




Effects of Non-Protein Amino Acids in Nectar on Bee Survival and Behavior

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Received: 9 July 2018 / Revised: 21 November 2018 / Accepted: 18 December 2018 / Published online: 6 January 2019
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Abstract

Nectar mediates complex interactions between plants and animals. Recent research has focused on nectar secondary compounds that may play a role in regulating some of these interactions. These compounds may affect the behavior of nectar feeders by interacting with their neurobiology. Non-protein amino acids (NPAAs) can constitute a large portion of the amino acid content of floral nectar, but their ecological function has, to date, not been investigated. In this study, we tested the effects of diets with low and high concentrations of γ -amino butyric acid (GABA) and β -alanine on the survival and behavior of *Bombus terrestris* and *Apis mellifera*. The most apparent effect on longevity was observed for *B. terrestris* workers that fed on high concentration of GABA, with longevity increased. By contrast, neither of the two NPAAs (at either concentration) had an effect on *A. mellifera* longevity. At the low NPAA concentration, only *B. terrestris* workers showed a difference in consumption, consuming more β -alanine solution than the other two solutions. By contrast, at the high NPAA concentration, only *A. mellifera* workers showed a difference in consumption, consuming more β -alanine solution. The effects of the NPAAs on behavior differed between the two species, with *B. terrestris* appearing more sensitive to the NPAAs than *A. mellifera*. After consuming NPAAs, *B. terrestris* showed changes in three (walking, flying, stationary) of the four behaviors recorded, although the effects varied with concentration and compound. In contrast, honey bees only showed a change in feeding behavior, with consumption of both NPAAs (at low concentrations) resulting in a decrease. Thus, pollinator intake of NPAAs may have important behavioral/ecological implications.

Keywords *Apis mellifera* · *Bombus terrestris* · GABA · Nectar · Pollination · Plant-pollinator interactions · β -Alanine · γ -Amino butyric acid

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s10886-018-01044-2>) contains supplementary material, which is available to authorized users.

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Introduction

Nectar is a chemically complex aqueous solution composed mainly of sugars, especially the disaccharide sucrose and its monosaccharide constituents, fructose and glucose (Nicolson and Thornburg 2007). The high sugar content of nectar enables insects to power their flight (Nicolson 2007). Amino acids are the most abundant nectar solutes after sugars. All twenty protein amino acids have been found in nectar (Nicolson and Thornburg 2007) and their influence on pollinator attraction has been investigated (Bertazzini et al. 2010; Petanidou 2007). Amino acids in nectar can also affect insect survival. For example, nectar rich in essential amino acids can reduce the lifespan of forager honey bees (Paoli et al. 2014). Several other substances have been identified in nectar, including lipids, phenols, alkaloids and volatile organic

compounds (González-Teuber and Heil 2009; Kessler and Baldwin 2007; Nicolson and Thornburg 2007). All these compounds positively or negatively affect nectar attractiveness for pollinators, with the effect depending on concentration and amount consumed, as well as on pollinator sensitivity (Adler 2000; Baker and Baker 1977, 1983; Faegri and van der Pijl 1979; Stevenson et al. 2017).

Besides the 20 standard protein amino acids and their post-translationally modified forms, thousands of non-protein amino acids (NPAAs) are known, including about 250 NPAAs in plants. NPAAs are involved in interactions with bacteria, fungi, herbivores and other plants (Huang et al. 2011; Vranova et al. 2011). A few NPAAs have been found in nectar, including γ -amino butyric acid (GABA), β -alanine, ornithine, taurine and citrulline (Nepi 2014). Although there has been no extensive study on the systematic distribution of NPAAs in nectar, they have been reported in 19 unrelated angiosperm species (Nepi 2014). NPAAs have been identified in all except one of 73 plant species of a Mediterranean phrygana community (Petanidou et al. 2006) and in all 82 species analyzed from the tribe Lithospermeae (Boraginaceae) (Nepi 2014). NPAAs content in nectar may vary both qualitatively and quantitatively, but GABA and β -alanine are generally the most common (Gottsberger et al. 1990; Nepi 2014; Petanidou and Smets 1996; Petanidou et al. 2006). Concentrations of GABA and β -alanine in nectar can vary in the range 0.57–750 μ M and 0.037–2.3 mM, respectively (Nepi 2014). It is interesting to note that honey bees and bumble bees generally visit plant species with nectar with high nectar GABA concentrations (Nepi 2014).

