Warming, CO_2, and nitrogen deposition interactively affect a plant-pollinator mutualism

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Abstract

Environmental changes threaten plant-pollinator mutualisms and their critical ecosystem service. Drivers such as land-use, invasions, and climate change affect pollinator diversity or species encounter rates. However, nitrogen deposition, climate warming and CO_2 enrichment could interact to disrupt this crucial mutualism by altering plant chemistry in ways that alter floral attractiveness or even nutritional rewards for pollinators. Using a pumpkin model system, we show that these drivers non-additively affect flower morphology, phenology, flower sex ratios, and nectar chemistry (sugar and amino acids), thereby altering the attractiveness of nectar to bumble-bee pollinators and reducing worker longevity. Alarmingly, bees were attracted to, and consumed more, nectar from a treatment that reduced their survival by 22%. Thus, three of the five major drivers of global environmental change have previously-unknown interactive effects on plant-pollinator mutualisms that could not be predicted from studies of individual drivers in isolation.

Introduction

Ecosystems worldwide are undergoing unprecedented environmental change (Assessment 2005) caused by drivers such as habitat fragmentation, deposition of anthropogenically-fixed nitrogen (N), biotic invasions, increasing atmospheric carbon dioxide (CO_2) and climatic changes. Each of these global environmental changes threatens biodiversity (Sala *et al.* 2000), though disruptions to crucial interactions between species may even precede biodiversity loss (Tylianakis *et al.* 2008). Moreover, it is becoming increasingly apparent that global change drivers can act synergistically or antagonistically, generating important interaction effects that cannot be predicted from the independent effect of each driver (Didham *et al.* 2007; Tylianakis *et al.* 2008; Schweiger *et al.* 2010). For example, nitrogen (N) deposition and temperature both affect plant physiological responses to elevated CO_2 (Reich *et al.* 2006; Zvereva & Kozlov 2006), with potential cascading effects on species that interact with plants (Tylianakis *et al.* 2008).

Although the arrangement of interactions within a whole community may confer stability to the system (Bascompte 2009), many pairwise biotic interactions can be disrupted by human changes to the environment (Tylianakis *et al.* 2008). However, such interactions form the basis of numerous ecosystem services on which human well-being depends. For example, animal pollination of plants is crucial for maintaining plant diversity (Ollerton *et al.* 2011), and provides an essential ecosystem service to humans through pollination of three quarters of global food crops (Klein *et al.* 2007). However, numerous studies have shown that environmental changes such as habitat modification and pesticide use (Aguilar *et al.* 2006; Brittain *et al.* 2010), plant and pollinator invasions (Lopezaraiza-Mikel *et al.* 2007; Aizen *et al.* 2008), or phenological mismatches due to climate change (Memmott *et al.* 2007) can potentially threaten this mutualism, either by reducing pollinator diversity or by altering the ability of native pollinators to encounter and successfully pollinate plants. Consequently, the decline of pollinators may

70 be paralleled by declines in the wild and agricultural plants that rely on them (Biesmeijer *et al.* 2006;
71 Potts *et al.* 2010; Cameron *et al.* 2011). Despite this widespread evidence to suggest that pollinators are
72 generally declining due to invasions, pesticides, phenological mismatches, and land-use change, the
73 results of previous studies are surprisingly variable (Tylianakis *et al.* 2008; Winfree *et al.* 2011). Some of
74 this variability may be due to the correlative, non-experimental approaches used (of around 670 studies
75 recently reviewed by Winfree et al. (Winfree *et al.* 2011), only a handful were experimental), which
76 cannot reveal mechanisms behind pollinator responses. Furthermore, studies testing effects of a single
77 driver cannot detect interactions. Yet, these and other examples of the effects of single global change
78 drivers on plant-pollinator mutualisms (Rusterholz & Erhardt 1998; Mevi-Schutz & Erhardt 2005;
79 Schweiger *et al.* 2010) raise concerns that a suite of drivers acting simultaneously could further alter this
80 relationship, and that changes to the attractiveness of flowers to pollinators or even the nutritional
81 quality of nectar could dramatically alter pollinator fitness and plant-pollinator mutualisms.
82
83 Here we examine the interactive effects of three global environmental change drivers (CO_2 enrichment,
84 N deposition and increased temperature) on a pollinator-plant mutualism, using a series of controlled
85 experiments to test hypotheses specific to various mechanistic pathways (Fig. 1). Single-driver studies
86 suggest that increasing atmospheric CO_2 levels (and its fertilisation effects) could affect plant-pollinator
87 interactions by altering plant morphology, phenology, and nectar production (Rusterholz & Erhardt
88 1998), thereby causing a general increase in reproductive investment and floral abundance (Jablonski *et*
89 *al.* 2002), even though gene suppression at elevated CO_2 may delay flowering (Springer *et al.* 2008). In
90 addition to higher carbohydrate levels from CO_2 enrichment, nectar volume and concentration may be
91 affected by temperature (Pacini *et al.* 2003) through more rapid evaporation as well as changes to plant
92 physiology. Furthermore, enhanced plant growth through nitrogen enrichment can drive increases in
93 flower abundance, duration, and size (Burkle & Irwin 2009b), and nitrogen enrichment can affect the

