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

C. Ruth Archer[a], Angela Köhler[a1], 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*

*Author for correspondence

Email: swnicolson@zoology.up.ac.za

Telephone: +27 (0) 12 420 5343

Fax: +27 (0)12 362 5242

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] Laboratory of Fundamental and Applied Research in Chemical Ecology (FARCE), University of Neuchâtel, Rue Emile-Argand 11, 2000 Neuchâtel, Switzerland

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.

Keywords: *Apis mellifera scutellata*, carbohydrate, essential amino acids, epigallocatechin-3-gallate; intake array

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. 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., 2014a): 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., 2014b).

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 Results). 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 (5.8x5.8x5.8cm) (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 Paoli et al. (2014). Cages contained two larger holes for inserting 2.0 ml Eppendorf tubes, each with three small holes through which bees could feed. One of these tubes was used to provide water and the other was used to provide one of the 16 liquid diets. We established two replicate cages, for each of the 16 treatments and three colonies. In this experiment, because we had only 20 workers per cage, survival was monitored for only two weeks. Otherwise, methods were as in Experiment 1.

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 AA concentration is that of all ten essential amino acids, each of which has the concentration given in the Individual AA (M) column. All amino acids were added to 1M 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 AA (M)	Individual AA (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

2.3. Statistical analysis

All analyses were conducted in R version 3.0.3 (R core development team., 2013). Colony and, where appropriate, replicate cage are included as random effects in all analyses.

2.3.1. Survival

To analyse survival we used the function "survreg" in the R "survival" package (Therneau, 2013), incorporating colony (Experiment 1 and 2) and replicate cage nested within colony (Experiment 2) as random effects using the "frailty" function. We determined which survival function - Weibull, Gaussian, exponential, extreme or logistic - best described the data by comparing model fit using "anova". For all analyses, a logistic distribution best described the data. We fit survival models including all explanatory variables (i.e. EGCG dose - Experiment 1 and 2, diet - Experiment 2) and interactions between them, before using backwards model simplification to assess their significance.

2.3.2. Regulation of total food intake on a single, nutritionally imbalanced diet

Nutrient intake was analysed per bee i.e. daily intake of each diet per cage was divided by the number of bees alive in that cage. Cumulative intake per bee is the sum of each of those daily values. We analysed cumulative intake per bee at day seven, when most bees were still alive to ensure that a few surviving honeybees did not disproportionately affect results. All bees in two cages died before day seven: these were excluded from analyses of total intake. Intake was analysed using a linear mixed-effects model using the package "lme" in R (Bates and Maechler, 2009) with diet type (EAA:C ratio) and supplementation (+EGCG/-EGCG) and the interaction between them as fixed effects. Colony and replicate cage were included as random effects and *post hoc* comparisons were made using the package "*lsmeans*" (Lenth, 2014) and Tukey adjustments.

2.3.3. Regulation of amino acid and carbohydrate intake on a single, nutritionally imbalanced diet

To examine how tightly worker bees regulated their intake of EAAs versus carbohydrate, we calculated the coefficient of variation (CV = SD/mean) of daily intake of each nutrient and compared them using a variance ratio test, where Fa,b = (CVa/CVb) or Fb,a = (CVb/CVa) whichever is the largest CV (CVa or CVb) (Zar, 1999). We compared the coefficient of variation for each nutrient (EAA versus C) within supplementation regimes and then compared the same

nutrient across supplementation regimes. We used CV (instead of variance or standard deviation) because dietary intake was greater in +EGCG workers than in –EGCG workers and variance increases directly with the mean (Zar, 1999). Colonies that died before day seven were included in these analyses of daily intake.

When individuals are constrained to a single, nutritionally imbalanced food they must compromise between eating too much of a nutrient or too little. We plotted average intake of EAAs on the x-axis and carbohydrate on the y-axis to create an intake array (see Simpson and Raubenheimer, 2012); the shape of this array reveals how animals manage trade-offs between over- and under-ingesting nutrients when fed nutritionally imbalanced diets (Raubenheimer and Simpson, 1997). We estimated the slope of the array for +EGCG and -EGCG animals. We used a linear mixed-effects model with cumulative intake of carbohydrate as the response variable, and cumulative EAA intake and supplementation status and the interaction between them as the explanatory variables, to see if slopes differed across +EGCG and –EGCG groups.

The intake array also allows us to locate the diet closest to that preferred by workers, which, assuming that animals regulate their nutrient intake to maximise the expression of a particular fitness determining trait or overall performance, should reflect the diet best for honeybee performance, called the intake target (Simpson and Raubenheimer, 2012). As in European bees, the shape of the intake array in this study is not a smooth line or arc but has a central hinge point. The location of this point indicates the intake target (Paoli et al., 2014). To identify this pivot point we first used a jack-knife method and fitted a linear regression to the intake array for animals that ate 0:1, 1:250 and 1:100 EAA:C. We sequentially added more amino acid biased diets, one at a time, and noted when the slope of the regression line switched from being positive to negative. This gave us an estimation of where the intake target might lie. We then fitted a third order polynomial for the intake array, extracted the equation and calculated the first and second derivation. We calculated the critical values for the second derivation ($y''(x)=0$) which gave us the inflection point. This is the point at which the curvature changes sign either plus to minus or vice versa. We then used the point (x value) obtained and entered it into the equation to calculate the corresponding y value and then the intake target.

