Nutrition affects survival in African honeybees exposed to interacting stressors

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Summary

1. Nutrition plays an important role in physiological stress resistance and by adjusting their intake of key nutrients, such as protein and carbohydrate, many animals can better resist stress.
2. Poor nutrition may contribute to the widespread and on-going declines of honeybee populations by increasing their vulnerability to abiotic (e.g. pesticides) and biotic (e.g. diseases) stressors. However, we do not know how nutrition affects stress resistance in social insects such as honeybees.
3. Here, we examined how exposure to the toxic secondary metabolite nicotine, a neurotoxin that shares structural similarities with the neonicotinoid pesticides, and low temperatures affected nutrient regulation in honeybees using the Geometric Framework of nutrition.
4. Groups of queenless, newly emerged worker bees were given diets containing specific ratios of protein and carbohydrate to determine, first, how toxin exposure and ambient temperature affected their nutrient intake and, secondly, how nutrition affected survival under stress.
5. We find that low temperatures and nicotine interacted to reduce survival in African honeybees that ate low protein, high carbohydrate diets. However, bees fed a high protein diet were better able to survive insult with these interacting stressors.
6. Although protein conferred a survival benefit in honeybees exposed to these dual stressors, when allowed to self-select their diet, caged workers did not shift their intake towards a higher protein food to improve their survival under these stressful conditions.
7. We discuss the possible constraints on nutrient regulation in honeybees and the role that diet could play in their decline.

Key-words: Geometric Framework, honeybees, nicotine, nutrition, pollinator declines, thermoregulation

Introduction

In nature, animals often encounter changes in their environment, such as fluctuating temperatures or elevated rates of predation, that induce stress (Buchanan 2000). Exposure to stressful conditions carries costs; in the extreme, pronounced or prolonged stress can disrupt individual homeostasis so severely that it causes mortality (Marshall & Sinclair 2010). However, even moderate stress exposure elicits physiological or behavioural responses evolved to increase survival under stress, and resources are often required to produce or maintain these responses (Kourtis & Tavernarakis 2011). Key resources may include energy or a specific nutrient, such as protein to synthesise detoxification enzymes or carbohydrate to fuel thermoregulation (discussed in Simpson & Raubenheimer 2012). This means that stress resistance often depends on the amount (e.g. Bozinovic *et al*. 2007) or ratio of particular nutrients that an individual eats (Lee *et al*. 2006; Povey *et al*. 2009; Hawlena & Schmitz 2010). Where stress resistance relies on a specific nutrient, stressed animals should consume more of that limiting nutrient to better mount a stress response without incurring reduced fitness as a result of trade-offs with other life-history traits (Simpson & Raubenheimer 2012). For example, how well *Spodoptera littoralis* and *S. exempta* invest in immune function depends on their protein intake, and so immune responses are improved in individuals that consume protein-rich diets (Lee *et al*. 2006; Povey *et al*. 2009). When offered a choice of diets, bacterially challenged caterpillars therefore eat food with a higher protein content than that selected by healthy larvae (Povey *et al*. 2009). Likewise, rats exposed to low temperatures increase their consumption of carbohydrate, while holding their protein intake constant, to meet the energetic

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demands of thermoregulation (Musten, Peace & Anderson 1974).

Although there are examples of solitary insects adjusting their nutrient intake to increase their survival under stress (e.g. Povey *et al*. 2009; Hawlena & Schmitz 2010), we do not know the extent to which social insects such as honeybees (*Apis mellifera*) vary their nutrient intake in response to stressful stimuli. At the colony level, honeybee foraging strategies are remarkably flexible; foraging decisions are influenced by interactions between nest mates, direct inspection of pollen cells and the presence of brood (Schmickl & Crailsheim 2004). Changes in one or more of these stimuli signal to foragers that the colony's nutritional requirements have changed (Schmickl & Crailsheim 2004). For example, honeybee larvae need to eat large amounts of protein for growth and development (Moritz & Crailsheim 1987) and so a higher concentration of larval pheromone stimulates increased pollen foraging (Pankiw 2004). Working in the opposite direction, depleted pollen stores trigger brood cannibalism (Schmickl & Crailsheim 2001) and the earlier onset of foraging (Janmaat & Winston 2000). However, honeybees also regulate their individual nutrition (Altaye *et al*. 2010) and we do not know whether, or how well, individual honeybees, buffered within the relatively stable environment of the hive, are capable of adjusting their individual nutrient intake to improve their survival following environmental stress.

It is vital that we understand the relationship between diet and stress resistance in honeybees because bees are experiencing widespread declines in abundance and diversity (Biesmeijer *et al*. 2006; Kosior *et al*. 2007; Cameron *et al*. 2011), and managed populations of honeybees have recently suffered heavy overwintering losses (e.g. vanEngelsdorp *et al*. 2008). This decline in wild and managed pollinators threatens agricultural output (Steffan-Dewenter, Potts & Packer 2005) and ecosystem function (Cox & Elmqvist 2000). Several interacting factors have so far been implicated in causing bee declines (Vanbergen & the Insect Pollinators Initiative 2013), including pesticides (Henry *et al*. 2012), the inability to regulate brood at the optimal temperature (Oldroyd 2007), intensive agriculture (Kremen, Williams & Thorp 2002) and diseases (Cox-Foster *et al*. 2007). A poor diet, resulting from low availability or diversity of floral resources in agricultural landscapes, has been repeatedly highlighted as one factor that could increase colony vulnerability to these other stressors (Cox-Foster *et al*. 2007; Brodschneider & Crailsheim 2010; Potts *et al*. 2010; Ratnieks & Carreck 2010; Vanbergen & the Insect Pollinators Initiative 2013). However, this hypothesis has not been tested directly.

We examined how intake of protein and carbohydrate affects the survival of caged honeybees (*A. mellifera scutellata* Lepeletier: Fig. 1) for fourteen days following emergence from the brood comb. Fourteen days is the period that honeybee workers remain within their natal colony. We exposed these workers to three doses of nicotine (0, 3 and 300 μM) and two temperatures (a low temperature of 30 °C and the optimal hive temperature of 35 °C) in a fully factorial design. Nicotine is a secondary metabolite produced by tobacco plants (Steppuhn *et al*. 2004). This highly toxic alkaloid (Detzel & Wink 1993) acts as an agonist of insect nicotinic acetylcholine receptors and so is an efficient defence against insect herbivores (Steppuhn *et al*. 2004). Nicotine is structurally similar to the neonicotinoids (Tomizawa & Casida 2005); long-lasting systemic pesticides that, in 2005, had a share of 16% of the agrochemical market and 77% of the insecticidal seed treatment market (Elbert *et al*. 2008). Neonicotinoids reduce honeybee foraging success (Henry *et al*. 2012), increase their susceptibility to pathogens (Alaux *et al*. 2010) and, at high doses, can reduce survival (Iwasa *et al*. 2004). Accordingly, neonicotinoids may play a role in honeybee declines. Resisting toxins such as nicotine may require specific nutrients; in some insects, dietary protein is needed for the production of detoxification enzymes (Berenbaum & Zangerl 1994). Similarly, surviving exposure to low temperatures may also rely on consumption of particular nutrients. Low ambient temperatures mean that honeybees have to invest heavily in metabolic heat production to regulate the temperature of their brood nest to around 34–35 °C (Kronenberg & Heller 1982; Jones *et al*. 2004). Brood reared at lower temperatures show morphological deformities (Himmer 1932) and impaired learning, communication and navigational abilities as adults (Tautz *et al*. 2003; Jones *et al*. 2005). The poor quality of these brood when they mature into foragers could reduce their survival and, in turn, help to drive colony collapse (Oldroyd 2007). However, thermoregulation is energetically expensive (Stabentheiner *et al*. 2003) and so is likely to require carbohydrate (Musten, Peace & Anderson 1974; Raubenheimer & Simpson 1997; Altaye *et al*. 2010). Therefore, we predicted that high protein intake will reduce mortality in honeybees exposed to nicotine, while carbohydrate consumption will improve survival in bees exposed to the cold and that bees will self-select their diet to improve their survival under stress.