94 concentration and composition of amino acids in nectar (Gardener & Gillman 2001), potentially altering
95 pollinator preferences (Gardener & Gillman 2002). We test for changes to flower morphology and
96 phenology, but do not test the known effects of these variables on pollinator visitation (Fig. 1).
97 Moreover, it is unknown whether the interactive effects of the global change drivers will alter nectar
98 chemistry in ways that affect pollinator visitation and nectar consumption, or how changes to nectar
99 may affect bee longevity, therefore we test these effects using controlled laboratory experiments.
100
101 We use pumpkin, *Cucurbita maxima,* as a model system because 1) it depends strongly on effective bee
102 pollination for fruit production (Hoehn *et al.* 2008), 2) cucurbits are cultivated over a wide geographic
103 range for their food and economic value, and 3) their large unisexual flowers produce enough nectar to
104 allow detailed biochemical analysis. We show that the three drivers have main and interactive effects on
105 various attributes of flower structure and nectar chemistry, and test experimentally whether altered
106 nectar chemistry affects attractiveness of the nectar to pollinators, as well as pollinator longevity. We
107 chose the bumble bee *Bombus terrestris* (L.) as a model pollinator because of its near global distribution,
108 importance for the pollination of both wild and cultivated plants (Velthuis & van Doorn 2006) and the
109 commercial availability of colonies to facilitate experimentation. Despite the importance of bumble bees
110 as generalist pollinators, many wild populations are facing decline (Goulson *et al.* 2008). We show that N
111 deposition, warming, and elevated CO_2 have previously-unknown effects on a pollination mutualism,
112 with interactions that could not have been predicted from studies of any of these drivers in isolation.
113

114 ## Materials and methods

115 Flower growth and nectar analyses
116 Pumpkin plants (*Cucurbita maxima* Var. “Little Cutie”) were grown in mineral soil in pots within custom-
117 built (60 × 60 × 60 cm) controlled-environment chambers under factorial combinations of three

different global environmental change treatments, each at one of two levels (ambient vs. elevated according to estimates for the end of this century). The three drivers were: CO_2 (360 vs. 700ppm), nitrogen (0.19 vs. 0.57g N added per pot (in the form of ammonium nitrate, elevated level equivalent to 100kg N ha^{-1}, or two years at typical global deposition levels), and temperature (19^{o} C or 23^{o} C, equivalent to the average summer temperature in the study region, and an elevated temperature value within the range predicted for the region by the end of the century). The 0.19g N per pot added to the control treatment was necessary to ensure that control plants were not too N limited to produce flowers (a preliminary trial in which we deprived plants of additional N produced no flowers). The factorial design produced eight treatment combinations, each with two replicate plants. The limit of two replicate plants was for logistical reasons, though the fully-factorial design maximised power, as effects were tested over an error term with degrees of freedom arising from the total number of replicates across all treatments (15 total d.f.). Nevertheless, low power would, if anything, provide a more conservative test, reducing the likelihood of finding any significant effects. Plants were watered daily with a small amount to keep soil moist, and grown under 'Grolux' tubes with a 16:8hr light:dark regime until they ceased flowering. The number of days to onset of flowering, flower diameter and total number and the sex of flowers produced were measured. In order to avoid contaminating the nectar with pollen, stamens were removed from the flowers (by cutting the tip of the flower at an appropriate point along its length) on the afternoon prior to flower opening. Cut flowers were not used for flower size analyses. Nectar was then extracted from open flowers using microcapillary tubes on the following morning, and the volume of nectar produced per flower was recorded. For nectar composition analyses, the first male flower from each plant was used. Sugar and amino acid composition and concentration of nectar were measured using High Pressure Liquid Chromatography (HPLC). Amino acids were derivatised using the AccQtag protocol (see Supplementary Material S1) and sugars analysed according to AOAC

141 International protocols (OMA, 18th Edition; method 977.20; analyses conducted by the Cawthron
142 Institute, Nelson NZ).
143
144 To test the response of nectar amino acid composition (presence and concentration of each amino acid)
145 to the three global change drivers, we first used a principal component analysis to reduce the number of
146 variables from concentrations of 21 amino acids to the four principal component axes that each
147 explained over 5% of the variance in the data. These four orthogonal axes explained a cumulative
148 86.93% of the variance in amino acid composition (see Table S2 Supplementary Material for factor
149 loadings). We then used the scores of these four axes as response variables in a multivariate analysis of
150 variance (MANOVA), with nitrogen, CO_2 and temperature treatments (current vs. elevated) as
151 predictors, and all interactions included. We also tested for changes to sugar composition using a
152 MANOVA with the same predictor variables, and the concentrations of sucrose, fructose and glucose as
153 the multivariate response. After testing the overall response of sugar composition to the global change
154 treatments, we tested for changes in the ratios of glucose to fructose, and of sucrose to fructose and
155 glucose combined, as these are known to be important determinants of attractiveness to pollinators
156 (Baker & Baker 1983). We tested for effects of the three global change treatments on these sugar ratios,
157 as well as total sugar and amino acid concentrations and various aspects of flower morphology and
158 phenology using generalised linear models. In the case of sugar ratios, and flower sex ratios, we used a
159 binomial error distribution with a logit link function. For the number of flowers per plant we used a
160 Poisson error and log link, and for the nectar volume, total sugars, total amino acids, time to flowering,
161 and flower diameter we used Gaussian (normal) errors. In all cases, initial models included all interaction
162 terms, and we simplified models by removing all non-significant interactions and main effects, retaining
163 only those main effects involved in higher order interactions to satisfy the principal of marginality. The
164 best-fitting model was obtained by minimising the Akaike Information Criterion (AIC), and final models

were checked for overdispersion. These analyses were conducted in the base and ade4 (Dray & Dufour 2007) packages of the R v.2.10.1 environment (R Development Core Team 2006).