3. Results

3.1. Experiment 1: effects of EGCG dose on survival of worker bees fed a single, pure carbohydrate diet

The dose of EGCG that bees were fed affected their survival (Fig. 1, Table 2, Supplementary Table 1). Both the 0.3 mM and the 0.5 mM EGCG diets improved survival relative to controls (i.e. no EGCG), while very high doses (2.2 mM) reduced it. The lowest EGCG dose (0.1 mM) had no perceptible effect on lifespan. Although bees fed 0.3 mM EGCG survived best, the positive effects of this EGCG dose on survival appeared to manifest later than those of a slightly higher dose (0.5 mM) (Fig. 1). Because we anticipated that survival in bees fed high EAA diets may be significantly reduced and mortality begin after about day seven (as in Paoli et al., 2014) we chose an intermediate dose between 0.3 and 0.5 mM for our second experiment in the hope that we would see pronounced, early acting effects on honeybee survival.

3.2.1. Experiment 2: the effects of amino acids, carbohydrate and EGCG on honeybee survival

The full survival model, including an interaction between EGCG and diet, provided a better fit than a simplified model excluding this interaction ($P = <0.001$) (Fig. 2). To better understand this interaction between diet and antioxidant supplementation, we analysed survival separately in bees that were fed EGCG and those that were not.

Models containing diet provided a better fit for –EGCG ($P = <0.001$, df = 10) and +EGCG ($P = <0.001$, df = 10) bees. In all cases, survival was best in workers that ate a pure sucrose diet (Fig. 2, Table 3). Amongst –EGCG bees, survival was particularly poor in bees fed 1:5, 1:75 or 1:100 EAA:C diets (Fig. 2, Table 3). EGCG supplementation improved survival in animals fed 1:250 and 1:100 EAA:C, but had minimal effects on the survival of bees fed any other diets (Table 3).

3.2.2. Experiment 2: regulation of total food intake on a single, nutritionally imbalanced diet

The total amount eaten over seven days was affected by both EGCG supplementation status ($L_{12,11} = 5.717$, $P = 0.017$) and diet type ($L_{12,5} = 25.685$, $P = <0.001$), however, there was no interaction between diet and supplementation ($L_{19,12} = 4.200$, P = 0.757) (Fig. 3). Bees fed

+EGCG diets ate significantly more (0.170 ± 0.005 mean ± SE g per bee) over seven days than –EGCG bees (0.160 ± 0.004 mean ± SE g per bee) (Fig. 3). *Post hoc* analyses showed that bees fed the 1:25 EAA:C diet ate less than bees fed 1:50, 1:75 and 1:100 EAA:C (Supplementary Table 2). Low intake of 1:25 EAA:C is one line of evidence that this diet is close to the intake target of honeybee workers (Paoli et al., 2014).

3.2.3. Experiment 2: regulation of amino acid and carbohydrate intake on a single, nutritionally imbalanced diet

The Coefficient of Variation (CV) for daily EAA intake was significantly greater than the CV for daily carbohydrate (carb) intake in both -EGCG (CV_{EAA} = 1.235 CV_{carb}= 0.205, $F_{47,47}$ = 6.015, P = <0.001) and +EGCG animals (CV_{EAA} = 1.344, CV_{carb} = 0.210, $F_{47,47}$ = 6.390, P = <0.001). The CV for daily EAA intake ($F_{47,47}$= 1.109, P =0.387) and for daily carbohydrate intake ($F_{47,47}$ = 1.024, P = 0.386) did not differ significantly between +EGCG and –EGCG.

The slope of the intake array for both –EGCG (β ± SE: -1.146 ± 0.419, r^2 = 0.553) and +EGCG workers (β ± SE: -2.108 ± 0.774, r^2 = 0.554) differed significantly (EAA:EGCG interaction: $L_{7,6}$ = 5.514, P = 0.019) (Fig. 4). There was a distinct "kink" in each array: analyses using the jack-knife method showed that the slope of the intake array was positive in animals fed high carbohydrate diets 0:1, 1:250, 1:100, 1:75 and 1:50 EAA:C (-EGCG: β ± SE: 6.932 ± 3.678, +EGCG: β ± SE: 2.129 ± 1.639), but switched to being negative when intake of the 1:25 diet was included (-EGCG: β ± SE: -2.909 ± 4.653, +EGCG: β ± SE: -4.028 ± 2.449). That worker bees switch their strategy of nutrient regulation between 1:50 and 1:25 EAA:C may suggest that the intake target lies in this region. However, fitting a third order polynomial line to each intake array (-EGCG: $y = 3212.2x^3 - 144.21x^2 - 0.4451x + 0.1651$, $r^2 = 0.5592$, +EGCG: $y = -6781.7x^3 + 302.03x^2 - 4.4409x + 0.1758$, $r^2 = 0.6014$) showed that the intake target was around 1:10 EAA:C for –EGCG bees and 1:19 EAA:C for +EGCG bees (Fig. 4).