Fig. 1. The study organism *Apis mellifera scutellata* (photographed by Ludwig Eksteen).

Materials and methods

ANIMALS AND CAGES

Frames containing capped worker brood were taken from each of four colonies of *A. mellifera scutellata* maintained at the

University of Pretoria apiary. These frames were transferred to the laboratory and incubated at 35 °C in constant dark to simulate the conditions within a hive. Groups of 100 workers were collected from the brood frame within 24 h of their emergence from the comb and then housed in standard polycarbonate hoarding cages (11 cm × 8·5 cm × 7 cm), which were closed at the front and back with movable slides and had a wire mesh base to allow ventilation (Köhler, Nicolson & Pirk 2013). At the front of each cage, beneath the moving slide door, was a plastic feeding frame with three round windows into which feeding vials could be inserted. These feeding vials were plastic tubes with a 2 cm window on the upper surface at the end furthest from the lid. This window allowed bees to enter the vial and collect food stored at the opposite end. Water was provided in a similar tube but with a smaller feeding window, and each cage contained a small piece of comb (5 cm × 5 cm) (Pirk *et al*. 2010). Cages were randomly allocated to a temperature treatment (30 or 35 °C), a nicotine dosage (0, 3, and 300 μM) and dietary regime.

In each experiment, bees were maintained under one of two temperatures and fed one of three doses of nicotine. Hence, we had six different treatment regimes. For the choice experiment, which is detailed below, one cage of 100 bees was established for each of four colonies and for each stressor combination and given one of two pairs of diets (0 : 1 vs. 1 : 1, 0 : 1 vs. 1 : 3 Protein (P) : Carbohydrate (C). This meant we had 48 cages in total. For the no choice experiment, bees from three colonies (the fourth having become infested with small hive beetle between experiments) were set up for each stress treatment with one of three diets, meaning that we had 54 cages.

EXPERIMENT ONE: CHOICE EXPERIMENT

We used the Geometric Framework of nutrition (Simpson & Raubenheimer 2012) to identify how stress affected the dietary optimum of newly emerged worker honeybees. The assumption behind this technique is that animals, when offered a choice of diets, eat a ratio of nutrients that should improve expression of a particular fitness determining trait or overall performance (Simpson & Raubenheimer 2012). This optimum (known as the intake target) is identified by providing nutritionally imbalanced diets and identifying the proportion of nutrients that animals choose (Simpson & Raubenheimer 2012).

Bees were fed diets composed of 0·5% agar solution, in a ratio of one part agar solution to two parts dry ingredients (33·3% water content). Using agar as a medium allowed us to uniformly incorporate both protein (P), in the form of casein (C7078; Sigma Aldrich, St. Louis, MO, USA), and sucrose as a source of carbohydrate (C) in a solid food (Table 1). Diets were stored at −20 °C prior to use. Each cage was provided with two feeding vials, one of which contained a sucrose only diet (0 : 1 P : C) and the other casein and sucrose in either a 1 : 1 P : C or a 1 : 3 P : C ratio. These diets were made containing 0, 3 or 300 μM nicotine as in the liquid diets used by Köhler, Pirk & Nicolson (2012a).

Table 1. The percentage by mass of each ingredient added to the diets. Nicotine was added with the water and agar where appropriate

Ratio P : C	Water + agar (%)	Casein (%)	Sucrose (%)
0 : 1	33·33	0	66·66
1 : 1	33·33	33·33	33·33
1 : 3	33·33	16·67	50·00
1 : 6	33·33	9·52	57·14

On the day of emergence from the brood comb, cohorts of 100 bees from each of the four colonies were transferred to a cage and given one block of each food type between 1.3 and 1.6 g, weighed to the nearest 0·1 mg, and water *ad libitum*. Food and water were replaced daily; uneaten food was stored at −20 °C until the end of the experiment, at which point it was dried to constant mass at 45 °C. Survival of bees was monitored, and dead bees were counted and removed from the cages each day. To calculate daily dry food consumption, control cages for each diet, containing a vial of water and pre-weighed blocks of food, were placed in incubators at the same time as the experimental cages. For each batch of food we made, 20 blocks of various sizes were weighed (wet mass), then dried and reweighed (dry mass). Plotting wet mass against dry mass allowed us to construct a regression equation for converting the wet mass of food provided into its original dry mass. By subtracting the dry mass of food remaining from the original dry mass, we could calculate consumption.

EXPERIMENT TWO: NO CHOICE EXPERIMENT

Methods were as in Experiment One, but instead of two vials of complementary foods, two vials of the same diet were provided to experimental animals. The aim of this experiment was to force bees to eat the diet similar to that they defended in Experiment One (1 : 6 P : C) or diets that contained more protein (1 : 3 P : C) or carbohydrate (0 : 1 P : C) than this target, to determine how these nutrients affected survival of stressed and control animals. Between Experiments one and two, one colony became infected with small hive beetles; from two full brood frames, we were able to collect only 900 bees of the necessary 1800 from this colony, and the bees were small and often malformed. Hence, we did not use this replicate for the second experiment. The remaining three colonies were the same across the two experiments.

STATISTICS

Choice experiment: nutrient intake

All analyses were conducted in R version 3.0.1 (R Core Development Team 2013). To analyse the final consumption of each diet or nutrient, we took a linear mixed modelling approach using the 'lme4' package (Bates, Maechler & Bolker 2013). The response variable 'consumption' was always analysed per bee surviving in the cage, and for any analysis of cumulative intake, we summed daily consumption per bee to account for diminishing survival. The random effect of 'cage' was included in our models whenever there were multiple measures for the same colony and treatment group (i.e. random vs. actual intake), and the random effect colony was always included unless the simpler model (excluding colony) had a significantly lower Akaike's information criterion (AIC) value than the full model (including colony) (Pirk *et al*. 2013). All models contained the explanatory variables nicotine dose and temperature, and the effect of each was assessed using backward model selection. This approach was used to test whether bees ate randomly from each diet type, whether intake of each nutrient differed from that expected had bees eaten at random (following the protocol outlined in South *et al*. 2011) and how stress affected the final consumption of protein and carbohydrate. To analyse daily intake of each nutrient, a linear mixed effects model with repeated measures was fitted and following model simplification, *post hoc* Tukey–Kramer tests were conducted in the 'multcomp' R package (Hothorn *et al*. 2013). For all analyses, if the residuals of the minimum adequate model were not normally distributed,

response variables were transformed using either a log, square root transformation or the function 'powerTransform' in the 'car' package R (Fox & Weisberg 2011).