Nepi (2014) described three different ways NPAAs may affect pollinator foraging behavior: i) by affecting the nervous system, ii) by regulating phagostimulation, and iii) by increasing flight muscle activity. Field observations have suggested that nectar with a high concentration of β -alanine may affect motor activity of bumble bees (Rossi et al. 2014). Concentrations of GABA higher than those reported in nectar have been also found to have negative effects on herbivorous insects, effectively functioning as deterrents (Huang et al. 2011 and references therein; Schoonhoven et al. 2005). Recent work has shown that survival, behavior and amino acid composition of hemolymph in *Osmia* bees are all affected by a diet containing twenty times more GABA and β -alanine than that found naturally in nectar (Felicoli et al. 2018).

The study reported herein focuses on the effect of GABA and β -alanine on the behavior and survival of honey bees, *Apis mellifera* L., and bumble bees, *Bombus terrestris* L., as both are considered model species in plant-pollinator studies. In particular, we investigated the effects of extended feeding on artificial nectar containing both low (similar to that naturally occurring in nectar) and high (20x higher) concentrations of the two NPAAs. We addressed the following questions: 1) Are β -alanine- and GABA-enriched sucrose solutions

preferred over a solution containing only sucrose? 2) Does ingestion of these NPAAs affect insect motor activity? 3) Is there any effect of either NPAAs on pollinator survival?

Methods and Materials

Study Species and Experimental Conditions Bumble bee colonies were maintained at 25 ± 1 °C and $40 \pm 5\%$ RH in continuous darkness and were fed ad libitum with fresh frozen pollen and sugar syrup. Colonies were purchased from Bioplanet srl, Cesena, Italy. A total of 90 individuals were collected from three colonies (each colony being a replicate) under red light and transferred in groups of 5 into 6 experimental cages per colony (each cage with 5 bees representing a treatment). Cages were plastic net cylinders (length = 25 cm, diam. = 16 cm) mounted horizontally with the ends closed by transparent plastic lids (Fig. S1). They were maintained at ambient temperature with a 14:10 hr L:D cycle. Since there is large variation in body size among bumble bee workers, very small (approximately < 0.10 g) and very large (> 0.35 g) individuals were excluded from the experiments (Sgolastra et al. 2017). We also excluded newly emerged and old bees, recognized by their almost white colour and lack of hairs, respectively.

Honey bee workers were obtained from colonies managed at CREA-AA (Council for Agricultural Research and Economics - Research Centre for Agriculture and Environment, Bologna, Italy) reared under standard beekeeping techniques. A total of 199 forager bees was collected from three hives in early summer using a funnel trap (Medrzycki 2013). The bees were anesthetized with a mixture of air and CO₂ (2:3) for 15 min. and transferred to cages (6 cages per colony) identical to those used for the bumble bees, in groups of at least 10 bees (OECD 1998; EPPO 2010). They were maintained at ambient temperature under a 14:10 L:D cycle for the duration of a test.

Artificial Nectar Solutions The control nectar solution contained sucrose only, while the test solutions contained sucrose enriched with either β -alanine or GABA, either at the maximum concentration (NAT) found in floral nectar (β -alanine = 2.3 mM; GABA = 0.75 mM; Nepi 2014), or at a 20x higher concentration (β -alanine = 46 mM; GABA = 15 mM); the latter was used so as to increase the likelihood of eliciting repulsion/attraction, mortality/survival and behavioral effects. Each cage was provided with one of the solutions. For bumble bees, the concentration of sucrose in all solutions was 20% w/v, whereas for honey bees it was 50% w/v. Solutions were administered ad libitum via tipless syringes mounted in the cages. New solutions were provided three times a week or when necessary. Sucrose and amino acids were purchased from Sigma-Aldrich (Milan, Italy).

Consumption, Survival and Behavioral Observations Once per day, we recorded the amount of each solution consumed by weighing the syringes. The amount was divided by the number of live bees in each cage to obtain individual daily consumption. Dead bees were removed once each day from the cages after recording mortality and were not replaced, to allow survival analysis. Behaviors were recorded daily through a scan sampling method at several observation periods. Each observation period was 5 min. Per cage, with at least 1 hr between successive observations. During each observation period, five scan samplings were made per cage. The total number of observation periods varied from 23 to 81 per cage, depending on bee survival among the different replicates. The scan sampling consisted of recording how many individuals in a cage were performing a specific behavior [flying, walking, feeding and not moving (stationary); see Appendix S1] at that moment. The number of times a behavior was performed was expressed as a percentage of the total, so as to allow for the different numbers of bees in cages. Consumption, survival and behavioral measurements were continued to the end of the experiment (ranging from 9 to 25 days for honey bees and from 13 to 46 days for bumble bees).

