Leaf N:P stoichiometry overrides the effect of individual nutrient content on insect herbivore population dynamics in a Tibetan alpine grassland

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ABSTRACT

Based on resource availability hypothesis, increasing exogenous nutrient inputs may favour herbivores by improving quality and quantity of host plants. Such plant-herbivore interactions have been demonstrated under nitrogen (N) addition, but less examined in the context of simultaneous inputs of N and phosphorus (P). Here, we conducted a multiple-nutrient addition experiment (Control, N addition alone, P addition alone, both N and P additions) in a Tibetan alpine grassland, to explore how N addition interacts with P to affect the larval density of the grassland caterpillar *Gynaephora menyuanensis*. We showed that P addition alone did not obviously affect the larval density, but P suppressed N-induced increases in larval density when N and P were simultaneously added. We further found that changes in leaf N:P of host plants induced by nutrient additions regulated larval density more strongly than leaf N or P content. Both increased plant productivity and shifted species composition under nutrient additions contributed less to the larval density. Interactive effects of N and P suggest that the simultaneous inputs of multiple nutrients may boost the carrying capacity of grasslands and reduce the risk of herbivorous insect outbreaks. The work also reveals that the resource availability hypothesis does not well predict the response of insect herbivore to all nutrient inputs, implying the necessity of incorporating N:P stoichiometry in this hypothesis.

1. Introduction

Atmospheric deposition and fertilizer application have significantly increased the inputs of nitrogen (N) and phosphorus (P) in most of terrestrial ecosystems since industrial revolution (Peñuelas et al., 2013; Zhang et al., 2018). As a crucial driver of ecosystem dynamics, plant-herbivore interactions may be profoundly influenced by the increases in exogenous nutrient inputs (Elser et al., 2000a; Burghardt, 2016). The resource availability hypothesis suggesting that plant species grow faster and resist less to herbivores under a higher resource level has been proposed since 1980 s (Coley et al., 1985; Hahn and Maron, 2016). Based on this hypothesis, nutrient additions generally improve the quality and quantity of host plants (Lind et al., 2017; Seabloom et al., 2021a) and reduce their defence investment (Sampedro et al., 2011; Han

et al., 2016), thereby favouring herbivore foraging and reducing the mortality of herbivores (Cease et al., 2016; Ebeling et al., 2021).

Nutrient additions can also change herbivore fitness through a shift in plant community composition (Giulio and Edwards, 2003; Basset and Lamarre, 2019), a phenomenon that has been commonly observed in fertilization experiments. For example, it has been documented that N addition causes the loss of legume species and reduces plant diversity (Duprè et al., 2010; DeMalach et al., 2016). Such changes in plant community composition may influence herbivore populations because of their feeding preference for specific plant species or functional groups (Song et al., 2018). Although a growing body of studies have indicated that nutrient additions influence herbivores via effects on quality, quantity and community composition of host plants, elucidating the relative importance of the pathways is still a key to predicting future

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herbivore population dynamics.

Resource availability hypothesis has been well demonstrated in studies of N addition (Sassi et al., 2012; Song et al., 2018), but it remains unclear whether the effect of P addition applies to this hypothesis (Elser et al., 2007; Cease et al., 2016). As a basic element, P participates the intracellular synthesis of phospholipids, adenosine triphosphate and nucleic acids (Elser et al., 1996), and organisms with high P content tend to have a faster growth rate (Elser et al., 1996; Schade et al., 2003). P enrichment also does not favour the production of chemical defences (Sampedro et al., 2011). The effects of P addition on plant-herbivore interactions may thus follow the resource availability hypothesis. However, simultaneous additions of P and N may have negative impacts on herbivore populations, given that herbivores need more time and energy to achieve stoichiometric homeostasis when feeding on too low or high N:P foods (Elser et al., 2000b; Berner et al., 2005; Zhang et al., 2014). Changes in N:P stoichiometry of herbivores' food also should be paid more attention in the context of simultaneous inputs of multiple nutrient resources.