Table 2. Survival of honeybees in Experiment 1. Results of the survival analyses of *A. m. scutellata* workers from three colonies fed one of four EGCG doses, for 21 days. Bees fed 0.63 M sucrose without EGCG were used as the reference treatment for comparison, i.e. the effects of supplementation were assessed relative to this control group. Colony was included as a random effect in these analyses, which were conducted using the "*Survreg*" package in R. The model performed a Likelihood ratio test on 4.3 df. For comparisons between doses results, see Table 1 in the Supplementary Information.

EGCG Dose (mM)	**Coef**	**Se(coef)**	**Z**	***P* value**
0.1	0.149	0.307	0.485	0.628
0.3	2.398	0.311	7.715	<0.001
0.5	0.757	0.297	2.553	0.011
2.2	-2.548	0.300	-8.489	<0.001

Table 3. The effects of EAA:C ratio on survival in caged honeybees. Because EGCG interacted with diet to affect survival, we analysed the effect of diet on supplemented (+EGCG) and un-supplemented (-EGCG) bees separately. In both instances we found a significant effect of diet. To determine where this significant effect lay, we "reshuffled" all survival analyses relative to a different baseline group, i.e. we first compared survival of animals fed all diets to those fed pure sucrose and then compared survival of animals fed all diets to those fed a 1:5 P:C ratio, etc. To account for this multiple testing approach, we have employed a Bonferroni correction whereby we class *P* values of less than 0.007 as significant (Shaffer, 1995). Values highlighted in bold are significant relative to this adjusted *P* value.

DIET (EAA:C)	0:1	1:250	1:100	1:75	1:50	1:25	1:10	1:5
-EGCG								
0:1	NA							
1:250	**<0.001**	NA						
1:100	**<0.001**	**<0.001**	NA					
1:75	**<0.001**	**0.002**	0.374	NA				
1:50	**<0.001**	0.215	**0.004**	0.060	NA			
1:25	**<0.001**	0.154	**0.003**	0.058	0.914	NA		
1:10	**<0.001**	0.392	**0.001**	0.018	0.670	0.571	NA	
1:5	**<0.001**	**<0.001**	0.554	0.751	0.024	0.021	**0.005**	NA
+EGCG								
0:1	**NA**							
1:250	0.055	**NA**						
1:100	**<0.001**	0.015	NA					
1:75	**<0.001**	**<0.001**	0.064	NA				
1:50	**<0.001**	**<0.001**	0.019	0.611	NA			
1:25	**<0.001**	**<0.001**	0.236	0.521	0.256	NA		
1:10	**<0.001**	**<0.001**	0.033	0.779	0.819	0.359	**NA**	
1:5	**<0.001**	**<0.001**	**0.002**	0.200	0.443	0.058	0.318	NA

Fig. 1. Survival of *A. m. scutellata* workers fed 0.63 M sucrose without EGCG (control) and with four EGCG concentrations over a period of three weeks. Open light grey circles represent survival of control workers (-EGCG), while filled blue circles represent survival of bees fed the diet containing EGCG. Survival of control workers is plotted in to facilitate comparison. N=3 worker groups from 3 colonies, with 100 individuals each. Error bars represent the standard error around the percentage of animals surviving at each time interval.