Bumble Bee Preferences

To test whether the global change drivers altered the attractiveness of nectar to pollinators, and to isolate the pathway through which this occurs, we synthesised nectar solutions and offered these to bumble bees in a choice experiment. Nectar solutions were generated by adding sugar and amino acid standards to water, in concentrations that mimicked the average concentrations of amino acids and sugars produced by the plants grown under each of the eight global change treatment combinations (see Table S1 Supplementary Material). We used synthetic nectar to ensure that any effects on pollinators were caused by the changes in nectar components that we measured (sugars and amino acids), thereby excluding the possibility that any unknown compounds in nectar were confounding these effects. We then tested the preference of bumble bees (12 colonies of *Bombus terrestris*; Zonda Resources, New Zealand) for synthetic nectar based on each of the global change treatments. The experimental nectar solutions were presented to the bees in a flight cage (175 × 175 × 175 cm) comprising three white polyester/nylon mesh sides and roof, one clear vinyl observation panel, and a black mesh floor panel (Bioquip Inc, USA). Nectar solutions were offered in an array (50 × 50 cm) containing eight randomised 'flower clusters' (each cluster comprised four artificial 'flowers' representing one of the eight nectar treatment combinations), with 7.5 cm between each cluster and its nearest neighbour. Artificial flowers were constructed from 1.5 ml clear centrifuge tubes with lids removed, containing 10 µl of 'nectar' solution embedded in a polystyrene base covered with green paper. All treatments were presented to the bees concurrently, and the position of each treatment cluster was randomised for each colony. Bee colony boxes were attached by a mesh tunnel to a flight cage, and a gate in the tunnel allowed us to control access of the bees to the flight cages. Flight cages

and bee colonies were kept in a glasshouse (night time min. temperature 15^{o}C, daytime max. 23^{o}C, good flight temperatures for bumble bees), both during and between experiments. All experiments were conducted between 10:00h and 12:00h on sunny days, with three colonies tested simultaneously (in separate cages) per day. All colony boxes had their internal sugar-solution feeder removed 36 hours prior to the experiment to encourage foraging, but still had access to internal 'honey pots' containing sugar solution and pollen they had stored.

Arrays were presented to a naive colony the afternoon prior to the experiment. The foragers were allowed access to the array for two hours to enable them to learn to forage on the experimental flowers. The array was then removed and the colony closed (foragers were able to return to their colony, but not exit the colony or access the nectar arrays). Arrays presented during this learning period had the same arrangement of treatment clusters as did arrays presented in the experimental period. To test the preference of the bumble bees, an array was set in each flight cage, and the colonies were opened to allow foragers to enter the flight cage. Bees were allowed to forage on the array for 10 minutes after the first forager landed on a 'flower'. The number of visits to each flower was recorded for the duration of the experimental period. After the 10 minute experimental period, the array was removed from the flight cage, and the volume of nectar consumed from each flower was recorded. Each colony was tested once in this manner. In each experiment at least 5 bees visited at least one experimental flower, and visitation rates and nectar consumption were pooled for the four flowers per treatment cluster. On average, each nectar treatment received between 2.4 and 4.5 visits per replicate (i.e. per 10 minutes of colony exposure), with an average of 29 visits across all treatments within a replicate.

We tested how visitation frequency and nectar consumption responded to global change driver-induced changes to nectar using generalised linear mixed effects models, conducted in the lme4 package (Bates & Maechler 2009) in the R environment. Response variables were either the proportion of available nectar consumed (using binomial errors) or the number of visits (using Poisson errors). The number of visits and the amount of nectar consumed were closely related ($P = 0.002$). Thus, the total amount of nectar consumed may represent a preference of bees for consuming a particular type of nectar, or it may simply represent a greater number of visits by bees with a constant rate of consumption per visit. To disentangle these possibilities, we also ran additional models with the amount of nectar consumed per visit as the response variable (Gaussian error structure). However, more than one bee could not forage on the same artificial flower at a time. Consequently, a situation where bees 'take turns' at feeding could lead to lower rates of consumption per visit, even though total consumption could be high. We therefore recommend a degree of caution in the interpretation of per-visit-consumption data. Day and colony identity were included as crossed random effects, to account for the possibility that multiple colonies tested on a single day may have responded to specific conditions (e.g., weather), and also to incorporate the non-independence of consumption of different nectar treatments by a given colony. We also included nitrogen, CO_2 and temperature treatments (current vs. elevated) and their interactions as fixed effects, simplifying models using the procedure outlined above. We used several additional models (replacing the fixed effects of drivers with those of sugar ratios or total sugar and amino acid concentrations) to understand better which specific aspects of nectar had the greatest effect on attractiveness to pollinators.

