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Antioxidant supplementation can reduce the survival costs of excess amino acid intake in honeybees

C. Ruth Archer [a,1], Angela Köhler [a,2], Christian W.W. Pirk [a], Vinette Oosthuizen [a], Zeno Apostolides [b], Susan W. Nicolson [a,*]

[a] Department of Zoology and Entomology, University of Pretoria, Private Bag X20, Hatfield 0028, South Africa
[b] Department of Biochemistry, University of Pretoria, Private Bag X20, Hatfield 0028, South Africa

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ABSTRACT

Over-consuming amino acids is associated with reduced survival in many species, including honeybees. The mechanisms responsible for this are unclear but one possibility is that excessive intake of amino acids increases oxidative damage. If this is the case, antioxidant supplementation may help reduce the survival costs of high amino acid intake. We tested this hypothesis in African honeybees (*Apis mellifera scutellata*) using the major antioxidant in green tea, epigallocatechin-3-gallate (EGCG). We first determined the dose-range of EGCG that improved survival of caged honeybees fed sucrose solution. We then provided bees with eight diets that differed in their ratio of essential amino acids (EAA) to carbohydrate (C) (0:1, 1:250, 1:100, 1:75, 1:50, 1:25, 1:10, 1:5 EAA:C) and also in their EGCG dose (0.0 or 0.4 mM). We found that bees fed sucrose only solution survived better than bees fed EAA diets. Despite this, bees preferred a diet that contained intermediate ratios of EAA:C (ca. 1:25), which may represent the high demands for nitrogen of developing nurse bees. EGCG supplementation improved honeybee survival but only at an intermediate dose (0.3–0.5 mM) and in bees fed low EAA diets (1:250, 1:100 EAA:C). That EGCG counteracted the lifespan reducing effects of eating low EAA diets suggests that oxidative damage may be involved in the association between EAAs and lifespan in honeybees. However, that EGCG had no effect on survival in bees fed high EAA diets suggests that there are other physiological costs of over-consuming EAAs in honeybees.

1. Introduction

In many species, the ratio of protein to non-protein energy that individuals consume is a central determinant of their lifespan (Simpson and Raubenheimer, 2009). For example, in ants (Dussutour and Simpson, 2009), crickets (Maklakov et al., 2008), flies (Lee et al., 2008) and bees (Pirk et al., 2010) survival is greatest in animals fed high carbohydrate, low protein diets. Similarly, excess intake of amino acids can reduce survival in insects (Paoli et al., 2014; Troen et al., 2007) and mammals (Miller et al., 2005). The physiological costs of eating these nitrogen rich nutrients are not well understood but may include the production of nitrogenous waste, which can have toxic effects (Wright, 1995), disruption of immune function (Povey et al., 2009) and interference with cellular signalling pathways (Simpson and Raubenheimer, 2009). One additional possibility is that overeating protein and amino acids elevates oxidative damage incurred during oxidative stress (Simpson and Raubenheimer, 2009).

Oxidative stress is the state that cells enter when the production of Reactive Oxygen Species (ROS) exceeds their antioxidant capacity. ROS are highly reactive and formed during normal cellular metabolism, primarily in the mitochondria (Barja, 2007). Although ROS have important physiological functions – for example, they act as signalling molecules (Veal et al., 2007) and are involved in immunity (Kohchi et al., 2009) – their high reactivity means that they may also cause cellular damage by oxidising proteins, lipids and DNA. This damage is costly: oxidative damage contributes towards age-associated diseases and may increase an individual's risk of death (reviewed in Speakman and Selman, 2011). This means that aerobic organisms must meet their functional demands for ROS (as in immune defence) while preventing the oxidative damage that high levels of ROS may cause.

* Corresponding author. Tel.: +27 (0) 12 420 5343; fax: +27 (0) 12 362 5242.
E-mail address: swnicolson@zoology.up.ac.za (S.W. Nicolson).
[1] Address: Max Planck Research Group, Modelling the Evolution of Ageing, Max Planck Institute for Demographic Research Konrad-Zuse-Str. 1, 18057 Rostock, Germany.
[2] Address: Laboratory of Fundamental and Applied Research in Chemical Ecology (FARCE), University of Neuchâtel, Rue Emile-Argand 11, 2000 Neuchâtel, Switzerland.

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Antioxidants, which neutralise ROS, help maintain this balance and a poor diet could, in theory, disrupt it. Diets high in protein or amino acids could push cells into oxidative stress by increasing ROS production from the mitochondria, impairing antioxidant defences against ROS or reducing the repair of oxidised molecules (López-Torres and Barja, 2008). In support of this idea, in rats, reduced intake of both protein and amino acids reduces oxidative damage (Ayala et al., 2007; Caro et al., 2008; Sanz et al., 2004, 2006). This appears to be because dietary manipulation lowers ROS production during electron transport in the mitochondria, both by reducing the concentration of mitochondrial respiratory complexes that generate ROS and also by changing the degree of electronic reduction of these complexes (the greater the level of reduction, the more ROS are produced) (Sanz et al., 2006). However, the mechanisms underlying low survival in insects, such as honeybees, fed amino acid rich foods are unknown.

Honeybees are the major pollinators of many food and wild plants and are therefore of great economic and ecological importance. However, honeybee populations are declining (vanEngelsdorp et al., 2008). A poor diet, due to land use changes reducing the availability and diversity of floral resources, may help drive these declines (Vanbergen and the Insect Pollinators Initiative, 2013). In keeping with this idea, nutrition is a key determinant of honeybee survival (Altaye et al., 2010; Archer et al., 2014b): African honeybees (*Apis mellifera scutellata*) fed high protein diets experience reduced survival (Pirk et al., 2010) and European honeybees (*Apis mellifera*) survive poorly when fed diets rich in essential amino acids (EAAs) (Paoli et al., 2014). To protect declining and threatened honeybee populations (Pirk et al., 2014) it is important that we better understand the association between diet and honeybee survival, and find ways of mitigating the costs of poor nutrition, especially in populations which are underrepresented in the literature (Archer et al., 2014a).

