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Contrasting effects of nitrogen fertiliser application on the performance of closely related grasshoppers through changes in plant nutrient concentrations

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Abstract

1. Global environmental changes mediated by anthropogenic processes can affect the nutrient status of plants, with important consequences for the performance and dynamics of insect herbivores that feed on them.
2. While it is well documented that insects from different feeding guilds (e.g., sap-feeders and leaf-chewers) can respond differently to altered food resources due to their distinct physiological and ecological characteristics, little is known about how ecologically similar insect species from the same feeding guild respond to changes in food nutrient status.
3. Using nitrogen (N) fertiliser, the authors examined the effects of N inputs on two sympatric grasshopper species, *Euchorthippus cheui* and *E. unicolor*, that share the same host food plant, *Leymus chinensis* grass. The authors examined the effects of fertilisation on the individual feeding behaviour, performance and abundance of the two grasshopper species.
4. The nutrient (protein) content of *L. chinensis* leaves was enhanced by fertilisation during the entire season. However, *E. cheui* and *E. unicolor* exhibited differing growth rates, development and body size responses to fertilisation.
5. *E. cheui* preferred *L. chinensis* leaves from high-N fertilised treatments, while *E. unicolor* preferred leaves from low-N fertilised treatments. Moreover, fertilisation increased the abundance of *E. cheui* but had no significant effect on the abundance of *E. unicolor* in the field.

6. The findings imply that effective management and conservation strategies for insects should target the needs of individual species rather than species groups or communities as a whole because nutritional and environmental requirements are often species-specific.

KEYWORDS

climate change, herbivore community, nitrogen deposition, plant quality, plant-herbivore interactions

INTRODUCTION

A suite of anthropogenic environmental changes is affecting plant community composition, plant traits and herbivore responses across much of the biosphere (Blois et al., 2013; Descombes et al., 2020; Harvey et al., 2020). Among the most widespread of these changes are the effects of agricultural practices, such as intensive use of fertilisers, as well as other processes, like climate change, which can have a major impact on natural food webs and biotic interactions (de Ruiter et al., 1994; Meunier et al., 2016; Renoirt et al., 2021). Effects of these processes can go in two directions: increased eutrophication through fertiliser or atmospheric nitrogen (N) deposition leads to increased plant nutrient concentrations (Decina et al., 2020; Galloway et al., 2008), which can benefit the performance of herbivores via enhanced food quality. Alternatively, elevated atmospheric carbon dioxide concentrations and higher temperatures may lead to increased plant biomass and nutrient dilution in plants, resulting in a reduction of nutrients per unit of plant mass and negatively affecting herbivores (Welti et al., 2020). Given these potentially opposing effects, a central question is how insect herbivores in nature may respond to them. Significant declines in insect abundance and biomass have been reported worldwide, and the cause is not immediately clear or there are multiple drivers of this decline (Dirzo et al., 2014; Hallmann et al., 2017; Sánchez-Bayo & Wyckhuys, 2019; Wagner, 2020; Young et al., 2016). It has been suggested that recent insect declines are being caused, among other factors, by alterations in plant nutrient concentrations (Joern et al., 2012; Pöyry et al., 2017; Welti et al., 2020; Zhu et al., 2019).

One of the key limiting nutrients in terrestrial ecosystems is nitrogen (N), and organisms in terrestrial systems are often N-limited, while herbivores should benefit from higher plant protein contents induced by N inputs (Mattson, 1980; White, 1993). While this pattern has generally been supported empirically, studies using a geometric framework methodology also show that some insect herbivores perform better and prefer low protein and high carbohydrate diets (Raubenheimer & Simpson, 1993; Simpson & Raubenheimer, 1993). One proposed mechanism to explain these exceptions is an imbalance of protein relative to carbohydrates. Depending on the physiology and ecological characteristics of organisms, and long-term adaptations to nutritional resources, insects from different feeding guilds can have different nutrient and energy requirements (Behmer, 2009, and references therein). For example, the N intake target of aphids is often much lower than that of leaf-chewers because phloem is typically

richer in sugars and poorer in N than leaves (Abisgold et al., 1994). Although earlier work showed that such differences in nutrient requirements may also vary between closely related species that share food resources (Behmer & Joern, 2008), knowledge on species-specific responses of taxonomically related insects (i.e., congeners) to changes in plant nutrient status is still scarce. Such knowledge is needed in order to understand to what extent we can generalise the impacts of global changes on the food quality of insects and other arthropods.

Although it has been established that plant nutrient concentrations may drive insect performance, on a large scale, links between nutrient status and insect abundance are often only based on correlations. For example, a recent study showed that nutrient content in prairie grasslands in North America has decreased over several decades and has been accompanied by a reduction in the biomass of native grasshoppers. However, the actual link between declining plant nutrient concentrations and grasshopper declines was not tested (Welti et al., 2020). Similarly, the argument that insects may decline with increased atmospheric N deposition in NW European heathlands is based on spatial correlations between plant nutrient concentrations, stoichiometry and observed insect abundances (Vogels et al., 2017). Therefore, to what extent insect abundances in the field are causally linked to shifts in plant nutrient content is not well understood. To acquire this knowledge requires experimental manipulation of plant quality in the field to test how insect populations respond, in combination with controlled feeding trials to test the responses of individuals. It is also important to conduct feeding trials using local plants in lieu of artificial diets, because this will help to understand the impacts of N fertilisation in the context of other plant chemical and physical properties.