Data Analysis Bee survival data were grouped by treatment and analysed by Kaplan-Meier survival analysis (Hosmer Jr and Lemeshow 1999). Survival curves were compared using Log rank tests among the three solutions and in pairwise comparison with Bonferroni correction.

Since controls showed inter-test variability, consumption and behavioral data were standardized by calculating the test:

control ratio for each measurement (i.e., dividing the test value by the mean of the control) to yield a consumption index, a walking index, a feeding index, a flying index and a stationary index. These data were tested for normality (Kolmogorov-Smirnov and Shapiro-Wilk tests). Consumption data were log10-transformed to achieve normality and analyzed by one-way ANOVA, followed by a Tukey post hoc test. Behavioral data were not normally distributed, even with transformation, and were analyzed by a Kruskal-Wallis test followed by a Mann-Whitney test with Bonferroni correction for differences among tests. Differences among treatments were analysed by independent sample *t*-tests or Mann-Whitney tests. All statistics were performed using STATISTICA software with the α -error set at 0.05.

Results

Survival Rate Analysis There were differences among the cumulative survival curves of *B. terrestris* fed with solutions at 20x (Fig. 1a, Log-rank $\chi^2_2 = 22.182$, $P < 0.001$) and natural (Fig. 1b, Log-rank $\chi^2_2 = 7.00$, $P = 0.030$) concentrations of NPAAs. Bumble bees fed with GABA solution at 20x concentration had a higher survival rate than those fed with control or β -alanine (at 20x concentration) (Table 1, Fig. 1a and b). Additionally, bumble bees fed with β -alanine solution at 20x concentration had a lower survival rate than those fed with the control (Fig. 1a). There were no differences in *A. mellifera* survival among treatments (Table 1, Fig. 1c and d).

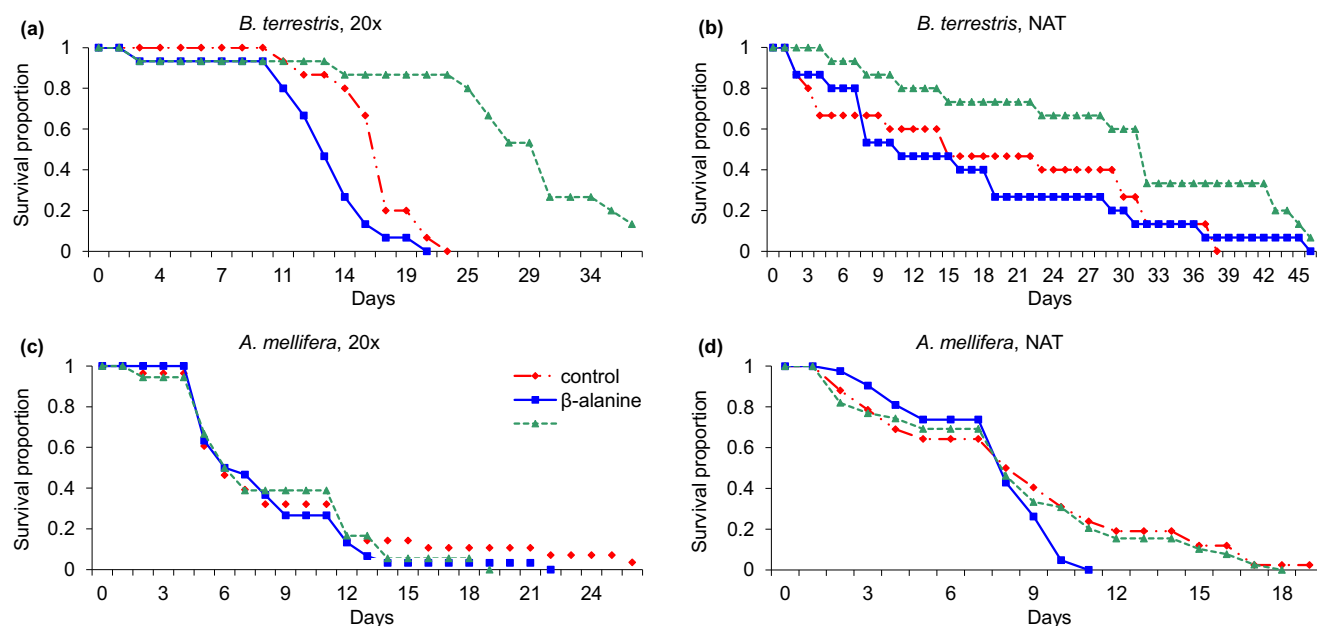


Fig. 1 Cumulative proportions of surviving bees fed with a control or different solutions of β -alanine and γ -amino butyric acid at two concentrations. **a** *Bombus terrestris*, 20x concentration of the amino acid found