Survival analyses

To analyse survival data, we fitted parametric survival models using the function 'survreg' in the 'survival' package (Therneau 2013), fitting colony and, for the choice trial, dietary pair, using the 'frailty' function. We fitted five models with different survival functions – Weibull, Gaussian, exponential, extreme, or logistic – and compared each model's AIC values to see which best described the mortality function. The risk of dying was calculated relative to survival of animals on a pure sucrose diet (no choice) in stress free conditions. To directly compare survival on different diets (1 : 3 and 1 : 6), the model output was rearranged and the 1 : 6 P:C diet used as a baseline for comparison.

Results

EXPERIMENT ONE: NUTRIENT REGULATION WHEN GIVEN DIETARY CHOICE

Non-random diet consumption

Analyses using a mixed modelling approach and backwards model selection showed that when given a choice between a pure sucrose diet (0 : 1 P : C) and one that contained equal amounts of protein and carbohydrate (1 : 1 P : C), bees ate more of the pure carbohydrate than the protein-containing diet ($L_{4,3}$ = 70·545, P < 0·001), irrespective of nicotine dose ($L_{7,5}$ = 0·203, P = 0·904), or temperature treatment ($L_{5,4}$ = 0·088, P = 0·767) (Fig. 2a, b). When bees were offered a 1 : 3 P : C diet, there was a marginally non-significant interaction between nicotine intake and diet ($L_{10,8}$ = 5·932, P = 0·052), but overall, bees ate significantly different amounts of each diet type ($L_{5,4}$ = 7·481, P = 0·006) (Fig. 2c,d).

We then analysed intake of protein and carbohydrate to determine how this strategy of diet intake affected consumption of each nutrient. Bees given a choice between a 0 : 1 and a 1 : 1 P : C diet ate significantly less protein than expected had they eaten at random from each food ($L_{4,3}$ = 53·609, $P \leq 0{\cdot}001$) and more carbohydrate ($L_{4,3}$ = 50·852, $P \leq 0{\cdot}001$) across all treatment groups (Fig. 3a,b). When offered a choice between the 0 : 1 and a 1 : 3 P : C diets, intake of protein and carbohydrate was affected by an interaction with nicotine: at high nicotine doses bees ate significantly more protein ($L_{10,8}$ = 6·824, P = 0·033) and less carbohydrate ($L_{10,8}$ = 7·450, P = 0·024) (Fig. 3c,d).

Nutrient intake targets

Analyses of the final intake of both carbohydrate and protein showed that control bees, reared at optimal temperatures and in the absence of nicotine, consumed a P : C ratio of 1 : 6·5 (Fig. 4). This total consumption of protein or carbohydrate per bee did not differ significantly between choice trials (*protein* $L_{5,4}$ = 0·722, P = 0·395;

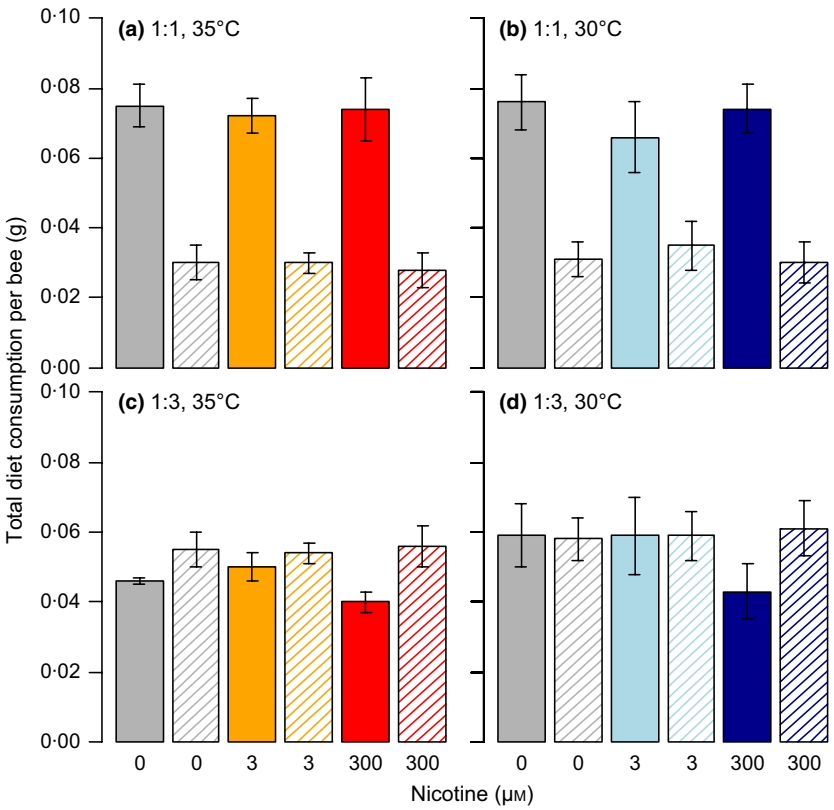

Fig. 2. The total consumption of each diet type per bee, averaged across experimental colonies. The sucrose diet (0 : 1 P : C) is represented by solid bars, while the 1 : 1 P : C (a, b) and the 1 : 3 P : C (c, d) diets are dashed bars. Graphs a and c show consumption of workers reared at 35 °C exposed to 0 μM (grey), 3 μM (orange) and 300 μM (red) of nicotine, while graphs b and d show intake of each diet at 30 °C and treated with 0 μM (grey), 3 μM (light blue) and 300 μM (dark blue) nicotine. Error bars represent standard error around the mean.