Bumble Bee Longevity

The effects of changes to nectar on pollinator fitness were tested by measuring the longevity of caged *B. terrestris* workers, each fed on one of the eight nectar treatments. Newly-emerged bees were collected

from a total of 16 colonies (Zonda Resources, New Zealand.), marked with a unique identifier tag (Eckroyd Beekeeping Supplies Inc. New Zealand), and placed into (30 × 30 × 30 cm) mesh cages. Each cage contained bees from a minimum of 5 different source colonies, and the experiment consisted of four replicates of each of the eight synthetic nectar treatments (Supplementary Table S2). One replicate was conducted with ten bees per cage, then due to limited supplies of colonies, five bees were used for the subsequent three replicates. Each cage had both water and nectar treatment feeders, and bees had access to both nectar and water *ad libitum*. The nectar in each cage was changed every three days to prevent fermentation. Cages were monitored daily for bee mortality. Because bees were unable to return to their source colony, the longevity we recorded may not represent their longevity under natural conditions when they have access to colony resources. However, the purpose of this experiment was to determine relative changes in longevity across treatments, so we were confident that keeping groups of bees isolated from other colony members would not bias any observed differences between treatments.

The effects of nectar treatments on bee longevity were tested using generalised linear mixed effects models in the lme4 package for R, with survival days of each bee as the response, normal (Gaussian) errors, and nectar treatments representing the eight combinations of global change drivers and their interactions as levels of a fixed factor. The number of bees per cage was included as a covariate to control for potential biases arising from one replicate of each treatment having 10 bees and the remaining replicates 5 bees. Cage number and the identity of the colony from which a bee was sourced were also included as crossed random effects, to account for the non-independence of bees within a cage, and for potential genetic (colony-specific) traits affecting longevity. This maximal model was simplified by removing non-significant fixed terms until no further reduction in AIC score could be obtained. Due to issues associated with calculating *P* values from mixed effects models with a Gaussian error structure (Bolker *et al.* 2009), we used Markov Chain Monte Carlo (MCMC) resampling to estimate

P values for this model and those testing consumption per visit above, though these gave qualitatively identical results to the less-conservative *P* values obtained from the traditional *t* test for each parameter estimate using the upper bounds of degrees of freedom. The MCMC procedure was carried out using the pvals.fnc function in the languageR package (Baayen 2010) for the R environment.

Results

Flower growth

The experimental environmental change drivers significantly influenced plant growth, C:N ratio (Supplementary Results S2.1), and flowering attributes such as phenology (timing of first flowers) and morphology (Supplementary Table S3a). The number of flowers produced by each plant increased with increasing nitrogen ($Z = 6.55$, $P < 0.001$) and temperature ($Z = 3.01$, $P = 0.003$), whereas elevated CO_2 slightly weakened the temperature effect ($Z = -1.79$, $P < 0.073$) and drove production of smaller flowers, with less nectar (effect of CO_2 on flower diameter $F_{1,11} = 5.06$, $P = 0.046$, nectar volume $F_{1,8} = 11.95$, $P = 0.0086$, number of flowers $Z = 0.25$, $P = 0.803$, Fig. 1). Elevated temperature (T) reduced the negative effect of CO_2 on the number of flowers produced (T x CO_2 interaction: $F_{1,10} = 5.08$, $P = 0.048$). Nitrogen addition increased flower diameter ($F_{1,11} = 6.02$, $P = 0.032$), whereas elevated temperature had a negative effect ($F_{1,10} = 16.13$, $P = 0.002$). Elevated temperature also caused a decrease in the ratio of female (fruit-producing) to male flowers ($F_{1,8} = 11.78$, $P = 0.040$). Furthermore, elevated nitrogen and temperature both accelerated the onset of flowering (by an average of 15.8 and 7.5 days respectively, T: $F_{1,8} = 26.03$, $P = 0.009$; N: $F_{1,8} = 5.90$, $P = 0.041$); whereas elevated CO_2 delayed the onset of flowering by an average of 10.8 days ($F_{1,8} = 12.12$, $P = 0.008$). Nectar volume was greater under elevated temperature ($F_{1,8} = 10.62$, $P < 0.012$), but lower under elevated CO_2 ($F_{1,8} = 11.95$, $P = 0.0086$), however, the positive effect of elevated temperature was greater at low nitrogen levels (negative N x T interaction: $F_{1,8} = 5.78$, $P = 0.043$; Supplementary Fig. S2.2a).