in nectar. **b** *B. terrestris*, natural concentration of the amino acid in nectar. **c** *Apis mellifera*, 20x concentration of the amino acid found in nectar. **d** *A. mellifera*, natural concentration of the amino acid in nectar

Table 1 Pairwise comparisons of survival of *Bombus terrestris* and *Apis mellifera* fed with control solution and solutions containing nectar (NAT) and 20xNAT concentrations of γ -amino butyric acid (GABA) and β -alanine

Treatments	<i>B. terrestris</i> 20x		<i>B. terrestris</i> NAT		<i>A. mellifera</i> 20x		<i>A. mellifera</i> NAT	
	Statistic	<i>P</i> value ^a	Statistic	<i>P</i> value ^a	Statistic	<i>P</i> value ^a	Statistic	<i>P</i> value ^a
Control vs β -alanine	-2.69	0.007	0.14	0.886	-0.15	0.884	-1.43	0.151
Control vs GABA	3.40	0.001	2.32	0.020	0.14	0.888	0.17	0.867
β -alanine vs GABA	3.64	<0.001	2.14	0.032	0.46	0.645	1.69	0.090

^aSignificance determined using Log rank test with Bonferroni correction. Bold indicates significant at $P < 0.016$

Consumption Analysis For bumble bees, there was a difference in consumption among solutions at natural NPAA concentrations ($F_{2,215} = 7.29$, $P < 0.001$), but not at 20x NPAA concentrations ($F_{2,146} = 1.02$, $P = 0.364$) (Fig. 2a). At natural concentration, bumble bees consumed more β -alanine solution (Tukey's post hoc, $P = 0.018$) than of the other solutions (Fig. 2a). Bumble bees also consumed more β -alanine ($t_{99} =$

4.06, $P < 0.001$) solution at natural concentration than they did of the 20x concentration (Fig. 2a).

Conversely, for honey bees there were no differences in consumption of the solutions at natural concentrations ($F_{2,83} = 0.01$, $P = 0.992$), but there was a difference at 20x concentration ($F_{2,88} = 4.06$, $P = 0.021$) (Fig. 2b). At 20x, honey bees consumed more β -alanine solution than the control solution (Tukey's post hoc, $P = 0.035$). In contrast to bumble bees, honey bees consumed more 20x β -alanine solution than natural β -alanine solution ($t_{51} = -2.45$, $P = 0.017$) (Fig. 2b).