Here, we present the results of an experiment testing the effects of N fertilisation on plant nutrient concentrations and plant defence, and this in turn on the preference, performance and abundance of closely related sympatric grasshopper species (*Euchorthippus cheui* and *E. unicolor*) in a semi-arid grassland where both species are native. We test three hypotheses. First, we test the hypothesis that *E. cheui* and *E. unicolor* respond to variable host plant quality induced by fertilisation in opposing ways. We expect this because the two grasshopper species have different peak emergence times. *E. cheui* occurs earlier in the growing season, hatching in mid-June with adults appearing in July. In contrast, its sister species, *E. unicolor*, occurs later in the growing season, with adults appearing in mid-August to early September (Ren, 2002). Both grasshoppers mainly feed on the dominant grass

species, *Leymus chinensis*, which makes up more than 70% of their diet (Data S1). The total N content of *L. chinensis* leaves gradually decreases over the growing season as plants develop (Zhu et al., 2020). Because the two grasshoppers appear at different times, we suggest that they might encounter *L. chinensis* of different quality, that is, early-season *E. cheui* is adapted to high-N food available early in the season and vice versa. In this scenario, we predict that *E. cheui* will react positively to an increase in plant nutrients induced by fertilisation, while *E. unicolor* reacts negatively. Our second hypothesis is that the responses of grasshoppers to fertilisation vary between males and females. We expect females require more nutrients and respond to fertilisation more positively than males because they have different physiological and nutritional requirements associated with reproduction. Finally, we test a third hypothesis that shifts in plant quality not only affect grasshopper preference and performance (an individual effect), but also potentially contribute to the abundance of two species (a population effect) in the field.

MATERIALS AND METHODS

Study site and insect herbivores

Our study was conducted at the Songnen Grassland Ecological Research Station in northeast China (44°45'N, 123°45'E). The field station is located in the Songnen grassland, which is the eastern region of the Eurasian steppe. In this grassland area, *Leymus chinensis*, a perennial grass, is the dominant plant species and accounts for 50%–80% of the total aboveground vegetation biomass (Wang & Ba, 2008). The grasshoppers *Euchorthippus cheui* and *E. unicolor* are the two most numerous insect herbivores in this area, annually accounting for >65% of all grasshopper individuals (Zhong et al., 2017). Both *E. cheui* and *E. unicolor* are univoltine (one generation per year) and pass through five nymphal stages to become adults. There is a general phenological difference between the species, with *E. cheui* being an early-season species and *E. unicolor* a late-season species. Therefore, we conducted our experiments in July and August, during the growing season, to coincide with the development of two species. The grasshoppers chosen are particularly suited for this research as both species are grass-feeders. Since grasses generally lack (or are at least low in) plant secondary metabolites, our fertilisation treatment would primarily affect plant nutrients and not secondary compounds (Tscharntke & Geiler, 1995). This allows us to focus on the effects of N on food quality.

Fertilisation experiment

In April 2016, we conducted an in situ field fertilisation experiment according to a completely randomised design with four levels of N fertiliser treatments. Sixteen independent 3 × 3 m plots were set up in a community dominated by grass *L. chinensis* (~90% relative cover) at 5 m intervals. Four-N level treatments (0, 5, 10 and 17 g N/m²/yr, hereafter referred to as “0 N, 5 N, 10 N and 17 N”) were randomly allocated to all

the plots with four replications. These levels of N addition have been shown to produce an effect on the quality of *L. chinensis* leaves (Q. Ma, unpublished data). The highest N level is similar to the fertilisation rates of most crops in this region (Cease et al., 2012). Nitrogen was applied as an aqueous solution of commercial NH₄NO₃ at two application times (Bai et al., 2010), with the unfertilized plots receiving an equivalent volume of water. Plots were fertilised in the early growing season (May 1), and the middle of the growing season (June 28), and 50% of N fertiliser was applied at each time.

Grasshopper preference in feeding trials

A series of paired-choice feeding experiments to measure the initial sensory preference by grasshoppers to different N fertiliser treatments. Feeding trials were all conducted in an environmentally controlled room (16:8 light: dark photo cycle, 28 ± 2°C and 30% relative humidity). *E. cheui* and *E. unicolor* were originally collected in the 5th instar (final nymphal instar) from field populations. Nymphs were kept in the lab on unfertilized *L. chinensis* leaves from the field for two days before using them in the feeding trials. At the beginning of the feeding trials, nymphs were separated according to sex and individually introduced into cages with 0.15 m diameter and 0.3 m height. They were kept overnight with sufficient water but no food to empty their guts and thus reach a similar physiological state between the individuals (Cease et al., 2012). Each nymph was offered a choice between leaves from different N fertiliser treatments (i.e., 0 N vs. 5 N, 0 N vs. 10 N, 0 N vs. 17 N, 5 N vs. 10 N, 5 N vs. 17 N, 10 N vs. 17 N) with up to 20 replications per species per sex. Fresh leaves were collected arbitrarily from field plots of each fertiliser treatment and were mixed and immediately placed in feeding experiments. Leaves (approx. 8 cm long) were placed into water-filled Petri dishes (0.1 m in diameter) through two slits on the lid, and each treatment had an equal size. The fresh weight of leaves was determined immediately before the experiments. Nymphs were left undisturbed until they fed substantially on at least one leaf (~40% removal) or up to a maximum of 30 h. The dry weight consumption of each leaf sample was calculated gravimetrically (Cease et al., 2012). Specifically, the dry weight eaten was calculated by subtracting the dry weight of uneaten leaves from an estimate of the original dry weight for a given sample. Original dry weight for each plant sample was estimated by multiplying the initial fresh weight by the dry:wet mass ratio estimate for each N treatment. For each species, the feeding trials were conducted within one-week, starting on 19 July and 18 August 2016, respectively, corresponding to the species' peak abundance in the field (*E. cheui* peaks in mid-July and *E. unicolor* in mid-August). All grasshoppers in the feeding trials were collected from unfertilized host plants in the field, leading us to assume that their nutritional status was similar.