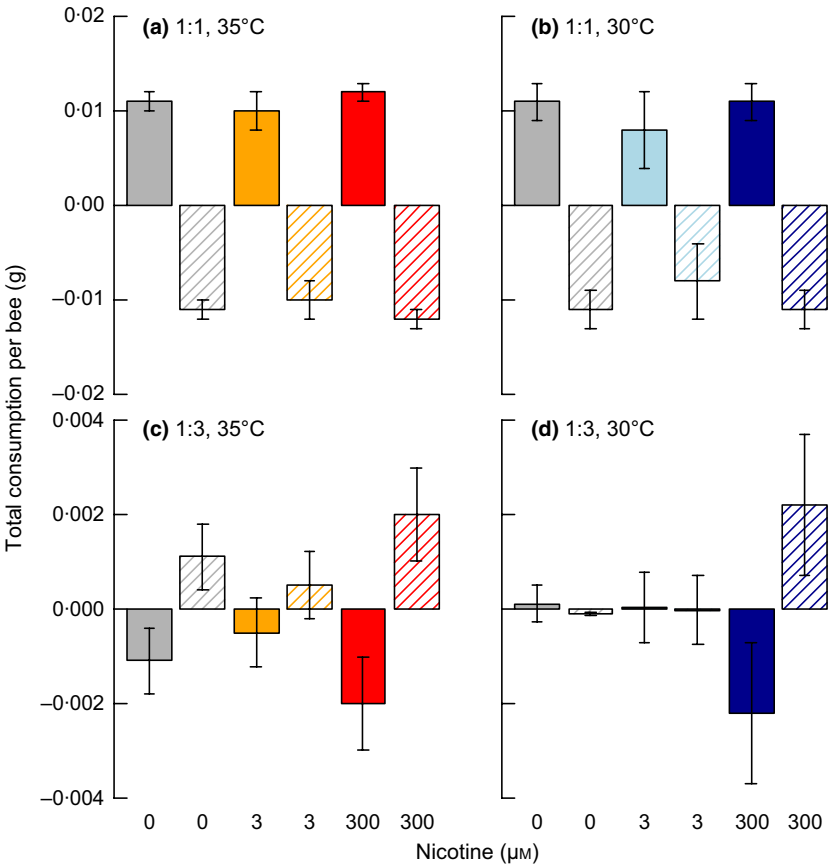

Fig. 3. Total consumption per bee of each nutrient relative to that if bees had eaten randomly (random consumption = 0), averaged across colonies. In this graph, the solid bars show intake of carbohydrate, while the dashed bars represent protein. Graphs (a) and (c) show consumption of workers reared at 35 °C exposed to 0 μM (grey), 3 μM (orange), and 300 μM (red) of nicotine, while graphs (b) and (d) show intake of each diet at 30 °C and treated with 0 μM (grey), 3 μM (light blue) and 300 μM (dark blue) nicotine. Error bars represent standard error around the mean.

carbohydrate $L_{5,4} = 0{\cdot}903$, $P = 0{\cdot}342$), meaning that bees reached the same nutritional end point irrespective of dietary pairing. This final intake was not affected by nicotine dose (*protein* $L_{7,5} = 0{\cdot}754$, $P = 0{\cdot}686$; *carbohydrate* $L_{7,5} = 1{\cdot}925$, $P = 0{\cdot}382$) or temperature regime (*protein* $L_{4,3} = 2{\cdot}185$, $P = 0.139$; *carbohydrate* $L_{4,3} = 1{\cdot}44$, $P = 0{\cdot}230$).

However, in honeybees, there are reasons to expect that nutrient demands differ according to age. Indeed, analyses of daily intake of protein and carbohydrate reveals a more complex pattern. Daily intake of protein and carbohydrate per bee was affected by a significant interaction between temperature and the age of bees (*protein* $L_{32,19} = 60{\cdot}830$, $P \leq 0{\cdot}001$; *carbohydrate* $L_{32,19} = 73{\cdot}013$, $P \leq 0{\cdot}001$) (Fig. 5). Nutrient intake increased for the first few days following emergence from the brood comb and then stabilised (for full results see the *post hoc* tests in the Supporting information). However, this pattern depended on whether the bees were reared at 30 or 35 °C. Protein consumption was significantly lower in newly emerged bees (1–2 days) than other age classes, while bees aged 3 days or less ate significantly less carbohydrate than older bees. Moreover, in the 30 °C treatment group, during the period of maximum intake, bees ate more than similarly aged or slightly older bees in the 35 °C treatment group.

The effect of age on nutrient intake suggests that we should characterise the intake target across discrete developmental stages, identified via the *post hoc* test output (see Supporting information). In bees reared at 35 °C, *post hoc* testing highlights two distinct phases of nutrient regulation; the nutrient intake of very young bees (1–3 days old) differs from that of older workers (4+ days). The daily ratio of P : C consumed in these very young animals (1 : 5·8) contains more protein than that selected by older workers (1 : 6·9). At 30°C, *post hoc* tests reveal three distinct periods of nutrient regulation, early (1–3 days), mid (4–10) and late (11–14), the daily intake ratios of these age classes are 1 : 6·0 P : C, 1 : 6·5 and 1 : 7·5 P : C, respectively.

Survival in bees allowed a choice of diets

In the choice experiment, survival analyses revealed an interacting effect of stress exposure on survival (Table 2, Fig. 6). Bees exposed to low temperature (30 °C) and fed a low dose of nicotine had poorer survival than control animals reared at 35 °C and fed diets without nicotine. Independently, neither of these stressors reduced survival; instead, a high dose of nicotine improved survival in bees at 35 °C.

EXPERIMENT TWO: SURVIVAL ON A SINGLE DIET

In one colony in the choice experiment, bees fed a 1 : 6 P : C ratio and exposed to high nicotine and low temperatures exhibited a pronounced die-off such that all bees were dead at day seven. Using parametric survival

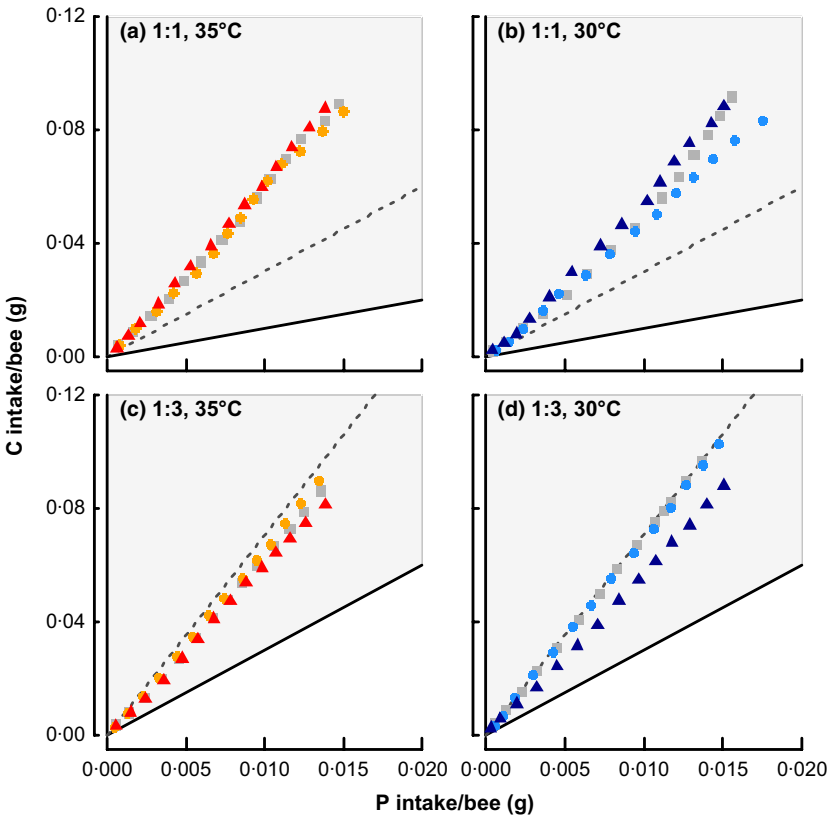

Fig. 4. Cumulative intake of protein and carbohydrate per bee when bees were offered a choice of a pure sucrose diet and one containing 1 : 1 P : C (a, b) or 1 : 3 P : C (c, d) at 35 °C (a, c) and 30 °C (b, d). Grey square symbols correspond to the intake of bees reared without nicotine, circles (orange at 35 °C and blue at 30 °C) represent bees fed 3 μM of nicotine, while dark triangles (red at 35 °C and dark blue at 30 °C) represent bees fed 300 μM of nicotine. Dotted grey lines represent random intake, while solid lines correspond to nutritional rails. The grey area shows the nutrient landscape bees could feed within.