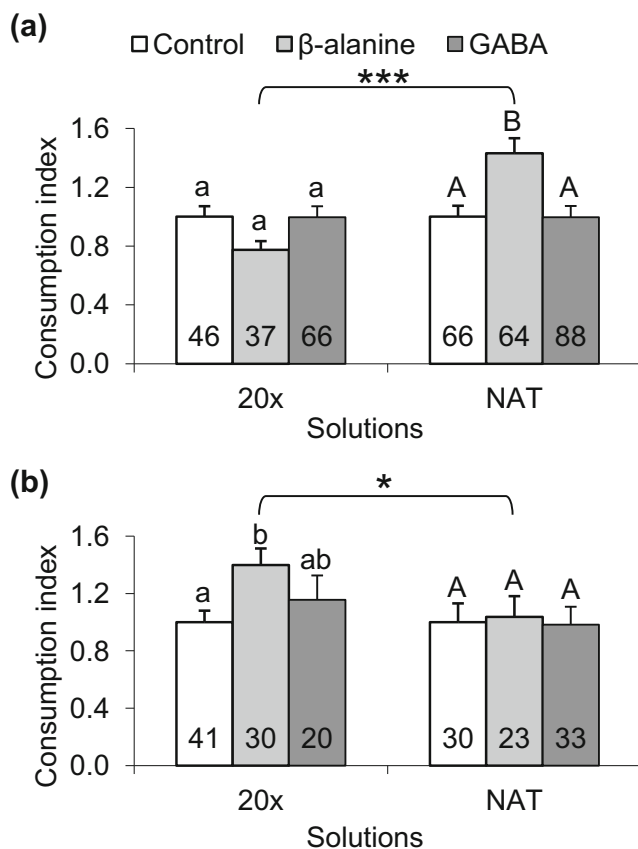


Fig. 2 Daily consumption index of control, β -alanine or γ -amino butyric acid (GABA) solutions. **a** by *Bombus terrestris* at concentration typically found in nectar (NAT), and 20xNAT. **b** *Apis mellifera*, NAT and 20xNAT. Numbers within histograms indicate the number of measurements. Values marked with different letters were different ($P < 0.05$) according to one-way ANOVA followed by Tukey post hoc test. Asterisks mark differences between 20x and NAT concentrations within solutions according to an independent sample *t*-test (* = $P < 0.05$ and *** = $P < 0.001$)

Behavioral Analysis At natural NPAA concentrations, the only behavioral difference observed for bumble bees was that bumble bees that consumed GABA solution had a lower flying index than those that consumed the other solutions (Fig. 3; Table 2). However, at the higher concentration, more differences were apparent. In particular, bumble bees that consumed β -alanine solution tended to have a higher walking index and lower feeding, flying and stationary indices than bumble bees that fed on control and GABA solutions (Fig. 3; Table 2). Next, we compared responses to the two concentrations of each NPAA. Bumble bees fed with the higher concentration of β -alanine had a higher walking index ($U = 2640$, $P < 0.001$) but lower flying and stationary indices than those fed at the natural concentration ($U = 1384$, $P < 0.001$ and $U = 2773$, $P = 0.008$, respectively), while bumble bees fed with 20x GABA solution had higher feeding and stationary indices ($U = 1347$, $P = 0.003$ and $U = 10,360$, $P = 0.026$, respectively) than those fed with the natural concentration (Fig. 3; Table 2).

Comparing the walking and flying indices of bumble bees fed β -alanine solution, we observed contrasting trends at the two concentrations trends. At the natural concentration, the walking index was marginally ($U = 4935$, $P = 0.072$) lower than the flying index, while at the 20x concentration, the walking index was higher than the flying index ($U = 579$, $P < 0.001$).

At natural concentrations of NPAAs, honey bees fed with either GABA or β -alanine had lower feeding indices than bees fed the control solution (Fig. 4b, Table 3). Other behavioral differences were relatively small, with GABA-fed bees having