Here, we conducted a long-term multiple-nutrient addition experiment to explore the effects of P addition alone and in combination with N on herbivorous insects in a Tibetan alpine grassland (Fig. 1a, b). We selected a common alpine grassland caterpillar, *Gynaephora menyuanensis* (Lepidoptera: Lymantriidae), as a research model. We tested two competing hypotheses: (i) similar to N addition, both P addition alone and in combination with N will favour the grassland caterpillar population based on the resource availability hypothesis (Fig. 2a); (ii) P addition will weaken positive effects of N addition when both nutrients are together added, because too high or low N:P stoichiometry is adverse to herbivore metabolism (Fig. 2b).

2. Materials and methods

2.1. Study site

The study site is located near the Haibei National Field Research Station on the northeastern part of the Tibetan Plateau (37°36′N, 101°19′E; 3200 m a.s.l.). The area is influenced by continental monsoon climates, with long, cold winters and short, cool summers. From 1981–2014, the mean annual temperature and precipitation were − 1.1 °C and 487.8 mm, respectively (Wang et al., 2020). The hourly minimum and maximum temperatures were − 37.1 °C in January and 27.6 °C in July. Approximately 84% of the precipitation fell in the growing season from May to September. The alpine grassland is dominated by *Stipa aliena* and *Elymus nutans*, mixed by *Kobresia humilis*, *Poa pratensis*, *Helictotrichon tibeticum* and *Gentiana straminea*. The soil is classified as Mat-Gryic Cambisols according to Chinese Soil Taxonomy, with pH and total N and P contents in the 0–10 cm layer of 7.8, 7.8 g kg⁻¹, and 0.77 g kg⁻¹, respectively.

G. menyuanensis is one of the main herbivorous insects in Tibetan alpine grasslands, exacerbates grassland degradation by reducing vegetation production, and increases livestock mortality by triggering mouth mucous membrane canker (Cao et al., 2015; Fig. 1c). It has been reported that outbreaks of grassland caterpillars occurred in approximately 17.6 million ha of alpine grassland during the early 2010 s (Hong et al., 2014).

2.2. Experimental design

In 2011, the nutrient addition field experiment was established in a homogeneous area of the alpine grassland (Fig. 1a). The experiment
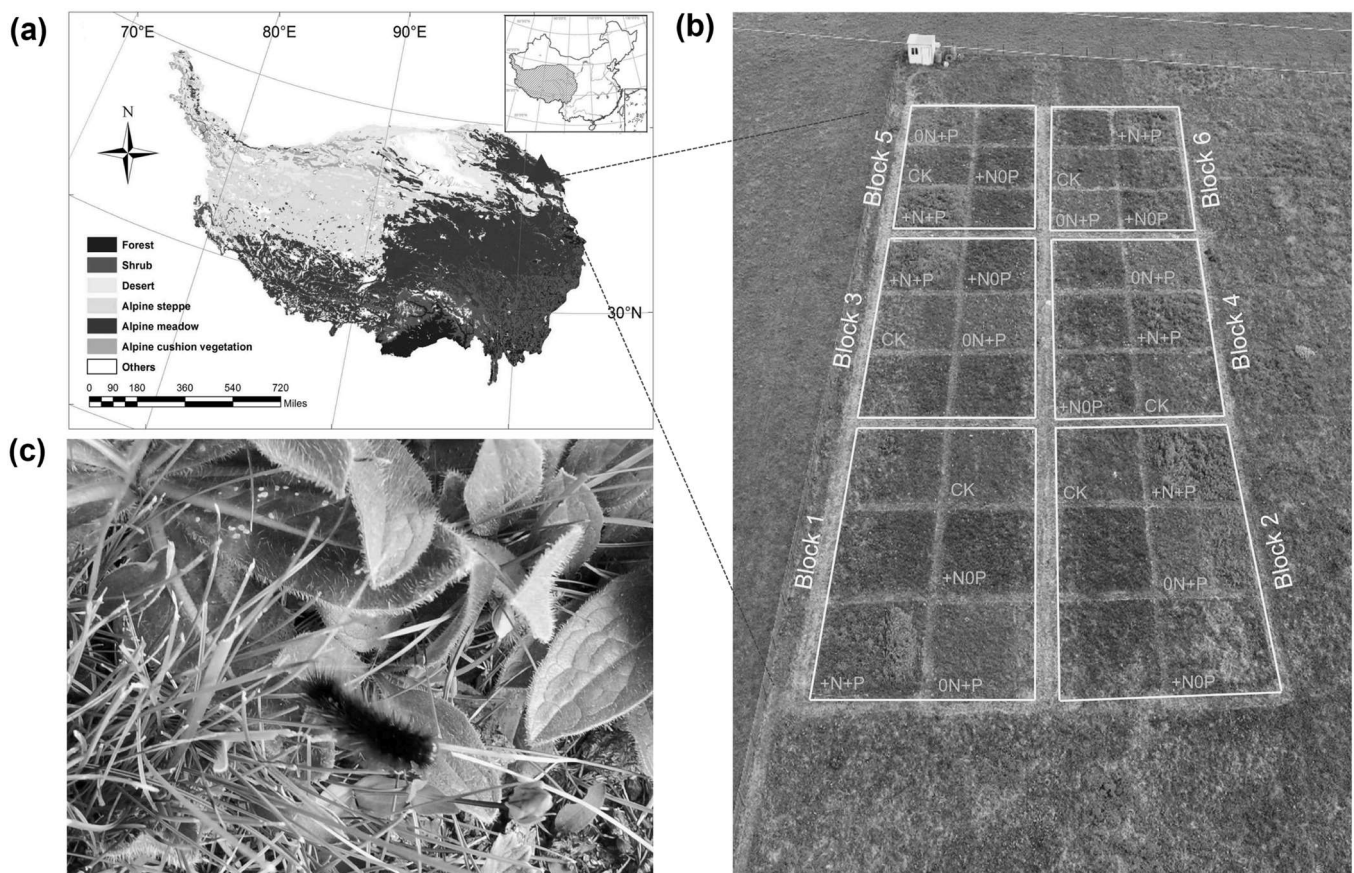