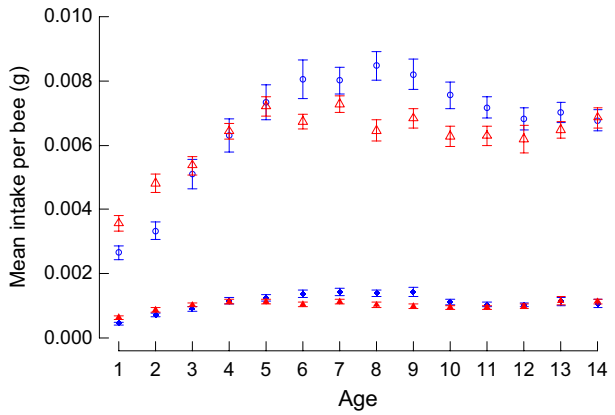

Fig. 5. Daily intake of protein (filled symbols) and carbohydrate (open symbols) at 30 °C (blue circles) and 35 °C (red triangles) per bee, averaged across all nicotine doses and colonies. Error bars represent standard error around the mean.

analyses, we analysed data including and excluding this replicate; results were qualitatively the same, but because the die-off was so severe relative to all other experimental cages, we excluded this cage in our final analyses.

Bees fed diets containing some protein (1 : 6 or 1 : 3 P : C) survived better than those fed sucrose solution alone (0 : 1 P : C) (Table 2, Fig. 7). Qualitatively, the risk of dying was lowest on the 1 : 6 P : C diet, which is closest to the intake target identified in Experiment One. Although eating the 1 : 6 P : C diet improved survival relative to the 1 : 3 P : C diet, this difference was only significant when bees were reared at 30 °C ($Z = 3{\cdot}134$, $P = 0{\cdot}002$) not 35 °C ($Z = 1{\cdot}068$, $P = 0{\cdot}209$). Independently, neither low temperatures nor nicotine reduced survival. The effect of nicotine on survival depended on the rearing temperature: at 35 °C, honeybees fed a high dose of nicotine had a lower risk of dying than those not treated with nicotine, but at 30 °C, bees fed nicotine had a greater risk of dying than the control bees. This effect was most pronounced in bees fed the 1 : 6 P : C diet, and in these animals, treatment with both stressors completely obliterated the survival advantage conferred by eating this nutrient ratio (1 : 6 P : C) rather than a pure sucrose food. Only bees fed the high protein diet (1 : 3 P : C) did not experience elevated mortality when exposed to nicotine and low temperatures.

Discussion

Although neither low temperatures nor nicotine consumption independently reduced survival in African honeybees, workers that were exposed to both stressors experienced elevated mortality. Only bees fed a high protein diet could tolerate this combination of stressors without experiencing reduced survival. Despite this, bees reared under these stressful conditions and offered a choice of diets did not eat more protein. Taken together, these results show that nutrition affects survival under stress but that honeybees do not mitigate the costs of stress exposure by flexible dietary choices. We discuss factors that may shape nutrient regulation in worker bees and then consider the mechanisms by which nutrition

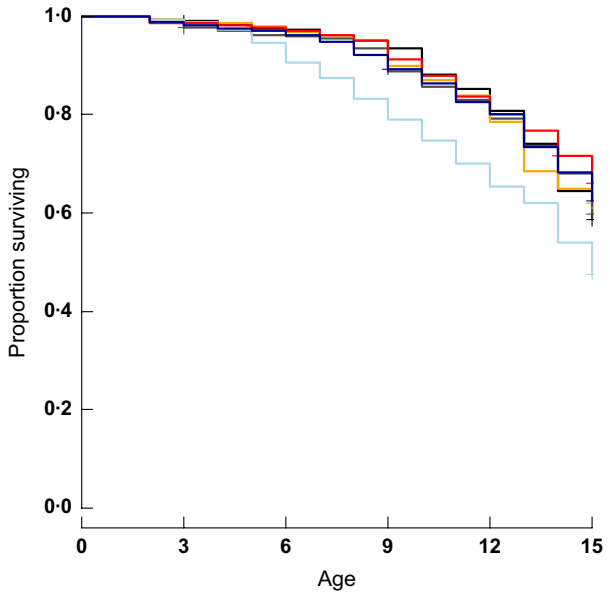

Fig. 6. Honeybee survival from Experiment One where bees were provided with a choice of diets (0 : 1 P : C and 1 : 3 P : C or 0 : 1 P : C and 1 : 1 P : C), while reared at one of two temperatures and fed 0 μM (35 °C – black, 30 °C – grey), 3 μM (35 °C – orange, 30 °C – light blue) and 300 μM of nicotine (35 °C – red, 30 °C – dark blue).

affects survival in stressed and unstressed animals. Finally, we discuss the possible constraints on nutrient regulation in honeybees and the role that diet could play in their current declines.

HOW AGE AND ENVIRONMENT AFFECT NUTRIENT REGULATION IN CAGED HONEYBEES

Like ants (Dussutour & Simpson 2009), honeybee workers are able to select from a choice of diets to regulate their intake of protein and carbohydrate independently. Irrespective of the choice of diets they were given, bees ate non-randomly from each food to consume a low protein to high carbohydrate diet (control bees – 1 : 6·5 P : C). Although this final intake target was consistent across choice trials within our study, it is more protein biased than that found previously (Altaye *et al.* 2010). However, in this earlier study, the most protein-rich food on offer for some choice trials was 1 : 10 P : C, meaning that honeybees could not reach a more protein-biased target than this when self-selecting their nutrient intake. Unfortunately, this means we cannot draw comparisons between the two results.

Although the final intake of protein and carbohydrate by worker bees was not affected by stress exposure, bees ate more food as they aged, and their peak intake was greater when they were exposed to 30 °C. Following emergence from the brood comb, young honeybees increased their daily intake of both nutrients, with daily protein intake stabilising before intake of carbohydrate and consumption of both nutrients plateauing earlier in warm conditions. This pattern of intake probably reflects the influence of age-dependent task specialisation or age polyethism. In honeybee colonies, newly emerged workers clean cells (days 1–3), while older workers (days 4–12) nurse brood (Johnson 2010). These nurse bees feed larvae protein-rich secretions from their hypopharyngeal glands, which develop from eclosion until around day nine (Crailsheim *et al.* 1992). The development of these glands requires protein (Crailsheim *et al.* 1992), which may explain the age-dependent increase in protein consumption we see in this experiment. Additionally, nurse bees ensure the brood chamber remains around 34–35 °C, which often entails investing in endothermic heat production (Stabentheiner, Kovac & Brodschneider 2010). Generating heat is