Here, we examine the relationship between EAAs, lifespan and antioxidant supplementation in caged *A. m. scutellata* workers. We aim to develop our understanding of how EAAs affect survival while examining the potential of antioxidant supplementation to improve honeybee survival and alleviate the costs of high EAA intake. We use the major antioxidant found in green tea, epigallocatechin-3-gallate (EGCG) (Aucamp et al., 2000). Bees may encounter this antioxidant in nature because Chinese beekeepers regularly move hives to tea plantations (Zheng et al., 2011) and secondary metabolites, such as EGCG, are often present in pollen and nectar (Detzel and Wink, 1993). Earlier studies using various animal models show that EGCG can improve survival under stress (Zhang et al., 2009), reduce oxidative damage (Kumaran et al., 2008) and protect against bacterial and fungal pathogens (Nakayama et al., 2011; Park et al., 2006). We first fed bees different doses of EGCG in sucrose solution to identify an EGCG dose that has positive effects on honeybee survival. We then measured dietary intake and survival in honeybees fed a single diet that varied in its EAA:C ratio. This also allowed us to compare the association between EAAs and survival in African honeybees and European bees, as we used a protocol developed for European subspecies (Paoli et al., 2014). We provided these diets either supplemented with 0.4 mM (+EGCG) or without (−EGCG) EGCG to test whether this antioxidant restores longevity in bees fed EAA rich diets.

2. Materials and methods

2.1. Experiment 1: effects of EGCG dose on the survival of worker bees fed sucrose solution (0:1 EAA:C)

To identify an appropriate dose of EGCG for supplementation, brood frames were collected from three different colonies of *A. m. scutellata* at the University of Pretoria experimental farm and incubated at 34 °C in constant darkness. On the day of their emergence from the brood comb, freshly emerged (<24 h) workers were caged in groups of 100 individuals. Each group received a diet consisting of 0.63 M sucrose solution and one of five EGCG doses (0, 0.1, 0.3, 0.5 and 2.2 mM). Diets were made every two weeks to ensure that the EGCG did not deteriorate and were frozen in aliquots at −20 °C and defrosted on the day of use. Each colony received all five diets; therefore, a total of 15 groups were fed for a maximum of 21 days in standard laboratory hoarding cages (Köhler et al., 2013) following standard procedures (Köhler et al., 2012). The liquid diet and water were provided fresh daily, when survival was also measured and dead bees removed from cages. To calculate consumption of diets we measured the mass change between the experimental diets provided and then retrieved twenty-four hours later. We repeated this process for control tubes of each diet, which we kept in the same conditions as experimental diets but in the absence of bees and used to estimate average evaporation. We subtracted average evaporation values from the observed differences in mass in experimental diets to calculate consumption.

2.2. Experiment 2: effects of nutrient ratio (EAA:C) and EGCG on the survival and consumption of worker bees

We used the ten amino acids identified as being important for honeybee health by de Groot (1953). As in Paoli et al. (2014), where the effects of eating different EAA:C ratios were examined in European bees, diets were made from equimolar concentrations of ten EAAs (methionine, tryptophan, arginine, lysine, histidine, phenylalanine, isoleucine, threonine, leucine and valine) and added to a 1.0 M sucrose solution (Table 1). All ten EAAs were added to the diets at the same concentration: for example, for the 1:10 diet, the total final concentration of the amino acids was 0.1 M, with each amino acid present at a concentration of 0.01 M.

Diets differed in their EAA:C ratio (according to the Geometric Framework of nutrition (Simpson and Raubenheimer, 2012)) in one of eight ratios: 0:1, 1:5, 1:10, 1:25, 1:50, 1:75, 1:100 and 1:250 EAA:C, calculated on a molar:molar basis. Each diet was then split into two batches, one of which was supplemented with 0.4 mM EGCG (the dose intermediate between the two best dosages for survival in Experiment 1, see Section 3). This resulted in 16 different dietary treatments, one for each ratio of EAA:C and each level of supplementation (+EGCG/−EGCG).

Freshly emerged honeybees were transferred in groups of 20 to plastic transparent cages with a volume of ~200 cm^3 (5.8 × 5.8 × 5.8 cm) (Plastilon Packaging, Rietfontein, South Africa), perforated with 30 equally distributed ventilation holes. We used 20 bees in this experiment as opposed to the 100 used in Experiment 1 to allow direct comparison with the study of

Table 1
Dietary amino acid concentrations used in Experiment 2. The P:C ratio is the ratio of amino acids to carbohydrate converted to the equivalent weight: weight ratio of proteins to carbohydrate used in past experiments on honeybee nutrition (e.g. Altaye et al., 2010). The total EAA concentration is that of all ten essential amino acids, each of which has the concentration given in the Individual EAA (M) column. All amino acids were added to 1 M sucrose solution. This solution was used for the 0:1 EAA:C diet. Based on a table in Paoli et al. (2014).

Diet (EAA:C)	P:C	Total EAA (M)	Individual EAA (M)
1:250	1:573	0.004	0.0004
1:100	1:230	0.01	0.001
1:75	1:153	0.015	0.0015
1:50	1:115	0.02	0.002
1:25	1:34	0.04	0.004
1:10	1:23	0.1	0.01
1:5	1:11	0.2	0.02