Fig. 1. Geographical location (a) and landscape (b) of the studied long-term multiple-nutrient addition experiment and photographs of *G. menyuanensis* (c) in an alpine grassland on the Tibetan Plateau.
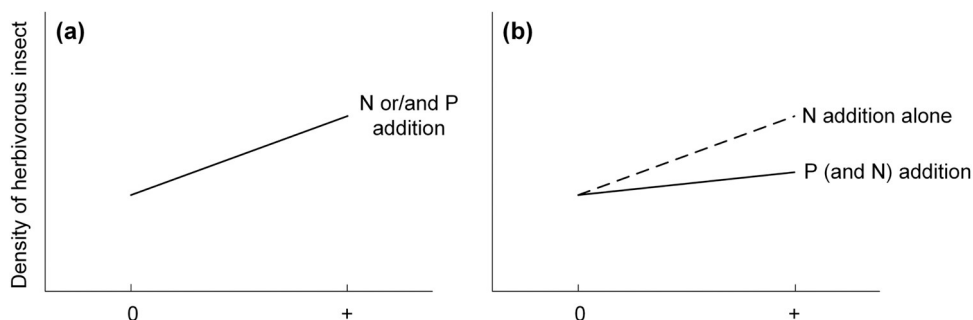
Fig. 2. Two competing hypotheses on effects of nutrient additions on insect herbivore population tested in this study. (a), we hypothesize that high nitrogen (N) or/and phosphorus (P) addition increases herbivorous insects based on the resource availability hypothesis. (b), we hypothesize that P addition alone and in combination with N addition may not produce a positive effect on herbivorous insects when causing too low or high N:P stoichiometry of host plants. 0, without nutrient addition; +, with nutrient addition.

followed a randomised block design with six blocks. In each block, four treatments were applied, which included control (0 N 0 P), N addition alone (+ N 0 P; urea, 100 kg N hm^{-2} a^{-1}, which is approximately tenfold background atmospheric N addition), P addition alone (0 N + P; superphosphate, 50 kg P hm^{-2} a^{-1}) and both N and P additions (+ N + P). The dose of nutrient addition follows previous studies in grasslands (Niu et al., 2010; Yang et al., 2012; Yuan et al., 2020), and simulates a high-level nutrient input on the Tibetan Plateau (Fang et al., 2012; Wang et al., 2017). It has been documented that the nutrient addition levels can relieve nutrient limitation on alpine plant growth (Ren et al., 2017; Luo et al., 2019). Each plot was 6 × 6 m. There were 2-m buffer zones between blocks and 1-m buffer zones between plots. The nutrients were added in a granular form in each experimental year. Especially, we distributed the nutrients to plots evenly after sunset in early June, July and August.

