-Clark, 1970; Hack, 1998; Hoback and Wagner, 1997; Kavanagh, 1987). Male crickets provided with greater access to nutritional resources have been shown to expend more energy on calling song than males with limited nutritional resources (Hunt et al., 2004), and the availability of protein in the diet has been found to underlie a resource allocation trade-off between calling song and lifespan in the congeneric cricket *Teleogryllus commodus* (Maklakov et al., 2008). Male *T. commodus* fed diets that stimulated increased energetic expenditure on calling song were found to have elevated levels of protein oxidation in whole-body lysates (Archer et al., 2015). However, dietary supplementation with vitamin E did not affect protein oxidation or antioxidant capacity (Archer et al., 2015).

Given that courtship song is more energetically expensive than calling song (Hack, 1998), we might have expected to see similar differences in ROS production and oxidative damage between our courtship treatment groups (Harman, 1956). There are several reasons why our manipulation may not have affected oxidative balance. First, increased metabolic rate may not necessarily result in increased ROS production. Numerous studies have found that increased metabolism has no effect on ROS production and can even decrease ROS production, depending on the flow of protons across the inner mitochondrial membrane during oxidative phosphorylation (Speakman and Garratt, 2014; Zhang and Hood, 2016). Thus, while stridulation might be energetically costly in terms of the macronutrient resources required to power wing

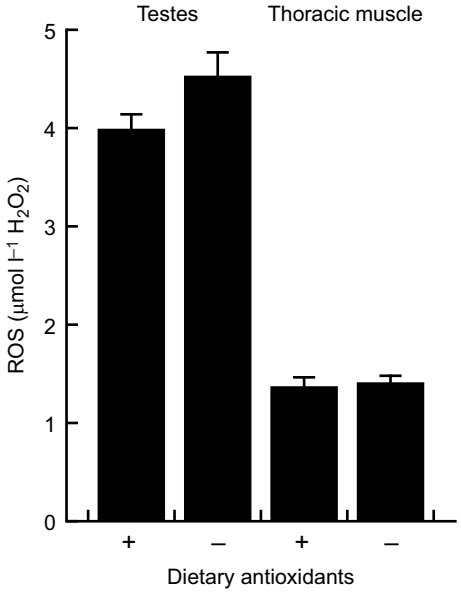

Fig. 2. Levels of reactive oxygen species (ROS) in tissue lysates of testis and thoracic muscle of male crickets, *T. oceanicus*, fed an antioxidant-rich (+) or -poor (–) diet. Data are means±s.e.m.

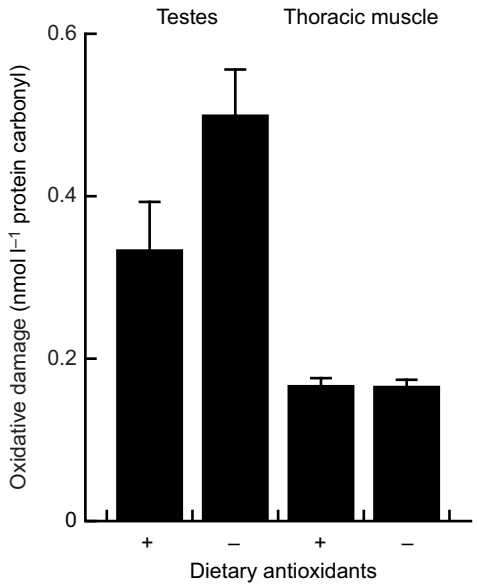

Fig. 3. Concentration of protein carbonyls in tissue lysates of testis and thoracic muscle of male crickets, *T. oceanicus*, fed an antioxidant-rich (+) or -poor (–) diet. Data are means±s.e.m.

movement, it need not impose an oxidative cost of reproduction. Second, males exposed to females may have optimized their courtship effort in order to maintain oxidative balance in the thoracic muscles (Metcalfe and Monaghan, 2013). The temporal properties of courtship song do vary across males, are phenotypically plastic in response to environmental challenges such as immune assault (Tregenza et al., 2006) and have been found to trade-off with ejaculated sperm quality (Simmons et al., 2010). Future work might therefore examine the qualitative aspects of courtship song produced by males fed these artificial diets. Finally, we exposed males to females for 3 nights when they were 10–13 days of adult age. It may be that this level of exposure was insufficient to detect oxidative costs of courtship, or that such costs accumulate with age or are greater in aged animals with lower residual reproductive value and which invest more heavily in courtship (Speakman and Garratt, 2014; Zhang and Hood, 2016). Indeed, Dowling and Simmons (2012) found age-dependent changes in sperm quality for males exposed to females; effects of courtship on sperm quality were found at 13 days but not 6 days of male age. Whether this was due to age per se or to the longer periods of exposure is unclear. Future work might vary age and the amount of time that males are exposed to females in order to determine the independent effects of these variables on oxidative balance in the testes and thoracic muscle.

The conceptual model of oxidative stress as a mediator of life-history evolution has assumed that reproduction has a general organism-wide impact on oxidative balance, with consequences for lifespan. A more complex model considers tissue-specific effects at different life stages (Speakman and Garratt, 2014). Our study suggests that the testes are especially vulnerable to oxidative stress. It might be that the oxidative costs of reproduction are confined to the testes, with effects on the proportion of lifespan during which males remain fertile, rather than on lifespan per se. We only examined the immediate oxidative cost of reproduction, and future work should consider the long-term maintenance of fertility and lifespan. Interestingly, in long-lived houbara bustards, *Chlamydotis undulata*, males with more extravagant sexual displays experience a faster rate of decline in spermatogenic function than males with less extravagant displays (Preston et al., 2011). Whether fertility decline is a consequence of testes-specific oxidative costs of reproduction warrants further study. Recently Maklakov and Immler (2016) argued that the current paradigm in life-history evolution is overly centred on the trade-off between lifespan and reproduction. They point out how apparent challenges to the lifespan–reproduction trade-off can be reconciled by considering the high cost of germline maintenance. We suggest that the oxidative defence of the germline will prove to be an important cost of reproduction, at least for males.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
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Data availability
Analyses reported in this article can be reproduced using data deposited in the University of Western Australia digital repository (Simmons et al., 2018): data_from_Simmons_et_al(.xlsx). http://dx.doi.org/10.4225/23/5b3b31a23d2be.

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