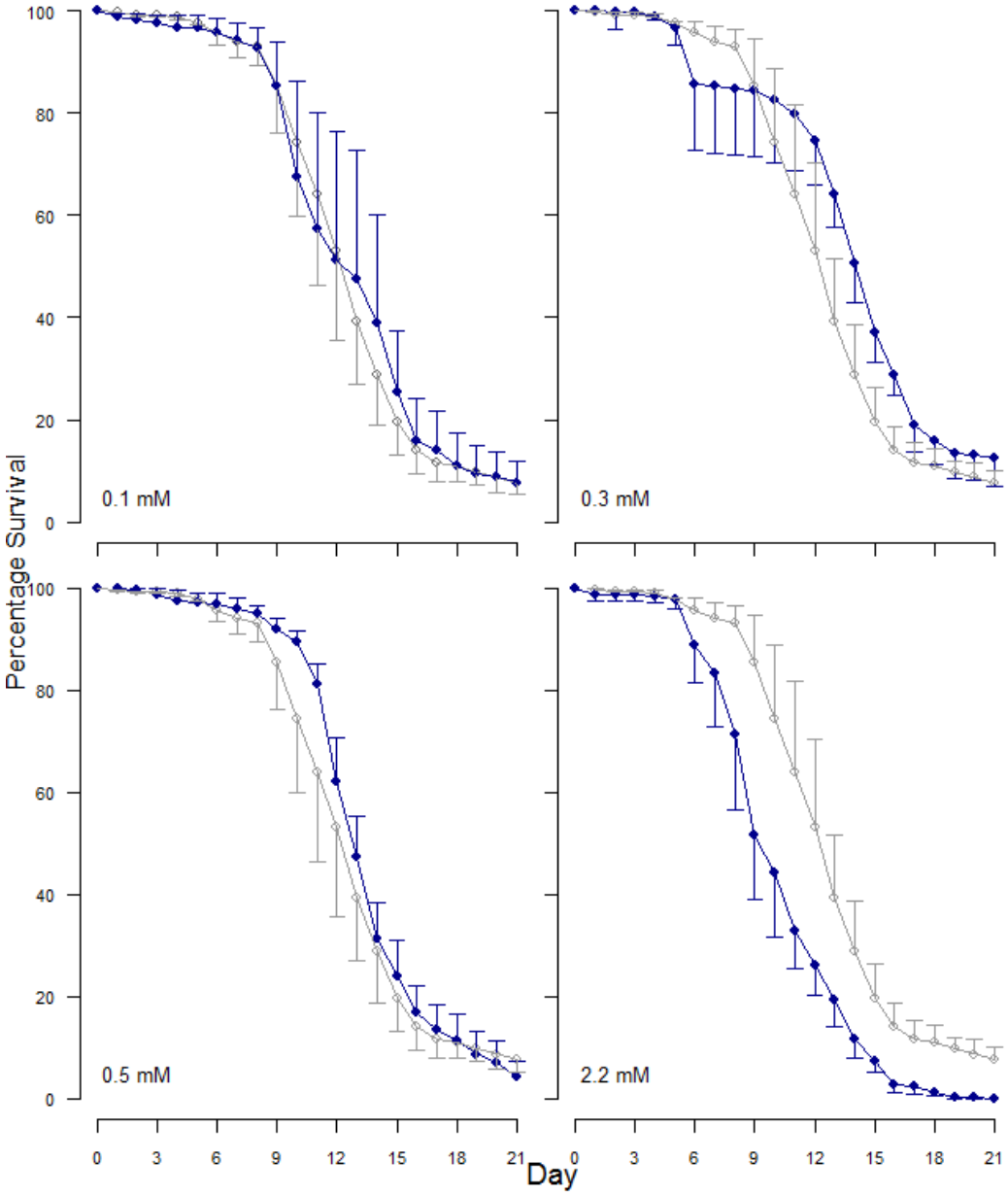


Fig. 2. Survival of *A. m. scutellata* workers fed one of eight diets that varied in their ratio of carbohydrate to amino acids (EAA:C) and were supplemented (+EGCG) or not (-EGCG) with EGCG. Symbols are as follows: open circles and solid lines = 0:1 EAA:C; open squares and dashed lines = 1:250 EAA:C; open triangles and dotted lines = 1:100 EAA:C; filled circles and lines with alternating dash and dot = 1:75 EAA:C; green inverted triangles and solid lines = 1:50 EAA:C, red open triangles = 1:25 EAA:C, blue open circles = 1:10 EAA:C, orange squares and dashed lines = 1:5 EAA:C