2.3. Measurement of larval density and its influencing factors

Previous studies have reported that the peak growing season is an optimum observation period for the density of *G. menyuanensis*, when the caterpillars are generally on the fifth-seventh instar with a long feeding time and large activity range (Song et al., 2018; Pan et al., 2019). In this study, we systematically surveyed the *G. menyuanensis* density in July of 2017, 2018 and 2019. We observed the larvae in two 2 × 0.5 m quadrats during the periods of 10:00–12:00 and 15:00–17:00 on sunny days when they were relatively active. We focused on four blocks (blocks 3–6; Fig. 1b), each plot was observed three times, and the average values were analysed (Pan et al., 2019).

In addition, we also measured the food quantity (i.e., aboveground plant biomass), food quality (i.e., ratio of graminoid to forb, community-level leaf N and P content and leaf N:P) and habitat conditions (i.e., soil temperature and moisture; light condition, indirectly represented by plant community height) of *G. menyuanensis*. Specifically, we measured the aboveground biomass by harvesting the aboveground parts of live plants in a 0.5 × 0.5 m quadrat, and the plant samples were further sorted to species, dried and weighed around July each year. We measured the leaf N content of twelve common species using an elemental analyser (2400II CHNS/O Elemental Analyzer, Perkin-Elmer, USA) and the leaf P content using molybdenum blue colorimetry. The species covered more than 75% of the aboveground community biomass, including four graminoids (*S. aliena, E. nutans, P. pratensis* and *K. humilis*) and eight forbs (*Oxytropis ochrocephala, Tibetia himalaica, Medicago archiducis-nicolai, G. straminea, Saussurea pulchra, Morina chinensis, Potentilla saundersiana* and *Aster diplostephioides*). We further calculated the community-level leaf N and P contents by multiplying the species-level leaf N and P contents by their production-based weights from 2017 to 2019. Plant community height was measured in July 2020 by averaging the plant height at the four corners and center of each plot. Soil temperature and moisture at the 5-cm depth were measured in the three examined years, with a portable thermometer (JM 624 Digital Thermometer, Jinming Instrument Co., Ltd., Tianjin, China) and a TDR moisture meter (IMKO, Ettlingen, Germany), respectively.

2.4. Feeding experiment

We conducted a feeding experiment in July 2020 based on previous studies on the feeding preference of grassland caterpillars (Yan et al., 2005; Song et al., 2018; Pan et al., 2019). We first collected larvae of *G. menyuanensis* from the studied grassland near the field experiment. The larvae were starved for 24 h and then assigned to four polyethylene cuboid incubators (17 cm length × 12 cm width × 5 cm height), each with 10 larvae. A total of 9 g fresh leaves from nine species (~1 g per species) were placed in each incubator, and the feeding experiment lasted 48 h. All these incubators were placed near the experimental platform during the day and taken back to the laboratory at night to avoid damage from freezing. Finally, the relative consumption of plants by the larvae was calculated by the difference between the initial and final weights. To eliminate the influence of leaf wilting on final weights, we set two controls with fresh leaves but without larvae in identical incubators. In this study, the larvae consumed approximately 0.5 g during the entire experimental period.

2.5. Statistical analyses

Repeated-measures ANOVA and Tukey's significance tests were used to examine the effects of nutrient additions on the larval density of *G. menyuanensis* and their food quantity, food quality and habitat conditions across years, in which N addition, P addition and year were treated as the main factors and block was treated as a random factor. One-way ANOVA was used to assess the difference in relative consumption among species. We used simple linear regression to investigate the relationship between environmental factors and larval density. In addition, we further used multi-model inference approach to quantify the relative importance of environment factors in regulating larval density (package 'MuMIn' version 1.46.0) (Burnham et al., 2011; Harrison et al., 2018). This approach is based on the corrected Akaike information criterion (AICc; AIC corrected for small samples), and has been widely used (Scheper et al., 2014; Deng et al., 2018). First, we performed a global model using the 'lmer' function (Table S1), and produced a full submodel set from the global model using the 'dredge' function. We then filtered eight top models using a cut-off of ΔAICc < 4 (Table S1) and obtained the model parameters based on the top models using the 'model.avg' function. Finally, we estimated the relative importance of each environmental factor by summing the Akaike weights across all the top models that contained the candidate factors. All statistical analyses were performed with R 3.6.1 (R Development Core Team, 2019).