284

285 Nectar composition

286 The drivers interactively affected various aspects of nectar chemistry (Supplementary Table S3b). Total
287 amino acid and sugar concentrations were not affected by the separate effects of temperature and N,
288 but the effect of temperature became positive under elevated N (T x N interaction: total sugars $F_{1,9}$=
289 6.18, P= 0.035, total amino acids $F_{1,11}$= 4.78, P = 0.049; Supplementary Figs. S2.2b and S2.2c). Elevated
290 CO_2 also made the effect of temperature on sugar concentration become positive (T x CO_2 interaction:
291 $F_{1,9}$ = 7.69, P = 0.022). Despite this change in total concentration, the relative composition of amino acids
292 did not change significantly under any of the global change treatments (all predictors were removed
293 during model simplification). There was a net effect of CO_2 on nectar sugar composition, but when the
294 concentrations of specific sugars were analysed individually, there was no effect of any drivers on
295 sucrose (the largest sugar constituent in nectar). However, there was a significant positive effect of
296 elevated CO_2 on the concentrations of glucose and fructose (glucose $F_{1,9}$ =6.13, P = 0.035; fructose $F_{1,9}$
297 =5.16, P = 0.049) though this effect was reduced by elevated temperature (T x CO_2 interaction: glucose
298 $F_{1,9}$ =11.95, P = 0.007; fructose $F_{1,9}$ =11.85, P = 0.007). There was also a negative interaction between
299 temperature and nitrogen (N x T interaction: glucose $F_{1,9}$ =11.62, P = 0.008; fructose $F_{1,9}$ =8.03 P = 0.02).
300 There were significant negative effects of both nitrogen and temperature on the ratio of glucose to
301 fructose (effect of N: $F_{1,8}$ =10.33, P = 0.0123, effect of T: $F_{1,8}$ = 21.58, P = 0.0017, and a nearly significant
302 positive effect of nitrogen on the ratio of sucrose to glucose + fructose (N: $F_{1,10}$ = 4.79, P = 0.053). In
303 contrast, CO_2 had a negative effect on the ratio of sucrose to glucose + fructose, and there was a
304 significant temperature x CO_2 interaction (CO_2: $F_{1,10}$= 7.885, P = 0.019, T x C: $F_{1,10}$= 5.39, P = 0.043).

305

306 Bumble bee preferences and longevity

There was a non-significant ($P < 0.07$) tendency for bees to visit the artificial flowers containing the nectar recipe from the elevated N treatment more frequently, and they consumed significantly more nectar from this treatment ($Z = 5.8$, $P < 0.0001$) (Supplementary Table S3c, Fig. 2). The effect of elevated nitrogen on consumption increased under elevated CO_2 (N x CO_2 interaction: $Z = 2.7$, $P = 0.006$) but was reversed under elevated temperature (N x T interaction: $Z = -5.8$, $P < 0.0001$). There was also a significant, positive three-way interaction ($Z = 2.6$, $P = 0.010$) among the drivers in their effect on the volume of nectar consumed by pollinators (Fig. 3). At ambient CO_2 levels, elevated nitrogen increased nectar consumption at ambient but not elevated temperatures. In contrast, at elevated CO_2 levels, increased levels of nitrogen had a positive effect on nectar consumption at both temperatures. Despite these effects on overall consumption by the colony, none of the drivers had significant main effects on the amount of nectar consumed per visit, though elevated N had a positive effect on consumption per visit at elevated CO_2 levels (N x CO_2 interaction: $P_{MCMC} = 0.025$).

Total consumption by the colony was negatively related to the concentration of sugars and amino acids ($Z < -6.3$, $P < 0.0001$ in both cases), though these negative effects were sub-additive (total sugar x total amino acid interaction: $Z = 6.9$, $P < 0.0001$). Consumption was also positively affected by the sugar ratios (glucose : fructose ratio: $Z = 3.81$, $P = 0.0001$, sucrose : (glucose + fructose) ratio: $Z = 2.63$, $P = 0.009$). The amount of nectar consumed per visit was not significantly affected by sugar ratios, concentration or amino acid concentration. In combination, these results suggest that the changes in total consumption by the colony were more strongly determined by changes in the number of visits, rather than in the amount of nectar consumed per visit.

Despite the higher visitation and consumption rates, nectar from the elevated N treatment significantly reduced bee survival by an average of eight days ($t_{1,9} = -3.78$, $P_{MCMC} < 0.001$). There was also a negative effect of elevated CO_2 on longevity ($t_{1,9} = -2.49$, $P_{MCMC} < 0.01$), which almost disappeared when elevated CO_2 was combined with elevated temperature, though this interaction effect was marginally non-

331 significant (CO_2 x temperature interaction: $t_{1,9}$ = 1.75, P_{MCMC} = 0.086). Finally, total sugar concentration
332 had a negative effect on bee longevity ($t_{1,6}$ = -2.03, P_{MCMC} = 0.032).
333
334 **Discussion**
335 Rapid environmental changes facing ecosystems worldwide are predicted to continue and accelerate
336 (Assessment 2005). We have demonstrated that plant responses to these conditions can have direct and
337 indirect effects on the mutual benefits associated with a pollination mutualism through various
338 pathways (Fig. S3). The three global change drivers – climate warming, nitrogen deposition, and carbon
339 dioxide enrichment – all had significant, non-additive impacts on both plants and their interactions with
340 pollinators. Critical plant reproductive traits were affected, with resulting impacts on pollinator
341 visitation, nectar consumption, and pollinator longevity. The high frequency of interaction effects we
342 observed among different drivers may partly explain the variability of previously-reported effects of
343 single drivers on characters such as flowering times (Springer & Ward 2007) and nectar volume
344 (Rusterholz & Erhardt 1998; Dag & Eisikowitch 2000).
345
346 The three drivers each affected flower timing, with CO_2 enrichment delaying flowering (possibly through
347 altered gene expression (Springer *et al.* 2008)), while N enrichment and increased temperatures both
348 accelerated the date of first flowering. Phenological mismatches between plants and pollinators are a
349 commonly-predicted result of climate change (Memmott *et al.* 2007; Hegland *et al.* 2009), potentially
350 driving pollen limitation in some early-flowering plants (Rafferty & Ives 2011). Our results suggest that
351 other drivers of environmental change (namely CO_2 enrichment and N deposition) may similarly disturb
352 flowering phenology.
353