Grasshopper performance in feeding trials

To assess the effects of fertilisation on *E. cheui* and *E. unicolor* performance, we determined survival, growth rate, body mass and body size

when fed on *L. chinensis* leaves from 0 N and 10 N (medium N level) fertiliser treatments. Nymphs in the 3rd instar were collected from field populations and kept on unfertilized leaves in the lab as described above. After two days of reaching the 4th instar, nymphs were separated according to sex and randomly assigned to one of the two N fertiliser treatments. Ten individuals were placed in a 0.3 m diameter and 0.3 m height cage with seven replications per species per sge with seven replications per species per sex. Fresh *L. chinensis* leaves were cut arbitrarily from the field plots and mixed as a bulk sample for each treatment. Then leaves were offered in small water-filled plastic vials, presented ad libitum, and replaced every other day to avoid changes in quality. Grasshoppers were feeding for three weeks during July (for *E. cheui*) and August (for *E. unicolor*) when all nymphs have moulted to adults. The survival rate was estimated by counting the remaining grasshoppers in each cage. Adult body size was determined by measuring the hind femoral length of grasshoppers using an Electronic Digital Calliper. The relative growth rate was calculated as $\ln(W_{\text{final}}) - \ln(W_{\text{initial}}) / \text{time}_{\text{days}}$; W_{initial} (initial body mass) and W_{final} (final body mass) was determined by pooling and weighing all individuals for each cage before the start and end of experiments, $\text{Time}_{\text{days}}$ was referred to the whole experiment periods.

Grasshopper abundance in the field

To assess the effects of fertilisation on the abundances of *E. cheui* and *E. unicolor* in the field, we set up a 1-m² quadrat in each of the 0 N and 10 N fertiliser treatment plot. Each quadrat was carefully enclosed using a 1.5 m high shading screen barrier. Then all grasshoppers were collected using a sweep net, and the numbers of *E. cheui* and *E. unicolor* were counted. Surveys were conducted on 20 July, and 25 August 2016, when field populations of *E. cheui* and *E. unicolor* are during its peak period, respectively.

Host plant quality

In order to test the effects of fertilisation on the quality of the host plant, we measured the nutrient concentrations, chemistry and biomechanical properties of *L. chinensis* leaves (carbon (C), nitrogen (N), protein, total non-structural carbohydrates (TNC), water, cellulose, total phenol content and toughness) on the same dates as the sampling for grasshopper density in the field. Protein and carbohydrates were selected because they have been shown to be the key macronutrients for herbivores (Raubenheimer & Simpson, 1993), though they were much less reported in the literature compared to plant C and N concentrations. This is problematic because while C and N can serve as proxies for plant nutrients, they also include a large portion of substances that are structural, indigestible and even toxic to herbivores (Le Gall et al., 2020). At each sampling, twenty *L. chinensis* individuals were collected randomly from the quadrat used to measure grasshopper abundance within each plot. Two of the uppermost fully expanded leaves that had not been damaged by herbivores were chosen and

weighed. Leaves were oven-dried for 48 h at 70°C and weighed for water content and the subsequent analyses. Dried leaves were then ground to fine powder using a ball-mill and analysed total C and N concentrations using a Vario MICRO cube Elemental Analyser (Elementar GmbH., Hanau, Germany). Protein, TNC, cellulose and total phenolics of *L. chinensis* were determined using their corresponding assay kits (Suzhou Keming Science & Technology Inc., Suzhou, China), and all procedures followed the manufacturers' instructions. Toughness was measured by another twenty fresh leaves collected from quadrats using a modified SY-S03 penetrometer (Shiya Inc., Shijiazhuang, China) by recording the force needed to penetrate a leaf with a rod.

Statistical analyses

To assess the effects of fertilisation and sampling time on the quality of *Leymus chinensis*, we used linear models with “N fertilisation” (4 levels: 0 N, 5 N, 10 N and 17 N) and “sampling time” (2 levels: July and August) as fixed factors and plant nutrients and defensive properties (C:N ratio, protein, TNC, water, cellulose, total phenolic and toughness) as dependent variables. Within each sampling time, linear models with “N fertilisation” as a fixed factor were performed to detect differences in the above-mentioned variables. Tukey multiple comparison analyses were used whenever significant differences were found. Differences between sampling times for each N application treatment were analysed using t-tests. To assess the effects of fertilisation on grasshopper performance, a generalised linear mixed model with binomial error structure was used to determine the effects of fertilisation on the grasshopper survival rate, in which “N fertilisation”, “species” and “sex” were included as fixed factors and “cage” was included as a random factor (Chen et al., 2018). Similar models with Gaussian error structures were repeatedly used to determine the effects of fertilisation on the relative growth rate, body mass and body size of the two grasshoppers (glmer function in the R lme4 package). To examine the effect of fertilisation on the abundance of grasshoppers, we used a linear model with “fertilisation” as a fixed factor and the abundance of *E. cheui* and *E. unicolor* as dependent variables. For paired-choice feeding experiments, the selectivity of grasshopper between different N treatments was estimated by a score of either win or loss that was assigned to each paired comparison (i.e., 0 N vs. 5 N, 0 N vs. 10 N, 0 N vs. 17 N, 5 N vs. 10 N, 5 N vs. 17 N, 10 N vs. 17 N). Wins or losses were assigned based on dry mass consumption of each sample (Cease et al., 2012). We analysed the proportion of wins: losses against the null prediction that there would be an equal probability for grasshopper to choose the plant from each fertilised treatment using chi-squared tests (Harvey et al., 2009). The package rcompanion was used to conduct post-hoc pairwise chi-squared tests whenever significant differences were found (McGreevy et al., 2018). We used paired-t tests to analysis the dry mass consumption of grasshoppers for different N treatments in each comparison group. The homogeneity of the variances was tested for all response variables using a Levene's test. We tested the normality of residuals using the

TABLE 1 Effects of fertilisation, sampling time, and their interactions on concentrations of nutrients and defensive traits in *Leymus chinensis* leaves