Table 2. Results of parametric survival analyses for both Experiment One (Choice experiment) and Experiment Two (No choice). Survival of bees reared at 35 °C in the absence of nicotine is used as a reference level (i.e. that which other treatments are compared against) for the choice experiment. For the no choice experiment, we use bees fed a pure sucrose diet (0 : 1 P : C) reared at 35 °C and not fed any nicotine to compare all other treatment groups against. Terms in brackets represent the factor levels against which the reference levels are compared. Eight bees escaped from the experiment, and these were included in the analyses, but their life span was 'censored', that is, the age they were last seen alive was included in the analyses

Explanatory variable	Coefficient	SE (coeff)	*Z*	*P* value
Choice experiment (4 colonies)				
Temperature (cold = 30)	−0·035	0·281	−0·124	0·901
Low nicotine	0·022	0·282	0·079	0·937
High nicotine	0·610	0·287	2·126	0·034
Cold*low nicotine	−1·910	0·393	−4·855	<0·001
Cold*high nicotine	−0·438	0·404	−1·084	0·278
No choice experiment (3 colonies)				
Temperature (cold = 30)	0·061	0·400	0·153	0·879
Low nicotine	0·324	0·407	0·795	0·427
High nicotine	1·305	0·421	3·099	0·002
Diet (1 : 6)	3·020	0·482	6·260	<0·001
Diet (1 : 3)	2·446	0·471	5·191	<0·001
Cold*low nicotine	−0·736	0·571	−1·290	0·197
Cold*high nicotine	−1·919	0·584	−3·288	0·001
Cold*diet (1 : 6)	4·964	0·919	5·403	<0·001
Cold*diet (1 : 3)	1·783	0·717	2·487	0·013
Low nicotine*diet (1 : 6)	0·430	0·695	0·618	0·537
High nicotine*diet (1 : 6)	−1·081	0·694	−1·557	0·119
Low nicotine*diet (1 : 3)	0·726	0·695	1·045	0·296
High nicotine*diet (1 : 3)	0·193	0·706	0·273	0·785
Cold*low nicotine*diet (1 : 6)	−2·499	1·204	−2·077	0·038
Cold*high nicotine*diet (1 : 6)	−6·695	1·160	−5·771	<0·001
Cold*low nicotine*diet (1 : 3)	−0·536	1·025	−0·523	0·601
Cold*high nicotine*diet (1 : 3)	−0·138	1·024	−0·134	0·893

As discussed in the methods, this analysis excludes the replicate for one colony, exposed to a high dose of nicotine and fed a 1 : 6 ratio of P : C, which experienced a high and rapid die-off before day 7.

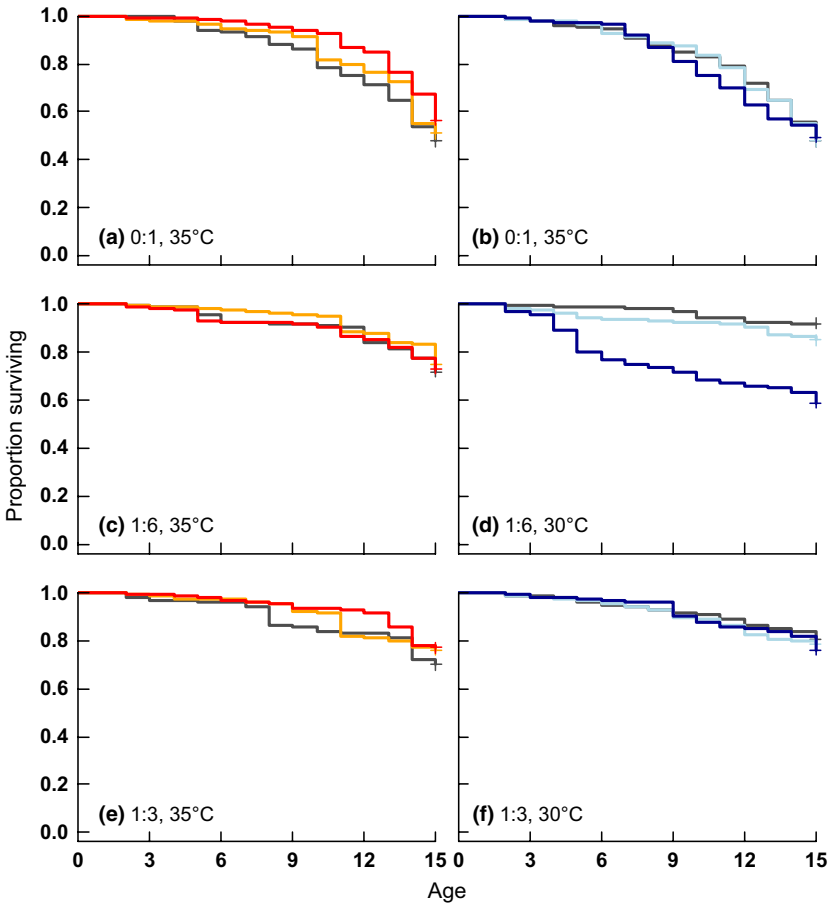

Fig. 7. Survival from Experiment Two, where bees were constrained to a single nutritionally imbalanced diet that contained protein to carbohydrate in a 0 : 1, 1 : 6, or 1 : 3 ratio. Figures show the proportion of bees surviving at 35 °C (a, c and e) and at 30 °C (b, d and f), fed 0 (grey), 3 (35 °C – orange, 30 °C – light blue), and 300 μM of nicotine (35 °C – red, 30 °C – dark blue). Each panel represents survival of bees fed a particular diet that is identified within the graph to facilitate interpretation, that is, 0 : 1 – a and b, 1 : 6 – c and d, 1 : 3 – e and f.

energetically very expensive (Stabentheiner *et al*. 2003) and so may explain the age-dependent increase in carbohydrate consumption, particularly in cold conditions when the energetic demands of thermoregulation are greater (Stabentheiner *et al*. 2003). Crucially, this age-dependent variation in nutrient intake suggests that bees can adjust their individual nutritional demands given their changing nutritional needs.

We measured nutrient regulation in groups of bees and therefore are unable to determine how well individuals regulate their intake. However, honeybee behaviours depend critically on social interactions. Social interactions are so important that small groups of bees may exhibit poor survival (Rinderer & Baxter 1978) and fail to perform behaviours commonly seen in larger groups (Hepburn 1986). Social interactions also affect feeding and foraging; adult bees regularly transfer food via trophallactic interactions (Crailsheim 1998), which may help communicate information regarding the profitability of food sources (Marco & Farina 2001). The influence of interactions with nest mates on honeybee performance and behaviour means that studying dietary regulation in individual bees is unlikely to produce meaningful data. Crucially, the age class of bees studied here is central in determining colony level nutrient regulation. They help feed larvae, the queen and workers and are responsible for retrieving food from returning foragers and storing it (discussed in Crailsheim 1998). Therefore, studying this age group offers insight into the changing nutritional requirements of a colony exposed to stress. However, future work needs to consider an even larger scale of organisation, the colony.