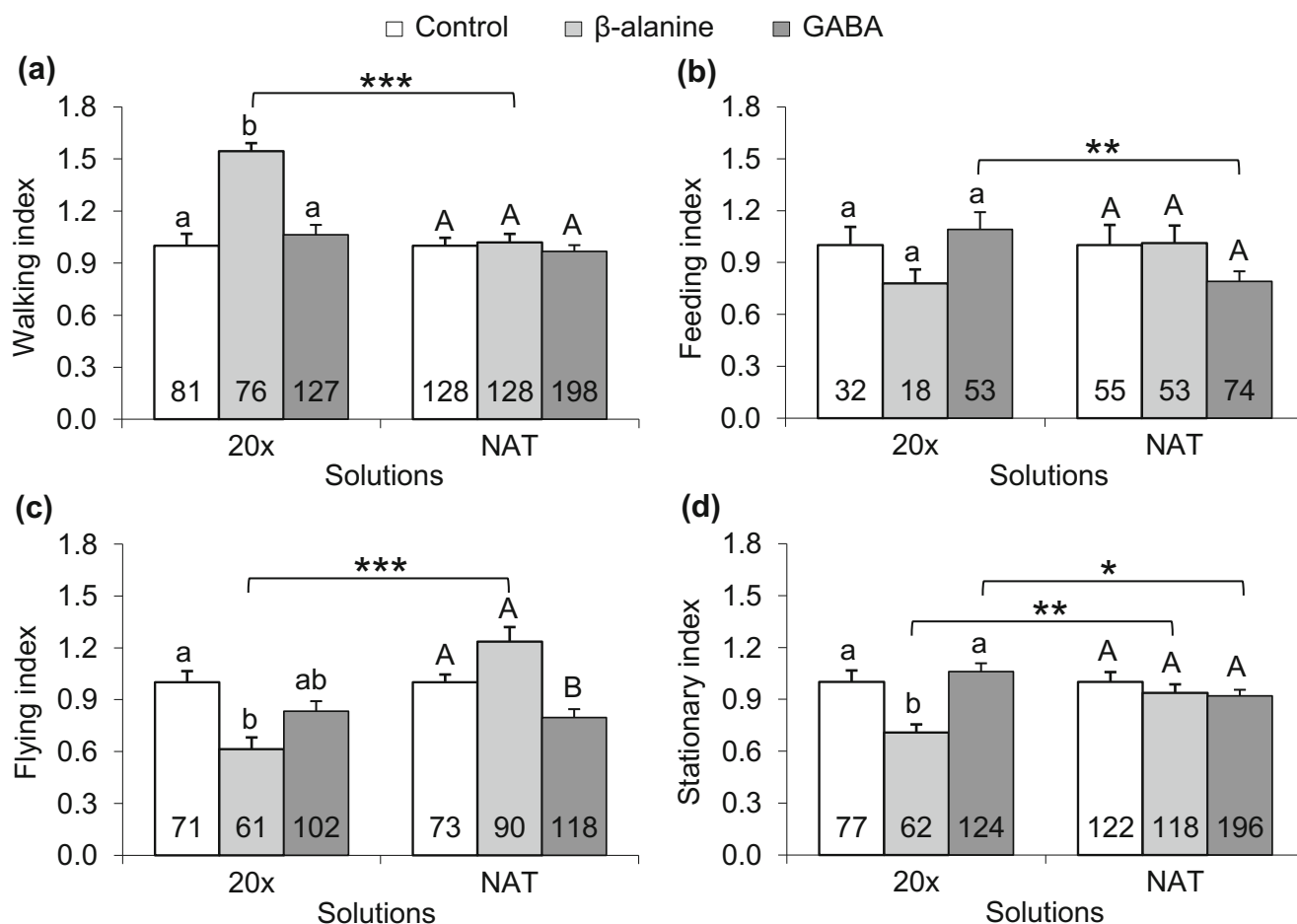


Fig. 3 Behavior indices of *Bombus terrestris* fed with control, β -alanine or γ -amino butyric acid (GABA) solutions at concentrations typically found in nectar (NAT) and 20xNAT. **a** Walking index. **b** Feeding index. **c** Flying index. **d** Stationary index. Numbers within histograms indicate the number of times that the behavior was observed. Values marked with

different letters were different according to Kruskal-Wallis test followed by Mann-Whitney *U*-test with Bonferroni correction. Asterisks mark differences between 20x and NAT concentrations within solutions according to Mann-Whitney *U*-test (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$)

a lower walking index but higher flying and stationary indices than β -alanine-fed bees (Fig. 4, Table 3). At the 20x NPAA concentration, the only difference among honey bees was that

Table 2 Differences in behavioral indices of *Bombus terrestris* fed with control, β -alanine and γ -amino butyric acid solutions at two concentrations (NAT = concentrations typically found in nectar, 20x = 20xNAT concentration)

Concentration	Behavior	H	d. f.	<i>P</i> value ^a
20x	Walking	30.24	2	< 0.001
	Feeding	2.57	2	0.276
	Flying	16.31	2	< 0.001
	Stationary	17.39	2	< 0.001
NAT	Walking	0.39	2	0.822
	Feeding	5.17	2	0.075
	Flying	19.55	2	< 0.001
	Stationary	1.85	2	0.396

^a Significance determined using Kruskal-Wallis test

GABA-fed bees had a lower stationary index than did control-fed bees (Fig. 4d, Table 3).

Comparing the NPAA solutions at the two concentrations, honey bees fed with β -alanine had higher feeding and flying indices ($U = 12$, $P < 0.001$ and $U = 574$, $P = 0.014$, respectively) at the higher concentration, while those fed with GABA solution had higher feeding and lower stationary indices ($U = 38$, $P < 0.001$ and $U = 2245$, $P = 0.002$, respectively) at the higher concentration.

Discussion

The results demonstrated that bumble bee survival and both bumble bee and honey bee behaviors were affected by dietary consumption of the NPAAsm GABA and β -alanine under our experimental conditions. The effects were species-specific and varied with amino acid concentration.