	Factors	Df	F	p
C:N ratio	Fertilisation	3.24	780.44	<0.001
	Time	1.24	0.8	0.38
	Fertilisation × Time	3.24	15.06	<0.001
Protein	Fertilisation	3.24	85.2	<0.001
	Time	1.24	82.69	<0.001
	Fertilisation × Time	3.24	29.17	<0.001
TNC	Fertilisation	3.24	2.00	0.14
	Time	1.24	26.10	<0.001
	Fertilisation × Time	3.24	2.35	0.10
Total phenols	Fertilisation	3.24	7.44	0.001
	Time	1.24	0.71	0.41
	Fertilisation × Time	3.24	2.36	0.10
Water	Fertilisation	3.24	1.47	0.25
	Time	1.24	6.67	0.02
	Fertilisation × Time	3.24	10.20	<0.001
Cellulose	Fertilisation	3.24	2.14	0.12
	Time	1.24	2.69	0.11
	Fertilisation × Time	3.24	4.79	0.009
Toughness	Fertilisation	3.24	20.68	<0.001
	Time	1.24	1.26	0.27
	Fertilisation × Time	3.24	4.66	0.01

Note: Leaf nitrogen (N), carbon (C), protein, total non-structural carbohydrates (TNC), total phenols, water, cellulose, and toughness were sampled from different N application treatments (0, 5, 10 and 17 g N/m²) in July and August 2016, respectively. Bold numbers indicate a significance of $p < 0.05$.

Shapiro-Wilks test. Untransformed data are presented in the figures. All statistical analyses were performed in the open source software R 4.1.0 (R Development Core Team., 2018).

RESULTS

Effects of fertilisation on host plant quality

Leaf concentrations of nutrients (i.e., protein, C:N ratio) and defensive traits (i.e., water, cellulose and toughness) varied significantly with fertilisation and sampling time (Table 1). Fertilisation significantly increased protein content ($F_{3,24} = 85.2, p < 0.001$, Table 1) and total phenols ($F_{3,24} = 7.44, p = 0.001$), but significantly decreased the C:N ratio ($F_{3,24} = 780.44, p < 0.001$) and toughness ($F_{3,24} = 20.676, p < 0.001$) of *L. chinensis* leaves. Without fertilisation, protein content of *L. chinensis* was significantly higher in July than in August ($t_{4,36} = 5.07, p = 0.006$, Table 2), but the C:N ratio ($t_{5,75} = -3.88, p = 0.009$) and leaf toughness ($t_{4,32} = -2.71, p = 0.05$) were lower in July than in August (Table 2).

Effects of fertilisation on the feeding behaviour of the grasshoppers

Consistent with our first hypothesis that grasshoppers respond to fertilisation in different ways, feeding selection experiments revealed that food preference and consumption on different N fertiliser treatments varied with species. *E. cheui* selectively fed on *L. chinensis* leaves from high-N fertiliser treatments, while *E. unicolor* preferred leaves from the low-N fertiliser treatments (Figure 1, Table S1). Moreover, the bigger the difference between N fertiliser treatments, the

TABLE 2 Effects of fertilisation on concentrations of nutrients and defensive traits in *Leymus chinensis* leaves. Values are means ± SE.

		0 N	5 N	10 N	17 N	F _{3,12}	p
C:N ratio	July	34.04 ± 0.56a	25.84 ± 0.37b	20.72 ± 0.34c	16.42 ± 0.44d	301.5	<0.001
	August	37.49 ± 0.69a	23.84 ± 0.13b	20.16 ± 0.28c	16.59 ± 0.28d	509.4	<0.001
Protein (%)	July	7.98 ± 0.2a	8.19 ± 0.23ab	8.84 ± 0.18b	9.85 ± 0.13c	19.68	<0.001
	August	6.88 ± 0.1a	9.71 ± 0.41b	12.18 ± 0.51c	13.07 ± 0.09d	69.25	<0.001
TNC (%)	July	4.07 ± 0.26	4.15 ± 0.33	4.09 ± 0.16	4.19 ± 0.22	0.049	0.985
	August	6.01 ± 0.69	6.29 ± 0.83	6.48 ± 0.50	4.38 ± 0.15	2.54	0.106
Total phenol (%)	July	1.68 ± 0.015	1.76 ± 0.039	1.78 ± 0.019	1.76 ± 0.033	2.453	0.114
	August	1.66 ± 0.03a	1.71 ± 0.06a	1.80 ± 0.02ab	1.87 ± 0.01b	6.367	0.008
Water (%)	July	44.10 ± 1.13a	38.73 ± 1.43b	42.53 ± 1.06ab	43.58 ± 1.22ab	3.972	0.035
	August	44.65 ± 1.24a	46.90 ± 0.21ab	43.16 ± 0.16bc	41.42 ± 0.45c	11.93	<0.001
Cellulose (%)	July	51.25 ± 3.66	60.97 ± 3.59	62.53 ± 3.17	54.94 ± 5.04	1.788	0.203
	August	60.83 ± 1.23a	58.42 ± 2.56ab	47.29 ± 3.96b	47.57 ± 2.18b	7.097	0.005
Toughness (N)	July	1.99 ± 0.21ab	2.26 ± 0.14a	1.96 ± 0.14ab	1.49 ± 0.14b	3.887	0.038
	August	2.64 ± 0.10a	2.49 ± 0.13a	1.64 ± 0.10b	1.38 ± 0.10b	31.93	<0.001

Note: Bold numbers indicate a significance of $p < 0.05$. Different lowercase letters indicate fertilisation had significant effect within each time ($p < 0.05$). The nutrient and defensive concentrations were expressed on a dry matter basis (% d.m.).

stronger the preference of grasshoppers would be. For example, when provided *L. chinensis* leaves from low-discrepancy paired groups (e.g., 0 N vs. 5 N, 10 N vs. 17 N), there was no detectable difference in the preference of grasshoppers between species and sex (Figure 1). But when provided leaves from high-discrepancy paired groups (0 N vs. 10 N and 0 N vs. 17 N), the preference of two grasshoppers was significantly different (0 N vs. 10 N, $\chi^2 = 18.27$, $p < 0.001$; 0 N vs. 17 N, $\chi^2 = 16.44$, $p < 0.001$; Figure 1b,c).