THE MECHANISTIC BASIS OF NUTRITIONAL EFFECTS ON STRESS AND SURVIVAL

Independently, neither nicotine nor low temperature exposure reduced worker bee survival. This was surprising; although adult bees are able to tolerate cold conditions by investing in endothermy (Stabentheiner, Kovac & Brodschneider 2010), honeybees have very few genes encoding detoxification enzymes and so are often susceptible to environmental toxins (Claudianos *et al*. 2006). Indeed, nicotine can cause mortality in adult worker bees (Detzel & Wink 1993; Köhler, Pirk & Nicolson 2012a). However, nicotine can also have a negligible (Singaravelan *et al*. 2006) or even a positive effect (Köhler, Pirk & Nicolson 2012a) on honeybee survival, although the effect of particular nicotine doses appears to depend on colony condition (Köhler, Pirk & Nicolson 2012a). There are two possible reasons for this. First, nicotine has both antibacterial (Zaidi *et al*. 2012) and antiviral effects (Yamashina *et al*. 2008) and so could improve honeybee health and survival. Alternatively,

the life-extending effect of nicotine may reflect a process called stress response hormesis (reviewed in Gems & Partridge 2008), whereby exposure to mild stressors induces a cascade of stress responses, some of which are associated with elevated somatic maintenance. In turn, this increased investment in the soma promotes survival. In *Caenorhabditis elegans* for example, brief exposure to a number of stresses, including heat and hyperbaric oxygen, can extend life span (Cypser & Johnson 2002).

Although the effects of nicotine or low temperatures were not individually lethal, these stressors interacted to cause elevated mortality in bees exposed to both. Such interactions between stressors are widespread; for example, sublethal or naturally occurring doses of neonicotinoid pesticides increase susceptibility to the gut parasite *Nosema* (Alaux *et al*. 2010; Pettis, Johnson & Dively 2012). Likewise, nicotine and *Escherichia coli* lipopolysaccharides interact additively to reduce life span (Köhler, Pirk & Nicolson 2012b). Characterising such interactions between stressors is an absolute priority in pollinator loss research (Vanbergen & the Insect Pollinators Initiative 2013) and we find that nutrition adds one additional layer to this already complicated picture.

Unstressed bees, if they ate a diet similar to the intake target (i.e. 1 : 6 P : C), had a significantly lower risk of dying relative to sucrose controls. Eating a 1 : 3 P : C diet also improved survival relative to sucrose controls, but to a slightly lesser extent. In bees exposed to the dual stressors of nicotine and a low temperature, survival was reduced in animals fed both the pure carbohydrate and the low carbohydrate diets. The only bees apparently able to withstand insult with these two stressors were those that ate high protein food. This suggests that protein conferred a survival benefit in bees challenged with these stressors, presumably by increasing their ability to mount a stress response. Protein may increase survival in bees treated with nicotine because bees may need a dietary source of amino acids to manufacture detoxification enzymes. This is the case in gypsy moth larvae, which when fed low protein diets had lower glutathione transferase activity (Lindroth, Anson & Weisbrod 1990). Likewise, parsnip webworm larvae can maintain their detoxification capacity on low protein diets only at the expense of reduced growth (Berenbaum & Zangerl 1994). In honeybees, ingesting nicotine is associated with upregulation of enzymes involved in detoxification and energy metabolism (Du Rand, unpublished data). While nicotine may increase a bee's demand for protein, at the same time it could reduce nutrient assimilation efficiency. There is some evidence for this in parasitoids of tobacco hornworm, where host nicotine intake appears to reduce the efficiency of nutrient assimilation in developing parasitoids (Bentz & Barbosa 1992).

Similarly, low temperature could reduce the rate of nutrient assimilation from protein digestion. In locusts (*Locusta migratoria*) fed kangaroo grass, protein was absorbed more efficiently at 38 °C than at 32 °C, while the reverse was true for carbohydrate (Clissold, Coggan & Simpson 2013). Additionally in honeybees, proteolytic activity is lower in workers surviving over winter than in those collected over summer (Crailsheim *et al*. 1993). Thus, the interaction effect of nicotine and low temperatures in our studies on life span could be explained if nicotine increased a bee's requirement for protein, while low temperatures and nicotine simultaneously reduced the digestibility of protein. This would exacerbate the consequences of deficiencies and cause elevated mortality on low protein diets.

As a result, we predict that protein deprivation, particularly over winter, when bees are surviving on stores that may be contaminated with pesticides (Chauzat *et al*. 2009), would carry severe costs. The importance of protein within the hive is illustrated by the behaviours elicited in honeybee colonies: if pollen stores drop below a finely tuned homeostatic set point, workers cannibalise brood (Schmickl & Crailsheim 2001) and begin foraging earlier (Janmaat & Winston 2000). Protein is likely to be particularly important in African honeybees, because African and Africanised honeybees, when compared with European subspecies of *A. mellifera*, readily abscond (non-reproductive swarming) and so require protein for investing heavily in brood production (Fewell & Bertram 2002) to allow rapid colony growth following absconding (Hepburn & Radloff 1998).

CONSTRAINTS ON NUTRIENT REGULATION IN HONEYBEES AND THE ROLE OF DIET IN THEIR DECLINE

Evidently, when exposed to nicotine and low temperatures, caged bees did not eat more protein to improve their survival. This is surprising given that solitary insects often exhibit flexible strategies of intake in response to stress exposure (discussed in Simpson & Raubenheimer 2012). This result may reflect that there may be comparatively weak selection for flexible nutrient intake in honeybees given that the hive is a relatively stable environment. Within the hive, temperatures are finely regulated and aspects of the hive environment appear to reduce the prevalence of pests and pathogens. For example, propolis, a resin collected by bees, has antibacterial properties (reviewed in Sforcin & Bankova 2011), and mathematical models predict that nest hygiene is an immediate and effective means of controlling diseases (Fefferman *et al*. 2007). Such colony level traits buffer the hive from environmental changes and so selection for flexible nutrient regulation to increase stress resistance may not be as strong in the honeybees as in solitary insects. However, more research is needed to see if inflexible intake is also seen in response to more severe stress exposure.

Recently, some authors have suggested that our increasing reliance on crop monocultures could lead to fewer and restricted floral resources and therefore malnutrition in bees, which could reduce the ability of colonies to

withstand stress (Cox-Foster *et al*. 2007; Brodschneider & Crailsheim 2010; Potts *et al*. 2010; Ratnieks & Carreck 2010; Vanbergen & the Insect Pollinators Initiative 2013). We find the situation is more complex; even when presented with nutritionally heterogeneous diets, the bees in this experiment did not adjust their intake to improve their survival when exposed to a lethal combination of nicotine and low temperatures. Further work is needed to understand the reasons for this and to characterise the relationship between nutrition and other stressors, in different subspecies and age groups of honeybees, to determine the role of diet in the decline of this important pollinator.

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Supporting information

Table S1. Summary of the *post hoc* test results for carbohydrate consumption across temperature regimes (30 and 35°C) in bees of different ages. Significant p-values are highlighted in bold.