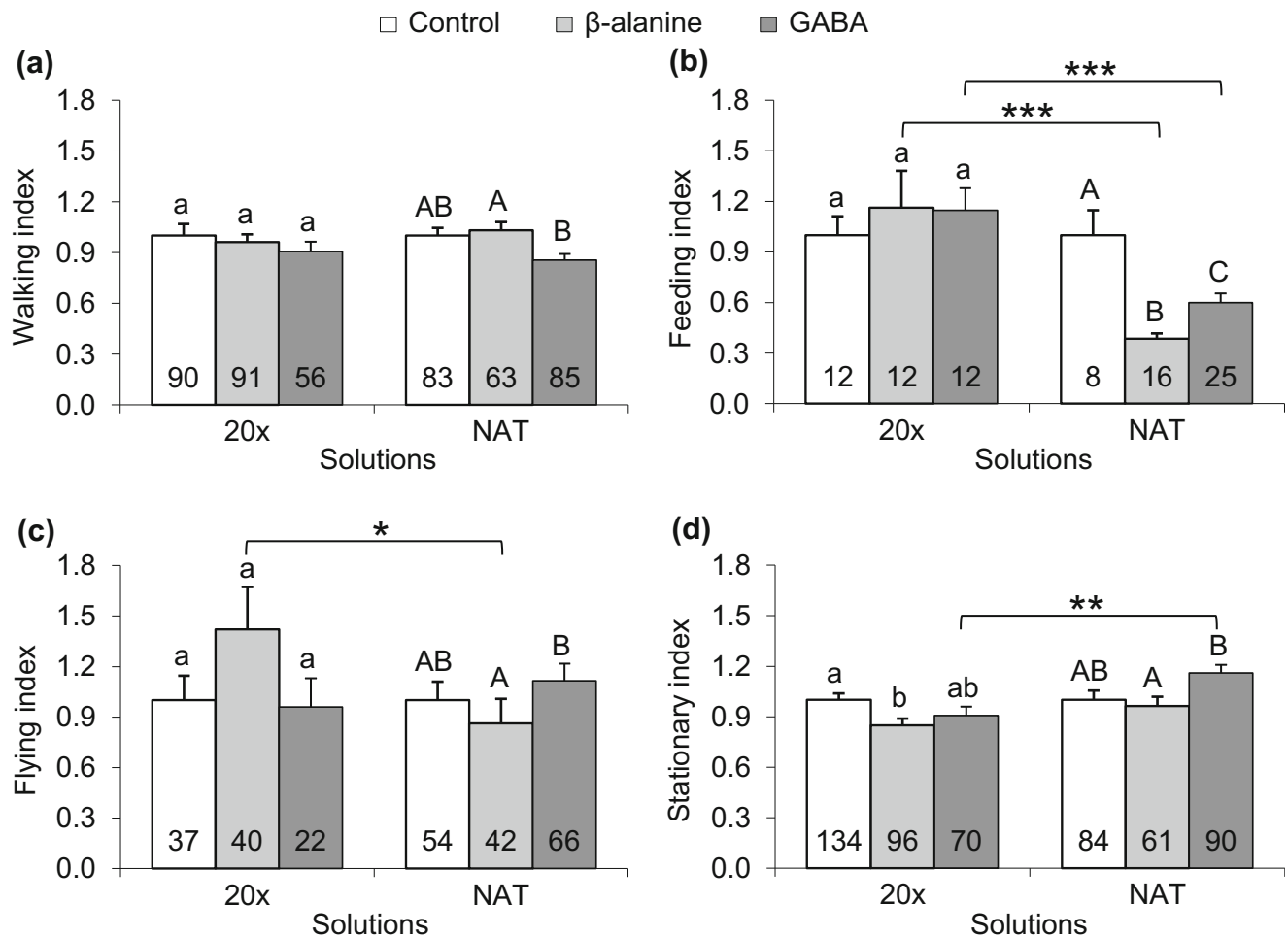


Fig. 4 Behavior indices of *Apis mellifera* fed with control, β -alanine or γ -amino butyric acid (GABA) solutions at concentrations typically found in nectar (NAT) and 20xNAT. **a** Walking index. **b** Feeding index. **c** Flying index. **d** Stationary index. Numbers within histograms indicate the number of times that the behavior was observed. Values marked with different

letters were different according to Kruskal-Wallis test followed by Mann-Whitney *U*-test with Bonferroni correction. Asterisks mark differences between 20x and NAT concentrations within solutions according to Mann-Whitney *U*-test (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$)

Table 3 Differences in behavioral indices of *Apis mellifera* fed with fed with control, β -alanine and γ -amino butyric acid solutions at two concentrations (NAT = concentrations typically found in nectar, 20x = 20xNAT concentration)

Concentration	Behavior	H	d. f.	<i>P</i> value ^a
20x	Walking	1.25	2	0.536
	Feeding	0.78	2	0.678
	Flying	1.64	2	0.441
	Stationary	7.50	2	0.024
NAT	Walking	6.45	2	0.040
	Feeding	20.33	2	<0.001
	Flying	6.93	2	0.031
	Stationary	7.01	2	0.030