Effects of fertilisation on grasshopper performance

Consistent with the hypothesis, the performance of *E. cheui* and *E. unicolor* responded differently to the fertilisation as indicated by the

interactive effects between fertilisation and species (survival rate, $\chi^2 = 38.52$, $p < 0.001$; growth rate, $F_{1,42} = 4.55$, $p = 0.04$; body mass, $F_{1,42} = 27.1$, $p < 0.001$; femoral length, $F_{1,42} = 32.30$, $p < 0.001$, Table 3). The performance of *E. cheui* was higher when reared on leaves collected from 10 N fertilised plots than on 0 N plots (survival rate, $\chi^2 = 15.49$, $p < 0.001$; body mass, $F_{1,24} = 9.66$, $p = 0.005$; femoral length, $F_{1,24} = 24.83$, $p < 0.001$; see Figure 2a,c,d), while the performance of *E. unicolor* was lower when confined to feed on 10 N fertilised leaves (survival rate, $\chi^2 = 21.27$, $p < 0.001$; body mass, $F_{1,24} = 36.39$, $p < 0.001$; femoral length, $F_{1,24} = 26.22$, $p < 0.001$; see Figure 2e,g,h). Contrary to our second hypothesis, there were no sex-specific responses for both species in response to N fertilisation, as indicated by no interactive effect between sex and fertilisation (Table 3).

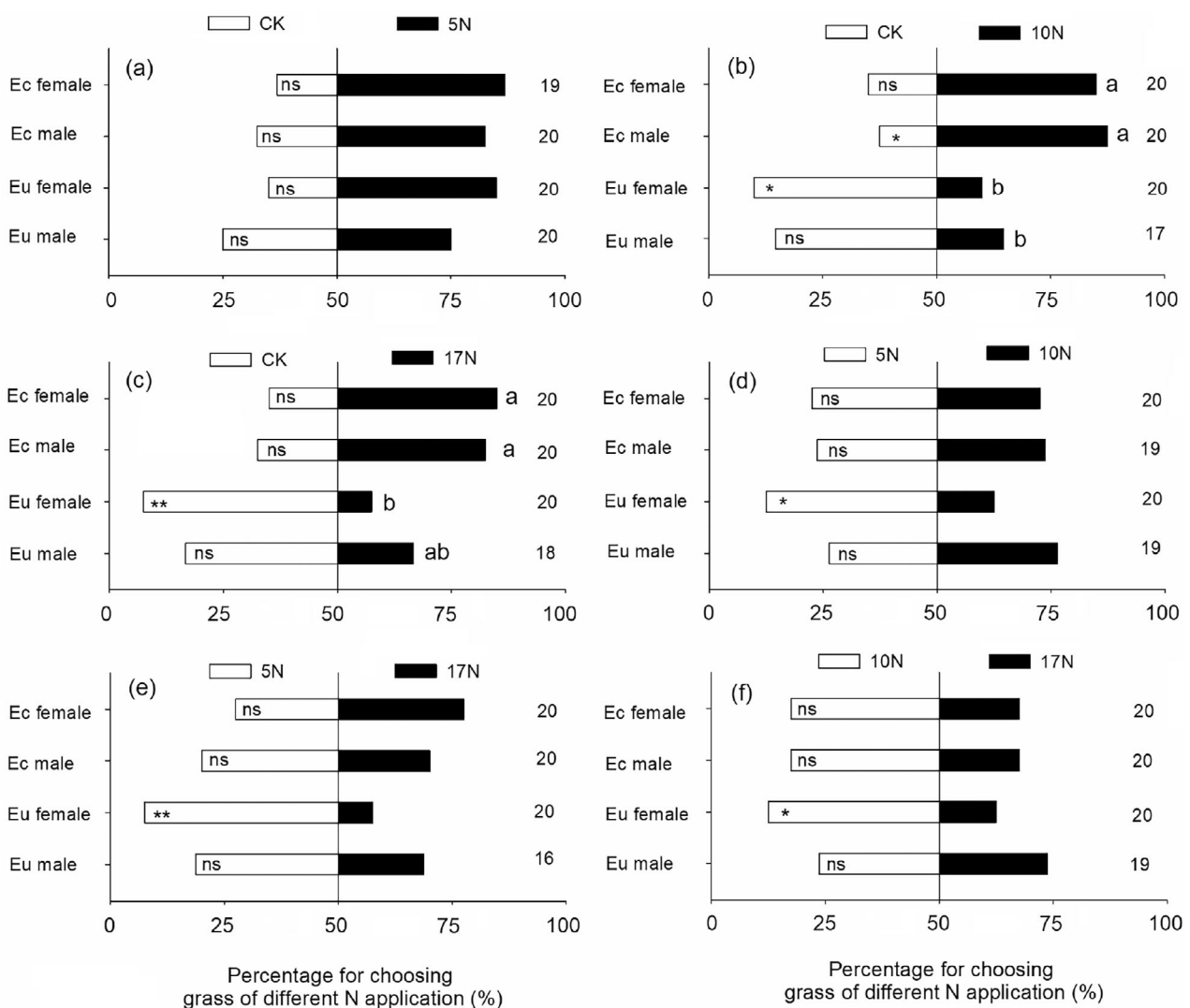


FIGURE 1 Percentage of grasshopper *Euchorthippus cheui* (Ec) and *E. unicolor* (Eu) selecting *Leymus chinensis* from different fertiliser treatment in paired-choice feeding experiments. Six paired *L. chinensis* leaves were compared: (a) 0 N and 5 N, (b) 0 N and 10 N, (c) 0 N and 17 N, (d) 5 N and 10 N, (e) 5 N and 17 N, and (f) 10 N and 17 N. Sample sizes are as indicated at far right column. Different lowercase letters adjacent to bar on right hand side indicate significant difference of food preference between two grasshopper species from different sexes ($p < 0.05$). Asterisks inside bar on left hand side indicate significant difference of preference by grasshoppers in each pairwise comparison (ns = not significant, * $p < 0.05$, ** $p < 0.01$).