Temp (°C)	**35**													
30	**Age (days)**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
	1	0.99	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
	2	1.00	0.38	**0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
	3	0.29	1.00	1.00	0.61	**<0.01**	0.18	**<0.01**	0.58	0.11	0.84	0.82	0.92	0.53	0.09
	4	**<0.01**	0.34	0.98	1.00	0.99	1.00	0.98	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	5	**<0.01**	**<0.01**	**0.02**	0.99	1.00	1.00	1.00	0.99	1.00	0.94	0.95	0.87	1.00	1.00
	6	**<0.01**	**<0.01**	**<0.01**	0.20	1.00	0.64	1.00	0.22	0.78	0.08	0.09	**<0.05**	0.25	0.81
	7	**<0.01**	**<0.01**	**<0.01**	0.24	1.00	0.71	1.00	0.27	0.83	0.11	0.12	0.07	0.30	0.86
	8	**<0.01**	**<0.01**	**<0.01**	**0.01**	0.73	0.11	0.83	**0.02**	0.18	**<0.01**	**<0.01**	**<0.01**	**0.02**	0.21
	9	**<0.01**	**<0.01**	**<0.01**	0.08	0.97	0.39	0.99	0.09	0.54	**0.03**	**0.03**	**0.02**	0.11	0.58
	10	**<0.01**	**<0.01**	**<0.01**	0.89	1.00	1.00	1.00	0.91	1.00	0.69	0.71	0.56	0.93	1.00
	11	**<0.01**	**<0.01**	0.07	1.00	1.00	1.00	1.00	1.00	1.00	0.99	0.99	0.98	1.00	1.00
	12	**<0.01**	**0.02**	0.42	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	13	**<0.01**	**<0.01**	0.17	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	14	**<0.01**	**0.02**	0.49	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table S2. Summary of the *post hoc* test results for carbohydrate consumption within temperature regimes (30 and 35°C) in bees of different ages. Significant p-values are highlighted in bold.

Temp (°C)	**35**														
30	**Age (days)**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
	1		0.21	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
	2	0.99		1.00	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.04**	**0.03**	0.08	**<0.01**	**<0.01**
	3	**<0.01**	**<0.01**		0.54	**<0.01**	0.08	**<0.01**	0.50	**0.04**	0.84	0.82	0.93	0.44	**0.03**
	4	**<0.01**	**<0.01**	0.26		0.97	1.00	0.91	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	5	**<0.01**	**<0.01**	**<0.01**	0.63		1.00	1.00	0.97	1.00	0.79	0.82	0.64	0.98	1.00
	6	**<0.01**	**<0.01**	**<0.01**	**<0.01**	0.99		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	7	**<0.01**	**<0.01**	**<0.01**	**<0.01**	1.00	1.00		0.93	1.00	0.66	0.69	0.49	0.95	1.00
	8	**<0.01**	**<0.01**	**<0.01**	**<0.01**	0.40	1.00	1.00		1.00	1.00	1.00	1.00	1.00	1.00
	9	**<0.01**	**<0.01**	**<0.01**	**<0.01**	0.89	1.00	1.00	1.00		1.00	1.00	1.00	1.00	1.00
	10	**<0.01**	**<0.01**	**<0.01**	0.21	1.00	1.00	1.00	0.83	1.00		1.00	1.00	1.00	1.00
	11	**<0.01**	**<0.01**	**<0.01**	0.90	1.00	0.88	0.93	0.15	0.61	1.00		1.00	1.00	1.00
	12	**<0.01**	**<0.01**	**<0.01**	1.00	1.00	0.23	0.29	**<0.01**	0.08	0.98	1.00		1.00	1.00
	13	**<0.01**	**<0.01**	**<0.01**	0.99	1.00	0.60	0.69	**0.04**	0.29	1.00	1.00	1.00		1.00
	14	**<0.01**	**<0.01**	**<0.01**	1.00	1.00	0.16	0.22	**<0.01**	**<0.05**	0.96	1.00	1.00	1.00	

Table S3. Summary of the *post hoc* test results for protein consumption across temperature regimes (30 and 35°C) in bees of different ages. Significant p-values are highlighted in bold.

Temp (°C)	**35**													
30	**Age (days)**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
	1	0.86	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
	2	1.00	1.00	0.20	**0.01**	**0.01**	0.13	**<0.01**	0.22	0.37	0.69	0.59	0.41	**0.01**	**<0.01**
	3	0.32	1.00	1.00	0.98	0.98	1.00	0.97	1.00	1.00	1.00	1.00	1.00	0.99	0.96
	4	**<0.01**	0.74	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	5	**<0.01**	0.21	1.00	1.00	1.00	1.00	1.00	0.99	0.97	0.80	0.87	0.96	1.00	1.00
	6	**<0.01**	**<0.01**	0.54	0.99	0.99	0.67	0.99	0.49	0.33	0.12	0.17	0.29	0.98	1.00
	7	**<0.01**	**<0.01**	0.18	0.82	0.80	0.23	0.85	0.16	0.08	**0.02**	**0.03**	0.07	0.79	0.87
	8	**<0.01**	**<0.01**	0.34	0.94	0.94	0.46	0.96	0.30	0.18	0.05	0.08	0.16	0.93	0.97
	9	**<0.01**	**<0.01**	0.46	0.98	0.97	0.58	0.98	0.41	0.26	0.09	0.12	0.23	0.97	0.99
	10	**<0.01**	0.93	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	11	**<0.01**	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	12	**<0.01**	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	13	**<0.01**	0.92	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	14	**<0.01**	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table S4. Summary of the *post hoc* test results for protein consumption within temperature regimes (30 and 35°C) in bees of different ages. Significant p-values are highlighted in bold.

Temp (°C)	**35**													
30	**Age (days)**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
	1		0.17	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
	2	**0.02**		0.98	0.31	0.33	0.94	0.26	0.99	1.00	1.00	1.00	1.00	0.34	0.22
	3	**<0.01**	0.48		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	4	**<0.01**	**<0.01**	0.65		1.00	1.00	1.00	1.00	1.00	0.97	0.99	1.00	1.00	1.00
	5	**<0.01**	**<0.01**	0.09	1.00		1.00	1.00	1.00	1.00	0.97	0.99	1.00	1.00	1.00
	6	**<0.01**	**<0.01**	**<0.01**	0.89	1.00		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	7	**<0.01**	**<0.01**	**<0.01**	0.40	0.97	1.00		1.00	1.00	0.95	0.98	1.00	1.00	1.00
	8	**<0.01**	**<0.01**	**<0.01**	0.69	1.00	1.00	1.00		1.00	1.00	1.00	1.00	1.00	1.00
	9	**<0.01**	**<0.01**	**<0.01**	0.82	1.00	1.00	1.00	1.00		1.00	1.00	1.00	1.00	1.00
	10	**<0.01**	**<0.01**	0.93	1.00	1.00	0.59	0.14	0.34	0.49		1.00	1.00	0.98	0.93
	11	**<0.01**	**<0.01**	1.00	1.00	0.95	0.14	**0.02**	0.05	0.10	1.00		1.00	0.99	0.97
	12	**<0.01**	**<0.01**	1.00	1.00	0.97	0.17	**0.02**	0.06	0.11	1.00	1.00		1.00	1.00
	13	**<0.01**	**<0.01**	0.91	1.00	1.00	0.63	0.16	0.37	0.52	1.00	1.00	1.00		
	14	**<0.01**	**<0.01**	1.00	1.00	0.95	0.14	**0.02**	0.05	0.09	1.00	1.00	1.00	1.00	1.00