354 Floral attributes such as the number of flowers and corolla size influence pollinator attraction to plants
355 (with bumble bees in particular preferring larger flowers (Goulson 2003)), and these attributes were
356 significantly affected by all three drivers. Furthermore, we found a number of main and interactive
357 effects of the drivers on the composition of nutrients in nectar (sugar and amino acid concentrations).
358 The subtle interactive effects of the drivers on nectar sugar concentration contrast with observations
359 that single drivers tend to affect the total amount of sugars in nectar through changes to nectar volume,
360 rather than sugar concentration (Rusterholz & Erhardt 1998; Dag & Eisikowitch 2000). However, total
361 sugar and amino acid concentration may be less important for stimulating or repelling insect nectar
362 feeding than are concentrations of particular amino acids (Petanidou *et al.* 2006). Alarmingly, the nectar
363 that was most frequently visited by bumble bees, and consumed in the greatest quantity by the colony,
364 was that from the elevated nitrogen treatments, which caused a significant reduction in bee longevity.
365 The elevated nitrogen treatment did not have higher concentrations of phenylalanine (which stimulates
366 feeding) or lower concentrations of amino acids such as asparagine, which behave as general repellents
367 (Petanidou *et al.* 2006)(Table S1). However, it did have higher sucrose to hexose (fructose and glucose)
368 ratios, which previous work (Petanidou 2005) and our results suggest can be more attractive to bees.
369 Acceptance of nectar is traditionally thought to be correlated with bee survival (Barker & Lehner 1974).
370 Thus, altered preference for nectar under rapidly changing environmental conditions may lead
371 pollinators to select less-nutritious floral resources, potentially reducing their fitness.
372
373 We found that sugar ratios can be altered by the global change drivers, and nectar sugar composition is
374 known to be related to the suite of pollinator species attracted to a particular plant (Petanidou *et al.*
375 2006) (*i.e*. pollination 'syndrome'(Perret *et al.* 2001)). For example, sucrose-dominated nectars, such as
376 that of *Cucurbita*, are preferred by large bees (Baker & Baker 1983), potentially explaining the
377 preference of *B. terrestris* for higher sucrose : hexose ratios. While we found no effect of the global

change drivers on sucrose concentration, the concentration of both glucose and fructose were increased by CO_2 and N, thereby reducing the ratio of sucrose to hexoses and attractiveness to bumble bees.

The fact that variation in the ratio of nectar sugars affects the type, number, species diversity, and duration of pollinator visits received by flowers (Baker & Baker 1983) carries strong implications for plant fitness, which depends on pollinator diversity and abundance (Hoehn *et al.* 2008). Previous work has found that elevated nitrogen alters the relative visitation frequency of different plant species by pollinators (Burkle & Irwin 2009a), and suggested that the effects of multiple interacting drivers on plant attractiveness to pollinators required further attention. Although we only found a marginally non-significant effect of N on pollinator visitation, we found a large number of interactions among the drivers in their effect on nectar consumption (Table S3C).

We found an interactive effect of elevated temperature and nitrogen on the total amino-acid concentration, and amino acids in nectar attract a variety of pollinators, such as butterflies, birds, bats, and bees (Erhardt & Rusterholz 1998). The amino-acid content of nectar is particularly important for strictly nectar-feeding pollinators, such as many butterflies, because they do not utilise pollen or insect prey as a source of protein. Thus, nectar amino-acid content affects butterfly fecundity (Mevi-Schutz & Erhardt 2005), and is higher in flowers that do not offer additional extra-floral protein sources (Baker & Baker 1986). While the composition of floral nectar amino acids is thought to be consistent within a species (Baker & Baker 1977), the total concentration is more variable (Gardener & Gillman 2001), and our results indicate that this concentration can be altered by interactive effects of global environmental changes. Although bumble bees require ongoing access to proteinaceous food for adult survival (Smeets & Duchateau 2003), we found no effect of amino acid concentration on bee longevity, despite the absence of pollen in the diet of caged bees in this study. This suggests that nectar amino acids may not

play a large role, though global change drivers may also affect bumble bee longevity through changes to pollen amount or nutritive value.