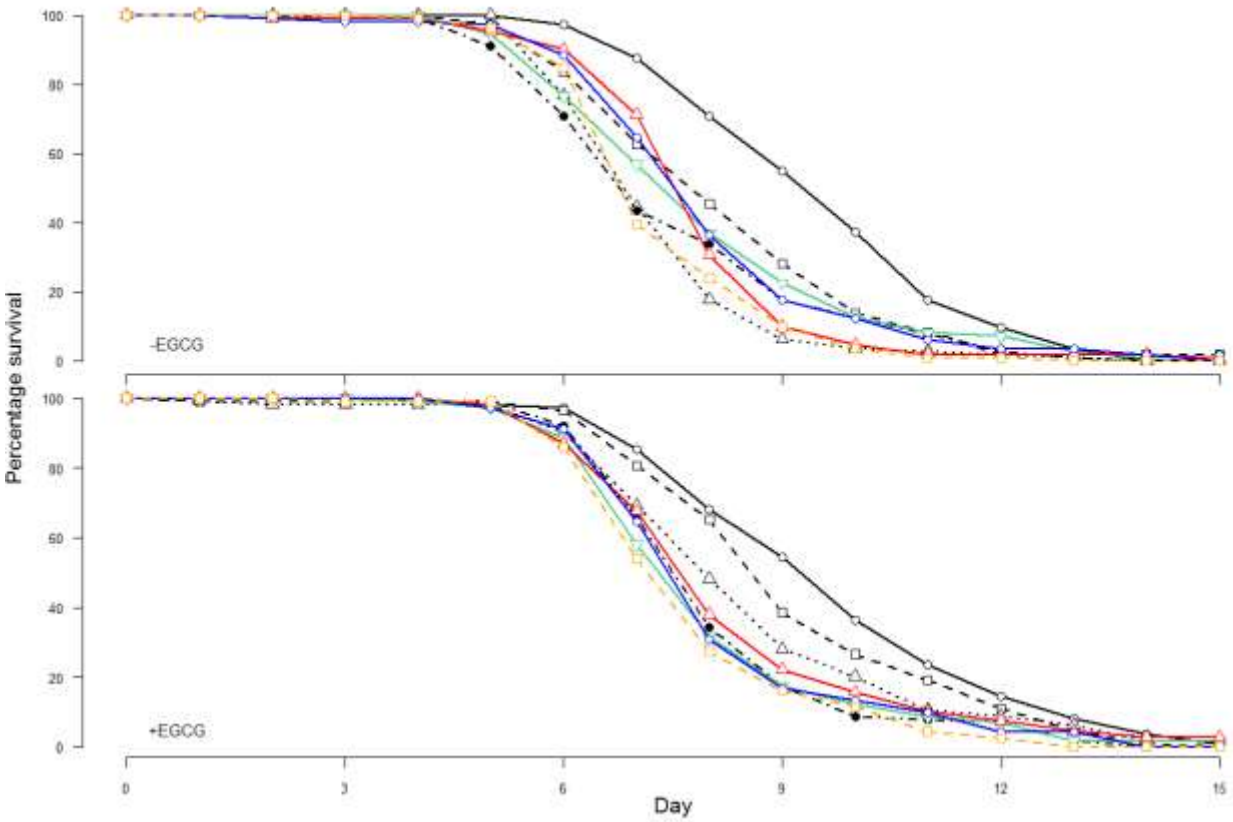


Fig. 3. Average total intake (g/bee) over seven days of each diet for -EGCG (filled symbols) and +EGCG (open symbols) bees in Experiment 2. Error bars represent standard errors around the mean. We found an overall positive effect of EGCG on intake but no evidence of a diet: EGCG supplementation interaction. Diet independently affected intake; *post hoc* tests show that the difference is driven primarily by bees fed diet 1:25 EAA:C eating less than bees fed 1:50, 1:100 and 1:75 EAA:C (Supplementary information Table 1).