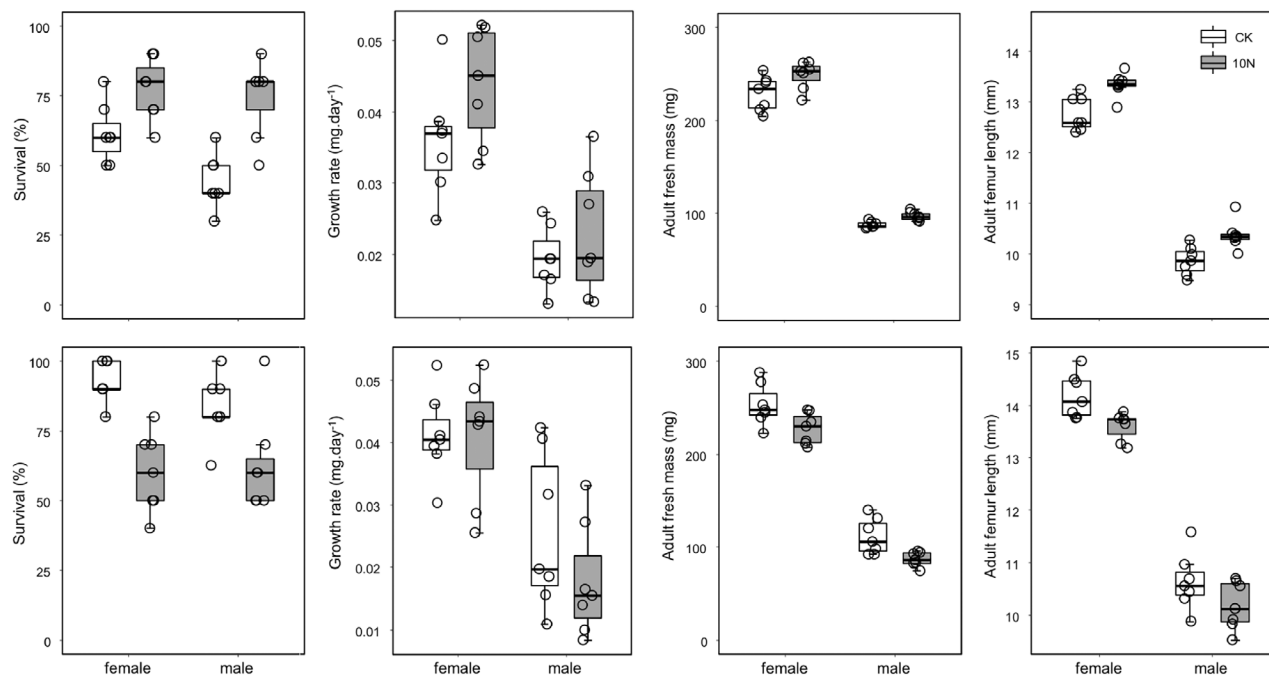


FIGURE 2 Effects of fertilisation on the performance of grasshopper *Euchorthippus cheui* and *E. unicolor*. The (a), (e) survival, (b), (f) relative growth rate, (c), (g) body mass and (d), (h) body size of female (open bars) and male (black bars) *E. cheui* and *E. unicolor* were monitored for three weeks. Grasshoppers were reared on *Leymus chinensis* leaves collected from 0 and 10 g N/m² fertilised plots. Values are means \pm SE.

Effects of fertilisation on grasshopper population density in the field

In July, fertilisation significantly increased the abundance of *E. cheui* by 94% ($F_{1,6} = 13.76$, $p = 0.01$, Figure 3). In August, however, there was no significant difference in the abundance of *E. unicolor* in the fertilised and unfertilized plots, but their abundance did tend to be lower in the fertilised plots albeit with a high variance ($F_{1,6} = 2.842$, $p = 0.143$).

DISCUSSION

Several anthropogenic processes associated with global change, and especially those that can affect the chemical environment, can affect plant traits, including nutrient concentrations and defensive compounds, in different ways. These processes are likely to have profound impacts on insect herbivores at the individual, population, and community levels. One of the most widespread and important effects of intensive agriculture is a heavy reliance on the application of fertilisers to enhance rapid crop growth (Snyder et al., 2009). Thus far, attention has focused primarily on the effects of nitrogen application and nutrient loading on communities of insects and other organisms in agricultural landscapes and ecosystems that border them, such as grasslands, where causation for demographic changes is sometimes inferred (Lawton et al., 2020; Vogels et al., 2017; Welti et al., 2020). However, less attention has been paid to understand how demographic responses in coexisting, closely related insects are driven by

differences in their preference and performance in response to fertiliser application. Our study demonstrates that fertilisation can have contrasting effects on the performance of different closely related grasshopper species, as well as on their feeding behaviour and on the abundance in the field.