The total economic value of crop pollination worldwide is estimated to be over €153 billion annually (Gallai *et al.* 2009), and 70% of agricultural crops depend on pollinators (Klein *et al.* 2007). There is growing concern about global decline in many pollinator species (Potts *et al.* 2010; Cameron *et al.* 2011), and our results highlight novel mechanisms through which human changes to the environment may alter plant-pollinator mutualisms. The high frequency of interaction effects we found indicates that current environmental changes will have manifold effects on pollination services, which cannot be predicted from studies of single drivers in isolation. These interactions among drivers may be as important as the main effects, and studies of multiple drivers will continue to reveal complex and unanticipated outcomes.

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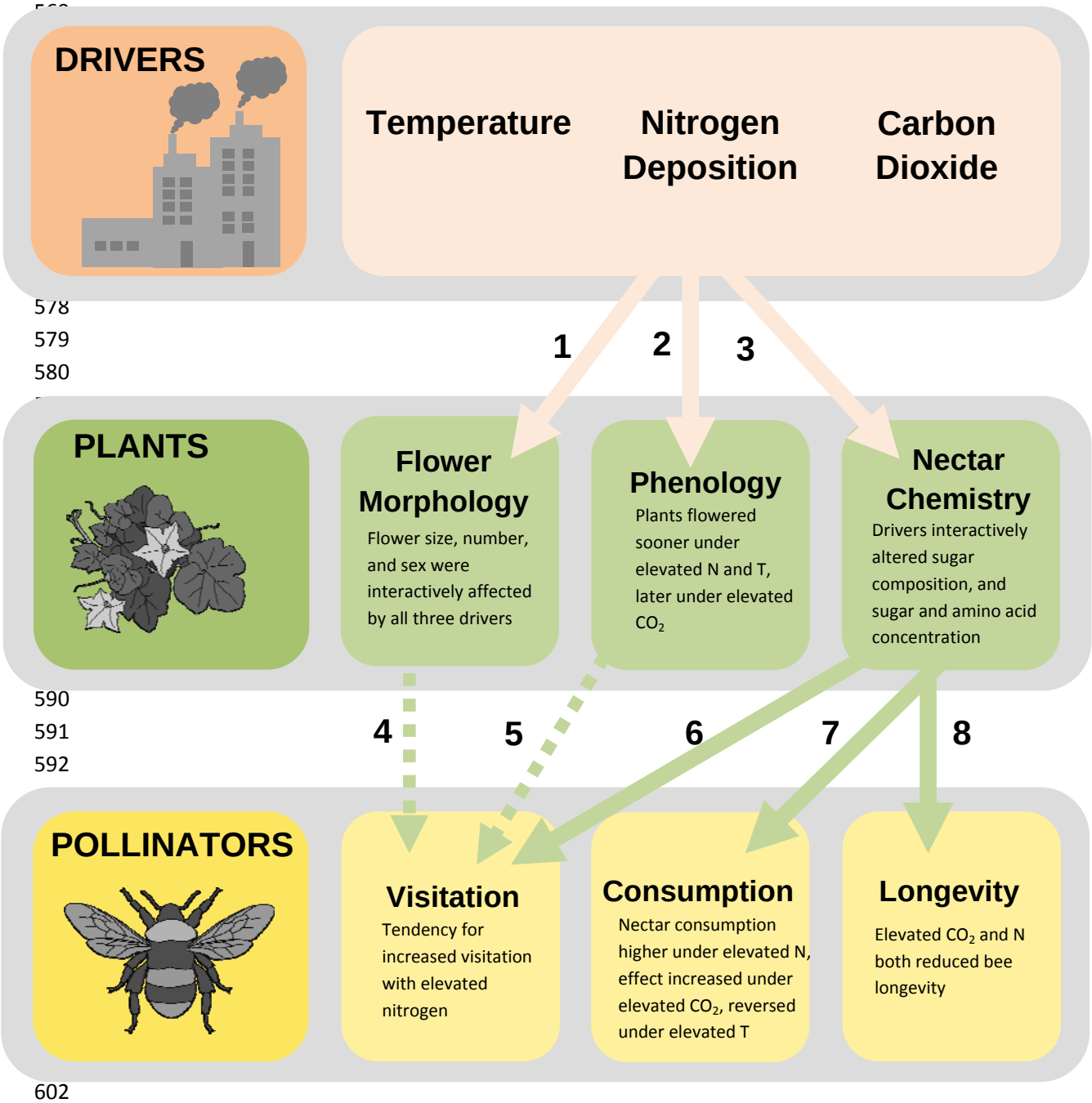
Figure 1: Pathways through which global environmental change drivers (climate, nitrogen deposition, CO_2 and their interactions) may affect pollination mutualisms. Dashed arrows represent pathways (**4**, **5**) examined in other studies (see Fig. S3 for further details), solid arrows represent pathways examined here. **1)** Multiple impacts of the drivers on flower size, abundance, nectar volume, and sex ratios. **2)** Flowering phenology was affected by all three drivers **3)** The drivers interactively affected nectar chemistry (sugar and amino acid concentration and sugar composition). Bees tended to more frequently visit **(6)** and consume nectar **(7)** from the elevated N treatment, and this effect interacted with CO_2 and temperature. **8)** Nectar from the elevated N and elevated CO_2 treatments significantly reduced bee longevity.