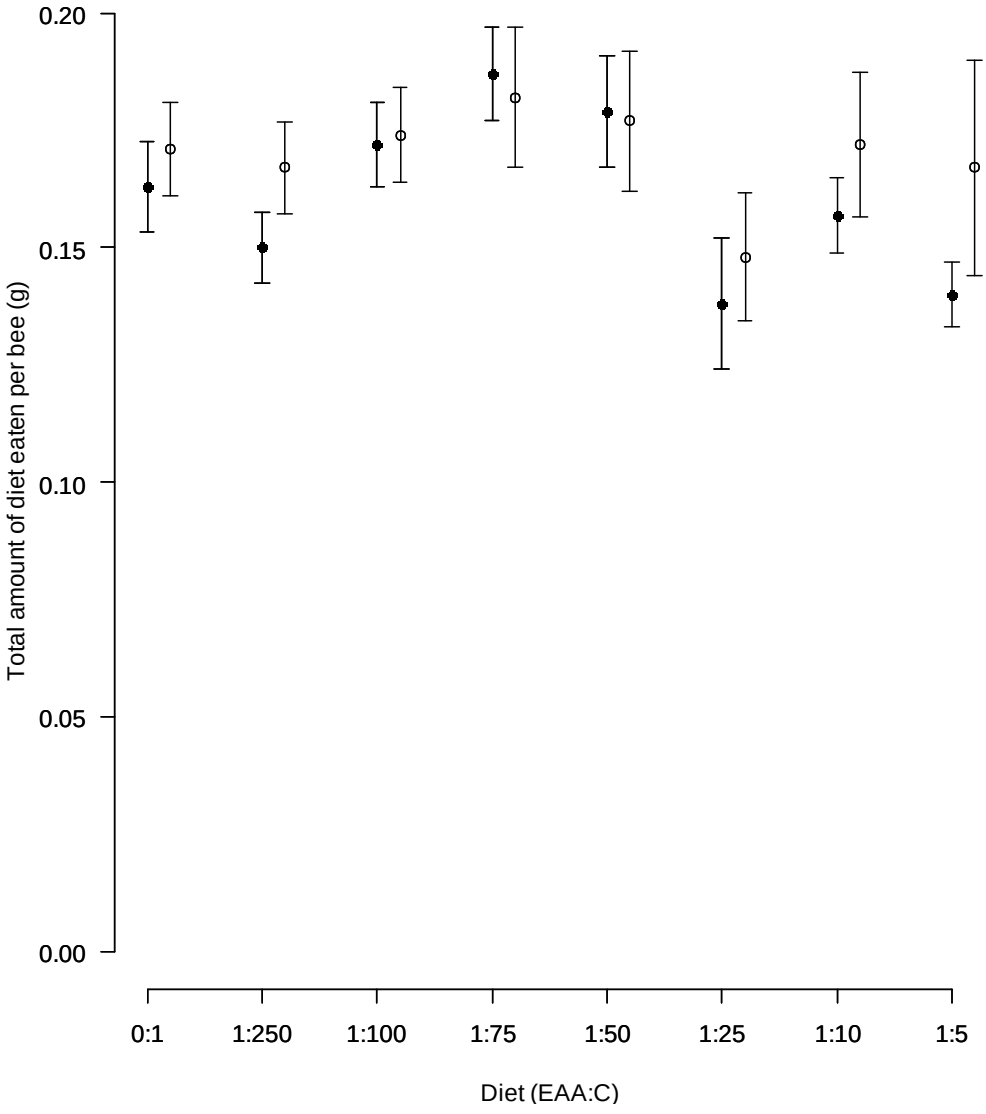
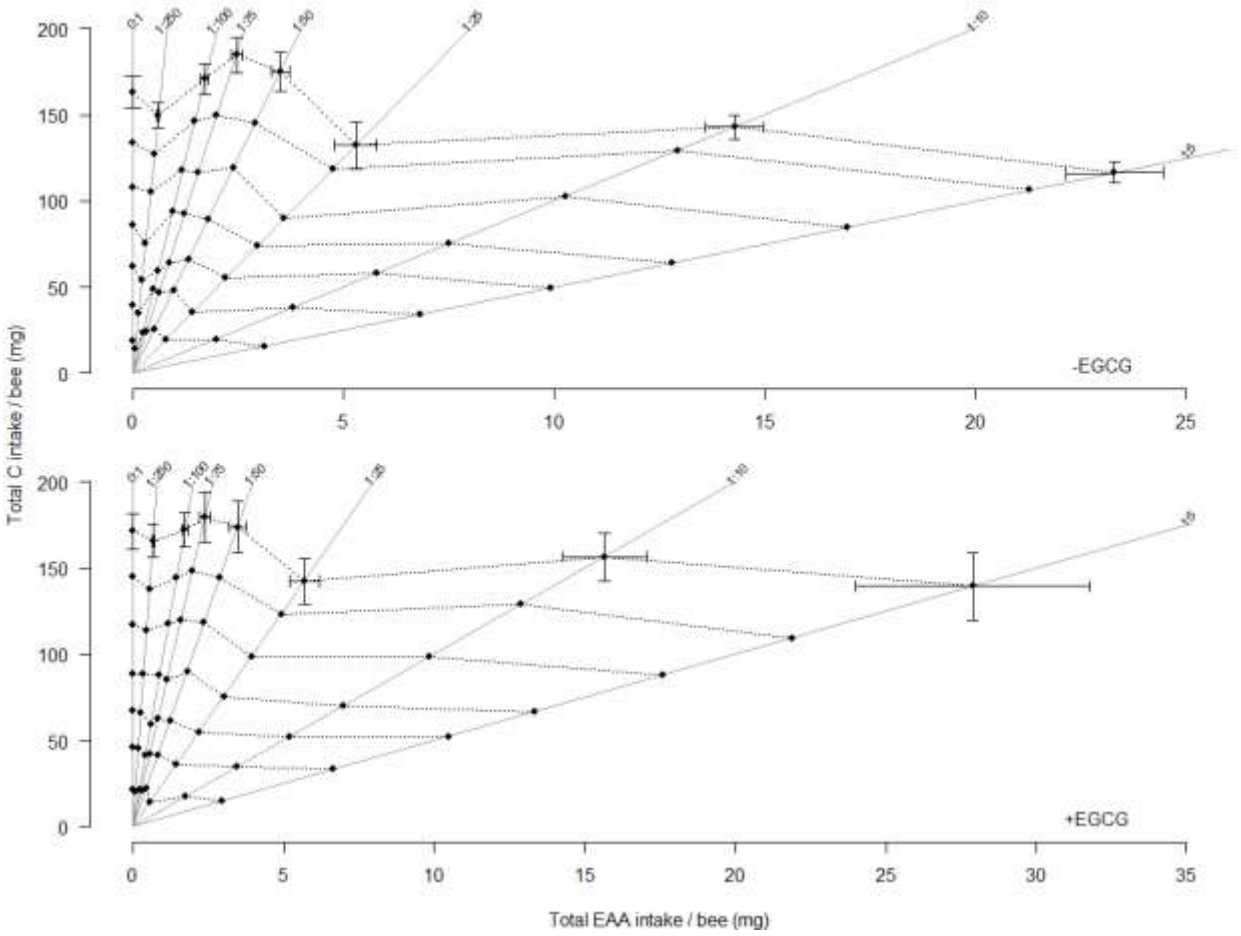