Fertilisation had contrasting effects on the closely related grasshoppers, which is in line with our first hypothesis. Our findings agree with earlier work by Zhu et al. (2020), who found species-specific responses of *E. cheui* and *E. unicolor* to livestock grazing, which they attributed to differences to plant quality. In the present study, we provide a mechanistic explanation for such a contrasting effect of plant quality on these two grasshoppers at both individual and population levels. We found that fertilisation promoted the leaf protein contents of *L. chinensis*, and the grasshopper *E. cheui* preferred and performed better on the leaves from the high-N fertilised plots while *E. unicolor* preferred and performed better on the unfertilized leaves. The contrasting responses of *E. cheui* and *E. unicolor* to fertilisation in our study suggest that closely related species can have very different nutrient preferences and physiological responses that transcend phylogeny. Our finding is in line with earlier work showing that closely related herbivores such as leafhoppers (Denno et al., 2000), grasshoppers (Behmer, 2009), and caterpillars (Audusseau et al., 2015) responded differently to changes in plant nutrient content. The existence of species-dependent nutritional requirements provides a mechanism that helps explain how these herbivores sharing the same overlapping diets might coexist in the field.

Although protein content appeared to play a key role in determining grasshopper feeding preference in our work, it is also important to

TABLE 3 Generalised linear mixed model testing the effects of species, sex, fertilisation and their interactions on the performance of grasshopper *Euchorthippus cheui* and *E. unicolor*

	Df	Chi-squared	p
<i>Survival</i>			
Species	1	3.55	0.06
Sex	1	3.09	0.08
Fertilisation	1	0.02	0.90
Species × Sex	1	0.02	0.90
Species × Fertilisation	1	38.52	<0.001
Sex × Fertilisation	1	3.44	0.06
Species × Sex × Fertilisation	1	0.46	0.50
<i>Growth rate</i>			
	df	F	p
Species	1.42	0.10	0.75
Sex	1.42	71.17	<0.001
Fertilisation	1.42	0.12	0.73
Species × Sex	1.42	0.003	0.96
Species × Fertilisation	1.42	4.55	0.04
Sex × Fertilisation	1.42	1.71	0.20
Species × Sex × Fertilisation	1.42	0.14	0.72
<i>Body mass</i>			
Species	1.42	1.17	0.29
Sex	1.42	1433.14	<0.001
Fertilisation	1.42	2.02	0.16
Species × Sex	1.42	0.49	0.49
Species × Fertilisation	1.42	27.10	<0.001
Sex × Fertilisation	1.42	0.34	0.57
Species × Sex × Fertilisation	1.42	0.58	0.45
<i>Body size</i>			
Species	1.42	37.10	<0.001
Sex	1.42	1201.63	<0.001
Fertilisation	1.42	0.02	0.89
Species × Sex	1.42	8.68	0.005
Species × Fertilisation	1.42	32.30	<0.001
Sex × Fertilisation	1.42	0.04	0.85
Species × Sex × Fertilisation	1.42	0.28	0.60

Note: The survival, relative growth rate, body mass and body size of female and male grasshopper were monitored for three weeks in the lab. Bold numbers indicate a significance of $p < 0.05$.

note that insects make feeding choices based on the balance of several nutrients (i.e., geometric framework, Raubenheimer & Simpson, 1993). The geometric framework states that organisms are predicted to have optimal intake targets for each nutrient, and deviations from optimal dietary nutrient ratios may result in fitness costs. In our system, fertilisation (10 N) increased leaf protein content by 11% in July and by 77% in August. This rapid increase of protein content in August can produce a large imbalance in the nutrients available to *E. unicolor* and may explain negative effects on its performance since ingesting excessive N has been shown to impose metabolic costs for

catabolising and removing nitrogenous wastes in many insect species (Tao et al., 2014; Zehnder & Hunter, 2009). There is evidence that insect herbivores have evolved a suite of behavioural and physiological traits to regulate the intake of nutrients to optimise their growth and survival (Berner et al., 2005; Clissold et al., 2013). This is especially true in grasshoppers, because their mouthparts contain large numbers of taste receptors with neurons sensitive to a range of nutrients, which can strongly favour their nutrient regulation (Behmer, 2009). Indeed, our feeding selection experiments revealed that both species choose nutritionally different foods, indicating that two species have different nutrient requirements.

To understand the reason behind the different nutrient requirements for the two grasshopper species, we need to take an evolutionary perspective. We suggest that the different nutrient requirements of two species are likely a result of their long-term adaptation to the varying nutritional environments. Under field conditions, the nutrient (protein) content of *L. chinensis* leaves decreased significantly between July and August, corresponding to the peak abundances of *E. cheui* (peaks in July) and *E. unicolor* (in August). Therefore, the phenological matches between the host plant and insect herbivores determine that two grasshopper species normally encounter host plants of different nutritional quality during their normal life cycles because of the displaced timing of phenology. This can explain why the early-season *E. cheui* preferred high-protein food available early in the season and reacted positively to the elevated plant nutrient content caused by fertilisation than the later-emerging *E. unicolor*. Joern and Behmer (1998) similarly showed that sympatric grasshopper species *Melanoplus sanguinipes* and *Phoetaliotes nebrascensis* exhibiting different phenological patterns responded differently to the artificial diets varying in nitrogen and soluble carbohydrate. In spite of this, anthropogenic processes can disrupt the phenology of interacting species, and tight evolutionary links between them may be broken because of a mismatch between food quality and food requirements (Van der Putten et al., 2010; Visser & Gienapp, 2019). The evolutionary and demographic consequences of phenological mismatches between insect herbivores and their resources deserve further attention.