Figure 2. Impact of nitrogen enrichment on a plant-pollinator mutualism. Changes to pumpkin (*C. maxima*) nectar constituents alter pollinator (*B. terrestris*) visitation to artificial flowers, nectar consumption, and longevity. The nectar from the elevated nitrogen treatment increased bumble bee visitation, and nectar consumption; however it decreased bee longevity.

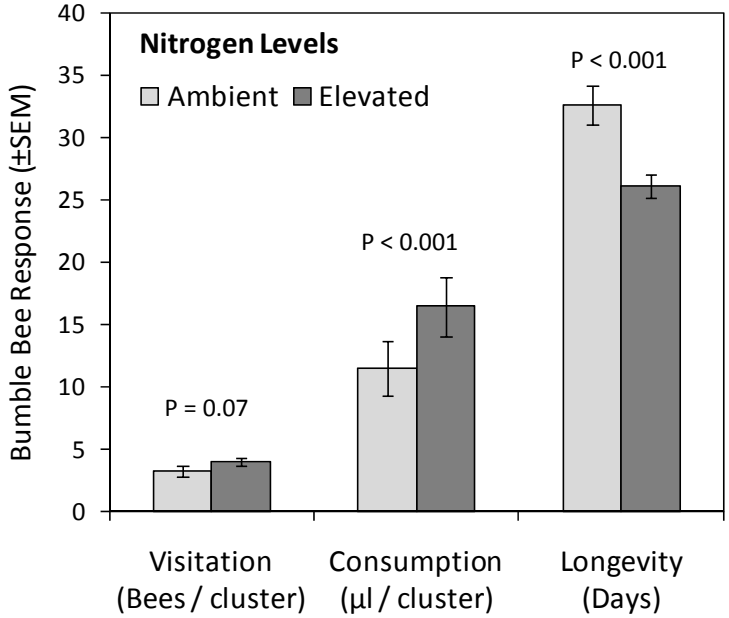
Figure 3. Interactive effects of drivers on nectar consumption. Interaction plots showing the impact of environmental change drivers (ambient vs. elevated levels of nitrogen, CO_2 and temperature) on the amount of nectar consumed by bumble bees. **(a)** ambient CO_2 levels (360 ppm) and **(b)** elevated CO_2 levels (700ppm). Mean values (± SEM) are calculated from parameter estimates (with an inverse link function applied) of a generalised linear mixed effects model, after controlling for random effects of bee colony and cage. Elevated nitrogen generally increased nectar consumption (significant main effect of N), however this effect was modified by both temperature and CO_2, as there was a significant 3-way interaction among the drivers.

Figure 1.


DRIVERS
Temperature
Nitrogen Deposition
Carbon Dioxide
1
2
3
PLANTS
Flower Morphology
Flower size, number, and sex were interactively affected by all three drivers
Phenology
Plants flowered sooner under elevated N and T, later under elevated CO_2
Nectar Chemistry
Drivers interactively altered sugar composition, and sugar and amino acid concentration
4
5
6
7
8
POLLINATORS
Visitation
Tendency for increased visitation with elevated nitrogen
Consumption
Nectar consumption higher under elevated N, effect increased under elevated CO_2, reversed under elevated T
Longevity
Elevated CO_2 and N both reduced bee longevity

610 **Figure 2.**

611



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615 **Figure 3.**

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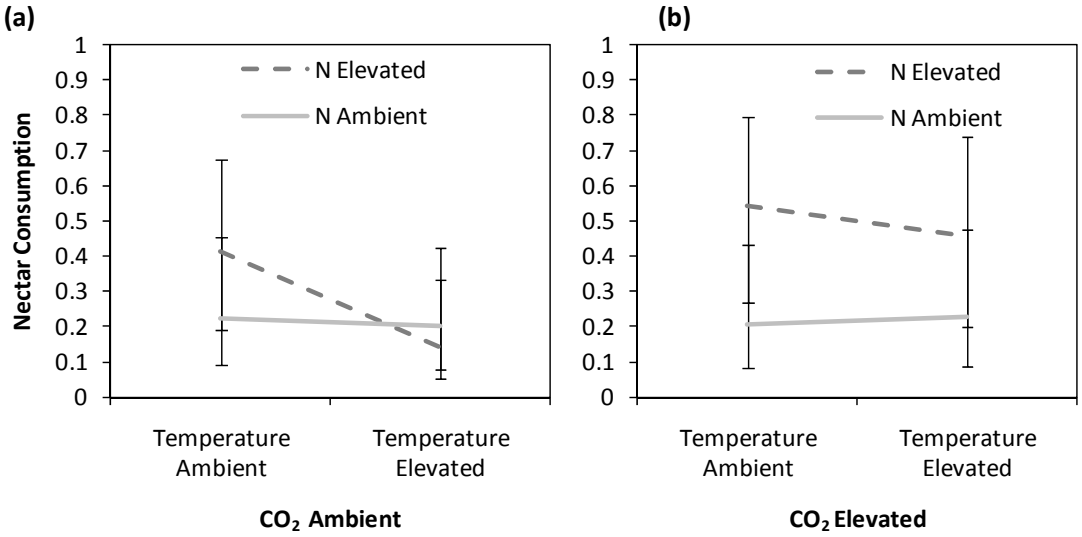

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