Fig. 4. Intake array of bees fed each of the diets in Experiment 2. Each grey line represents the ratio of EAA:C that bees were fed; bees could not consume diets that deviated from these ratios but could consume more or less of each diet type. Intake represents total cumulative consumption of each nutrient per bee over seven days following emergence, averaged across colonies and replicates. Error bars, which are only presented for the final intake values, correspond to standard errors around the mean. The shape of the intake array (i.e. lines that connect points of average intake) shows how bees trade off over and under eating EAAs or carbohydrates when provided with a single nutritionally imbalanced food. The key point that may be taken from this figure is that the array pivots around 1:25, suggesting that the intake target lies in this region (Paoli et al., 2014).



4. Discussion

Caged African honeybees survived best when fed sucrose-only solutions, while the addition of amino acids to their diet resulted in a shorter lifespan. Antioxidant supplementation improved honeybee survival but only when provided at intermediate doses and in bees fed low EAA diets. Here, we discuss the potential mechanisms underpinning these results and, more generally, what our data tell us about the dietary demands of African honeybees.

Bees fed sucrose only (0:1 EAA:C) survived better than bees fed diets containing EAAs, even at low doses. This result is surprising as bees often eat nitrogen rich foods; worker bees routinely ingest amino acid rich pollen (Human and Nicolson, 2006) and may even cannibalise brood under conditions of protein deficit (Schmickl and Crailsheim, 2001). Amino acids are vital for honeybee growth, maintenance and development, for example, foraging bees need to preserve and maintain flight muscles that experience wear and tear during flight (Roberts and Elekonich, 2005) and EAAs are vital building blocks for this protein turnover and repair. Despite this, honeybees of all age classes experience toxic effects of EAA intake (Paoli et al., 2014). Interestingly, we did not see a simple negative association between the EAA content of diet and honeybee survival; instead, particularly low survival was seen in worker bees fed high (1:5 EAA:C), intermediate (1:75 EAA:C) and rather low (1:100 EAA:C) EAA ratios. The association between carbohydrate, EAAs and honeybee survival is clearly complicated and the mechanisms underlying this result are unclear.

Although EAA intake reduced overall survival, African bees survived comparatively well on the 1:10 EAA:C diet that severely reduced survival in young European honeybees (Paoli et al., 2014). This suggests that African bees can tolerate higher EAA concentrations than European bees. This may reflect ecological differences between subspecies: African honeybees readily abscond (exhibit non-reproductive swarming) in poor conditions such as during nectar scarcity and so must invest heavily in brood production to allow rapid colony growth (Hepburn and Radloff, 1998). This requires protein; accordingly honeybees of African origin are more likely to produce pollen foragers (Danka et al., 1987) than European subspecies. This higher

demand for dietary nitrogen may mean that African bees are better able to survive on nitrogen rich foods.

Survival did not depend only on the ratio of nutrients that bees ate but also on their consumption of the antioxidant EGCG. In bees fed sucrose, a low EGCG dose (0.1 mM) had no significant effect on survival. Intermediate EGCG doses (0.3-0.5 mM) improved worker survival, while a high dose (2.2 mM) reduced it. Negative effects of high antioxidant doses have been observed in other species, such as in *Drosophila* fed vitamin E (Driver and Georgeou, 2003). These negative effects may suggest that consuming excessive amounts of antioxidants can disrupt optimal ROS homeostasis and inhibit the important functions of ROS to the detriment of survival (Dickinson and Chang, 2011).

The effects of EGCG on honeybee survival depended on their diet. EGCG only improved survival in bees fed low EAA diets (i.e. 1:250, 1:100 EAA:C). This result may reflect an interaction between specific nutrients and EGCG; sugars can help preserve secondary plant compounds with antioxidant activities (Peinado et al., 2010) while different EAAs can either inhibit or enhance the ability of EGCG to scavenge pro-oxidants (Huang et al., 2010). Alternatively, EAA rich diets may elevate ROS production so severely that the antioxidant dose we used could not protect against oxidative damage. High EAA intake may also have other deleterious effects on survival that EGCG could not ameliorate, for example, the accumulation of nitrogenous waste or disruption of cellular signalling. In summary, that EGCG improves survival only in bees fed low EAA diets suggests that oxidative damage may help mediate the association between nutrition and longevity but that other factors likely play a role. Further work that directly assays ROS production, oxidative damage and antioxidant protection in bees is needed to confirm our preliminary support for this idea.