^a Significance determined using Kruskal-Wallis test

Effects on Bee Survival Our results indicate that a diet enriched with GABA increases the lifespan of bumble bees, with the effect most apparent at a concentration of 20x concentration the normal amount found in nectar. Diets with an imbalance of protein amino acids can affect the longevity of adult insects (Bown et al. 2006; Grandison et al. 2009; Paoli et al. 2014; Vrzal et al. 2010). Essential amino acid supplementation in a limited caloric diet decreased the lifespan of *Drosophila melanogaster* without reducing its reproductive fitness (Emran et al. 2014; Grandison et al. 2009; Lee et al. 2008) while, conversely, restriction of amino acids in a carbohydrate-rich diet extended its lifespan (Min and Tatar 2006). The same results were found in similar experiments on cockroaches (Hamilton et al. 1990) and crickets (Maklakov et al. 2008). With regard to social insects, similar results have been reported for honey bees (Archer et al.

2014; Paoli et al. 2014; Pirk et al. 2010), ants (Cook et al. 2010; Dussutour and Simpson 2009) and bumble bees (Bogo 2016; Stabler et al. 2015). The only insect study that considered the effects of a NPAA, showed a GABA-induced reduction in larval growth and survival in the oblique-banded leafroller, *Choristoneura rosaceana*, (Bown et al. 2006).

GABA is the principal inhibitory neurotransmitter (Breer and Heilgenberg 1985) and is involved in stress responses in both vertebrates (Grønli et al. 2007) and invertebrates (Stevenson 1999). In insects, GABA limits excessive and potentially disruptive excitation and probably antagonizes octopamine in arousal pathways (Stevenson 1999 and references therein). The role of GABA in stress-protection and reduction of over-excitement may explain the increased lifespan of caged bumble bees. The experimental conditions (e.g., small cages) used in our study may have induced stress in bumble bees, with the dietary GABA in the diet helping alleviate these stresses.

Our results suggest that honey bees are less sensitive to the two NPAAs tested. Indeed, survival of honey bees was not affected when fed with natural or 20x concentrations of GABA or β -alanine. This difference in sensitivity between these species has already been observed for other chemicals, such as insecticides (Sgolastra et al. 2017), and may possibly be ascribed to different metabolic processes in workers of the two species, according to their behavioral and life cycle traits. Social species, in general are considered to be less susceptible to environmental stressors than are solitary species, due to “superorganism resilience” (Straub et al. 2015). This buffering ability is influenced by colony size. Since bumble bee colonies have many fewer individuals than honey bee colonies, they are not considered a true “superorganism” and are, therefore, expected to be less resilient. Thus, bumble bee workers may be more influenced than honey bee workers by environmental components such as nectar composition.

Effects on Consumption and Behavior Of the two NPAAs, only addition of β -alanine appeared to have an effect on consumption of sugar solution. However, this effect differed between the two species, with bumble bees showing greater consumption of solution with β -alanine at the natural concentration than at the 20x concentration, while honey bees consumed more at the 20x concentration (compared to the natural concentration). In terms of behavior, bumble bees appeared more sensitive to the NPAAs than did honey bees. Bumble bees showed changes in three (walking, flying, stationary) of the four behaviors after consuming NPAAs, although the effects varied with concentration and compound. In contrast, honey bees only showed a change in feeding, with consumption of both NPAAs (at natural concentrations) resulting in a decrease in the feeding index.

Conclusions Following this study on the effect of diets enriched with the NPAAs β -alanine and GABA has been studied with regard to bumble bee and honey bee survival and behavior, future work should focus on the role of β -alanine and GABA on insect longevity and behavior under more natural conditions, as well as investigate any synergistic effects of the two compounds. More information on the role of NPAAs in pollinator physiology, neurobiology and behavior is essential to understand the ecological importance of nectar NPAAs to both pollinator and plant.

Acknowledgements We thank Joan Rabassa Juvanteny and Mariachiara Zanichelli for laboratory assistance. Dr. Bogo was supported by a research fellowship from the University of Bologna and Council for Agricultural Research and Economics - Research Centre for Agriculture and Environment (CREA-AA).

Author Contributions MN, MG, LB and AF conceived the ideas; GB, LB, AF and SS designed the methodology; GB, LB, MB and MG collected the data; GB, LB and MN analysed the data; GB, LB, MN, AF, SS, MB and MG led writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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