In addition to shifting the nutrient status of host plants, nutrient enrichment can also alter plant chemical and physical defence that can have substantial impact on feeding preference and performance in insect herbivores, including grasshoppers. In our case, increases in high foliar protein contents were accompanied by increases in foliar total phenolics, which may also underlie the negative responses of the grasshopper, *E. unicolor*. Furthermore, there can be direct chemical interactions between nutrients and secondary compounds, such that the magnitude of the influence of secondary compounds is also dependent on the ratio of protein and carbohydrate in the plant. For example, the negative effects of tannic acid on African grasshoppers increased as the protein: carbohydrate ratio of the diet became more imbalanced (Simpson & Raubenheimer, 2001). In our system, we also suspect that excess protein in August may lead to higher toxicity of total phenols for *E. unicolor* during post-ingestion periods. In addition, grasshoppers may also select food based on its water content because the ingestion of free water can increase the uptake of carbohydrates

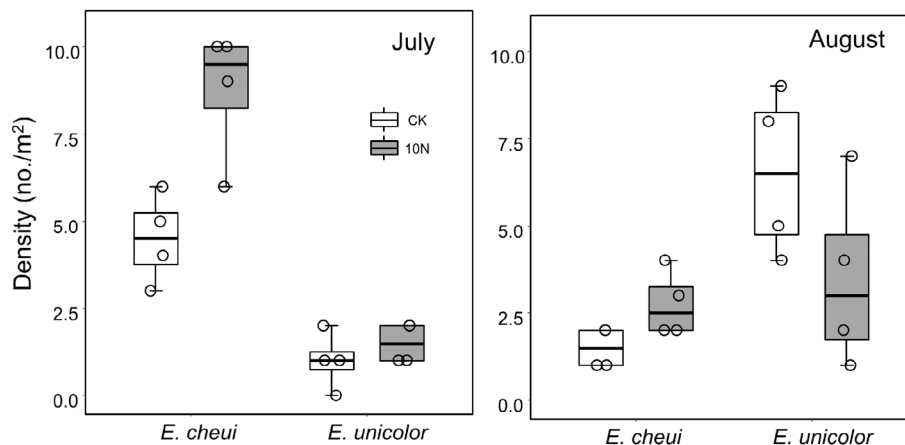


FIGURE 3 Effects of fertilisation on the density of grasshopper *Euchorthippus cheui* and *E. unicolor* in the field. The abundance of two species in a 1 m² enclosure in 0 g N/m² and 10 g N/m² fertilised plots were counted in July and August, respectively. Values are means \pm SE.

from food (Clissold et al., 2014). Finally, plant biomechanical properties such as leaf toughness (Clissold et al., 2009) and silica content (Massey et al., 2006) are a major determinant of grasshopper food choice and performance. We did not measure silica content in *L. chinensis* leaves, but the decline in *E. unicolor* performance has also been accompanied by a reduction in leaf toughness and concentration of structural carbohydrates and cellulose, implying that plant physical defence was unlikely an important factor driving the responses of the grasshoppers in our study. It should be noted, however, that the plant physical defences may result in a reduced rate and ratio of nutrient supply from food plants and thus indirectly affect insect performance (Clissold et al., 2006). As we were unable to fully disentangle all of the underlying mechanisms contributing to grasshopper food choice and performance, future studies in which plant chemical and physical defences are experimentally manipulated are required.

Contrary to our second hypothesis, we did not find clear sex-specific responses of *E. cheui* and *E. unicolor* to changes in plant quality. This finding was unexpected because females typically require more protein than males to optimise reproductive output (Bunning et al., 2016). The discrepancy may be caused by the fact that the grasshoppers investigated in our study were in their nymphal stage, which may have different nutrient requirements compared with sexually mature adults. Lee et al. (2013) demonstrated that mated female fruit flies (*Drosophila melanogaster*) preferred a more protein-rich diet compared with males and virgin females. However, a recent study reported that N fertiliser could decrease the survival and reproduction of adult female locusts (*Oedaleus senegalensis*) because females have higher energetic demands for migration (Le Gall et al., 2020). Additionally, sex differences in nutrient assimilation efficiency may affect the way how fertilisation influences feeding and performance in males and females. To test this hypothesis, future studies should include direct measures of consumption, excretion and growth efficiency of herbivores for different sexes. Furthermore, our data were limited to a single nymphal instar and short-term experiments cannot always capture the fertilisation effect on the performance. Thus, additional

studies are needed to understand how the nutrient demands of different grasshoppers shift over the course of entire lifetime.

We found that *E. cheui* has a higher abundance in the fertilised plots while the abundance of *E. unicolor* does not show a clear change. This result is partially consistent with our third hypothesis. We suggest that these results may be attributed to the high mobility of grasshoppers, allowing them the opportunity to move freely within the plant canopy to feed within and between plants (Behmer, 2009). Thus, we would expect that grasshoppers would shift their diet to alleviate imbalances in single dietary components, which was not possible in the lab experiments but could have occurred in the field. This may be partly why a significant decrease in *E. unicolor* abundance was not observed under N enriched conditions in the field. In addition, other possible effects of fertilisation, for example, shifts in predator communities, microclimate, and sward structure which change the composition and biomass of vegetation, may in turn affect the field measurements of grasshopper abundance (Cease et al., 2012). Altogether, our study shows that when plant nutrients change as a result of fertilisation, it does not automatically cause identical changes in closely related grasshoppers (or potentially other insect herbivores) because species may show different adaptations and thus different preferences for either high or low nutrient concentrations. Our findings imply that the responses of insect herbivores from the same guild to an altered food nutrient status may be more complex than we thought. Therefore, in order to establish an effective management and conservation strategy, the effects of environmental changes (e.g., N deposition) on insects should be assessed species-specifically.

AUTHOR CONTRIBUTIONS

Yu Zhu and Quanhui Ma contributed equally to this work. Yu Zhu, Quanhui Ma, Zhiwei Zhong and Deli Wang designed experiments; Yu Zhu and Quanhui Ma performed the experiments; Yu Zhu, G.V. and C.C. analysed the data; and Yu Zhu, Quanhui Ma, Zhiwei Zhong, Elisabeth S. Bakker, Jeffrey A. Harvey, G.F. Ciska Veen and Ming Jiang drafted the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Data S1. Supporting Information.

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