By measuring how much of each diet honeybees ate, we can also infer something about nutrient regulation in African bees. We find that honeybees confined to a single diet regulated their intake of carbohydrate more tightly than their amino acid intake. This means that bees were more likely to over-eat EAAs to gain extra carbohydrates than to over-eat carbohydrates to gain EAAs. This strategy is unusual in species where high nitrogen intake reduces survival

(Behmer, 2009; Simpson and Raubenheimer, 2009) and is more common in predators, which have evolved on nitrogen rich foods (e.g. Jensen et al., 2012). However, a similar result has been observed in ants, whereby foragers retrieve very similar quantities of carbohydrates when fed a range of nutritionally imbalanced diets (Dussutour and Simpson, 2009). Strikingly, these ants manipulated the food that they collected by removing carbohydrates and disposing of extra protein in waste pellets (Dussutour and Simpson, 2009). Bees may adopt a similar strategy of nutrient regulation and in hives, convert excess protein to bee bread; but this hypothesis remains to be tested.

Our analyses do not allow us to pinpoint the intake target with a high degree of certainty: bees ate least of the 1:25 EAA:C diet, suggesting this is closest to their preferred intake, but fitting a third order polynomial equation to our intake arrays suggests that bees may prefer to eat a diet richer in EAAs. Conducting a choice experiment (sensu Simpson and Raubenheimer 2012) would allow us to more accurately identify dietary optima. However, it is clear that the intake target is more protein biased than that best for honeybee survival (0:1 EAA:C). This may indicate that honeybees prefer diets that contain sufficient nitrogen to allow the physical development associated with nursing larvae (e.g. hypopharyngeal gland development - Crailsheim et al., 1992). Furthermore, this intake target contains more carbohydrate than that preferred by bees fed diets containing protein in the form of casein (between 1:6.5 P:C - 1:12 P:C) (Altaye et al., 2010; Archer et al., 2014a). This is presumably because free amino acids provide a more readily accessible form of nitrogen that is absorbed more quickly and efficiently than proteins (Rønnestad et al., 2000). Finally, the intake target of African bees is more biased towards amino acids than that of European bees (1:50 EAA:C) of a similar age (Paoli et al., 2014). This further illustrates that the dietary demands of European and African honeybees differ.

In terms of using antioxidants to extend lifespan and mitigate the costs of eating poor diets in the field, we find that honeybees may obtain some moderate benefit from EGCG. This may be particularly true in the field because in addition to its antioxidant properties EGCG also inhibits the growth of *Paenibacillus larvae*, the causal agent of American foulbrood, *in vitro* at

concentrations higher than 0.28 mM (Flesar et al., 2010), similar to the EGCG concentrations we found to prolong worker survival. EGCG can also be administered in the form of green tea, as we have established in a 6-day test run on a total of 120 caged workers from two colonies: green tea was readily accepted at concentrations of up to 1% (one tea bag per cup, brewed for 5 min) with 0.63 M sucrose (Köhler et al, unpublished data). Honeybees may even be attracted to tea, as they have been shown to prefer low doses of caffeine over sugar-only solutions (Singaravelan et al., 2005). However, the negative effects of high doses of EGCG on honeybee survival show that this is an intervention to be used with caution. Furthermore, because the effects of EGCG are evident only in bees that eat low EAA diets, this is unlikely to be an efficient means of improving survival if bees, since bees normally have access to dilute nectars but protein rich pollens.

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Supplementary Table 1. ***Post-hoc analyses of survival across dietary treatments in Experiment 1.*** The methods used to do conduct these analyses are detailed in the main manuscript. To account for this multiple testing, we have employed a Bonferroni correction whereby we class values of less than 0.0125 as significant. Values highlighted in bold are significant relative to this adjusted *P* value.

EGCG dose (mM)	0.0	0.1	0.3	0.5	2.2
0.0	NA				
0.1	0.628	NA			
0.3	**<0.001**	**<0.001**	NA		
0.5	**0.011**	0.044	**<0.001**	NA	
2.2	**<0.001**	**<0.001**	**<0.001**	**<0.001**	NA

Supplementary Table 2. Variation in food intake in Experiment 2. The results of *post hoc* tests using the package lsmeans in R, asking how total intake of foods varied across the dietary treatments used in Experiment 2. Only *P* values are presented and those that are significant highlighted in bold font.

DIET (EAA:C)	0:1	1:250	1:100	1:75	1:50	1:25	1:10	1:5
0:1	NA							
1:250	0.974	NA						
1:100	0.999	0.874	NA					
1:75	0.719	0.145	0.920	NA				
1:50	0.912	0.311	0.990	0.999	NA			
1:25	0.095	0.626	**0.039**	**0.000**	**0.001**	NA		
1:10	1.000	0.996	0.998	0.537	0.786	0.186	NA	
1:5	0.774	0.999	0.546	**0.032**	0.089	0.921	0.905	NA