3. Results

3.1. Effects of nutrient additions on larval density and environmental factors

N addition effect on larval density of *G. menyuanensis* was modulated by P addition across the three years ($P < 0.01$; Table 1). Specifically, N

Table 1
Summary of repeated-measures ANOVA on the effects of nitrogen, phosphorus, year and their interactions on larval density of *G. menyuanensis*.

	df	F	P
Nitrogen	1, 12	6.65	0.02
Phosphorus	1, 12	28.75	< 0.001
Nitrogen × Phosphorus	1, 12	9.83	0.009
Year	2, 24	13.99	< 0.001
Nitrogen × Year	2, 24	4.00	0.03
Phosphorus × Year	2, 24	2.78	0.08
Nitrogen × Phosphorus × Year	2, 24	1.53	0.24

addition increased the larval density by 89% when P was not added, but did not affect larval density when P was simultaneously added (Fig. 3).

There were significant interactive effects of N and P additions on plant community height and aboveground biomass ($P < 0.05$; Fig. 4c, h; Table S2). N addition increased plant community height by 21% when P was not added. The increase was more obvious (122%) when P addition was together applied. The aboveground biomass was also highest when N and P was simultaneously added. In contrast, we did not find any significant interaction of N and P on other environmental factors. N addition increased leaf N content and leaf N:P ($P < 0.001$; Fig. 4d, f), marginally improved the ratio of graminoid to forb ($P = 0.07$; Fig. 4g), but decreased soil temperature ($P = 0.02$; Fig. 4a; Table S2). P addition increased leaf P content but decreased leaf N:P and ratio of graminoid to forb ($P < 0.05$; Fig. 4e-g; Table S2).

3.2. Relative importance of plant quantity, quality and community composition to changes in larval density under nutrient additions

The larval density decreased with an increase in community-level leaf P content ($P = 0.003$; Fig. 5e) but increased with an increasing community-level leaf N:P across all treatments ($P < 0.001$; Fig. 5f). We did not observe any significant relationship between larval density and ratio of graminoid to forb (Fig. 5g), although the feeding experiment showed a higher preference of *G. menyuanensis* for graminoids than for forbs (Fig. S1). The multi-model inference approach further revealed

that leaf N:P was the most important factor for explaining the variations of larval density under nutrient additions (Fig. 5i; Table S1).

4. Disscussion

Ecologists have devoted much effort to elucidate the response of plant-herbivore interactions to exogenous N input, and demonstrated that N addition stimulates insect herbivore populations (Lind et al., 2017; Ebeling et al., 2021). Our study further provided an empirical evidence of how P addition and in combination with N affect insect herbivore density. We found that P addition alone did not affect larval density of *G. menyuanensis*, but P suppressed N-induced increases in larval density when N and P were added together. We further found that leaf N:P was a more important driver than individual nutrient content of host plants, and both increased ratio of graminoid to forb and enhanced plant productivity contributed less to the changes in larval density under nutrient additions. These findings will enrich our understanding of plant-herbivore interactions under global environmental changes.

4.1. Leaf N:P dominated the effects of nutrient additions on larval density

It has been predicted that nutrient additions favour herbivores by accelerating plant growth and reducing antiherbivore defence based on the resource availability hypothesis (Sassi et al., 2012; Robinson and Strauss, 2018). Our result that N addition alone increased the larval density of *G. menyuanensis* supported the prediction and was consistent with previous findings from another alpine grassland (Song et al., 2018) and sub-Arctic heath (Richardson et al., 2002). Additionally, the result was also in line with the fact, that is, the growth of insect herbivore is generally nutrient limited because of lower N and P contents of host plants than their body tissues (Zehnder and Hunter, 2009; Tao and Hunter, 2012). However, we found that P addition alone had limited effect on larval density. Given that P-induced reductions in chemical defence investment of host plants do not affect the population of herbivorous insects (Zehnder and Hunter, 2009; Sampedro et al., 2011), we speculated that the defence investment of host plants and the P content of herbivore body were not necessary to dominate the population
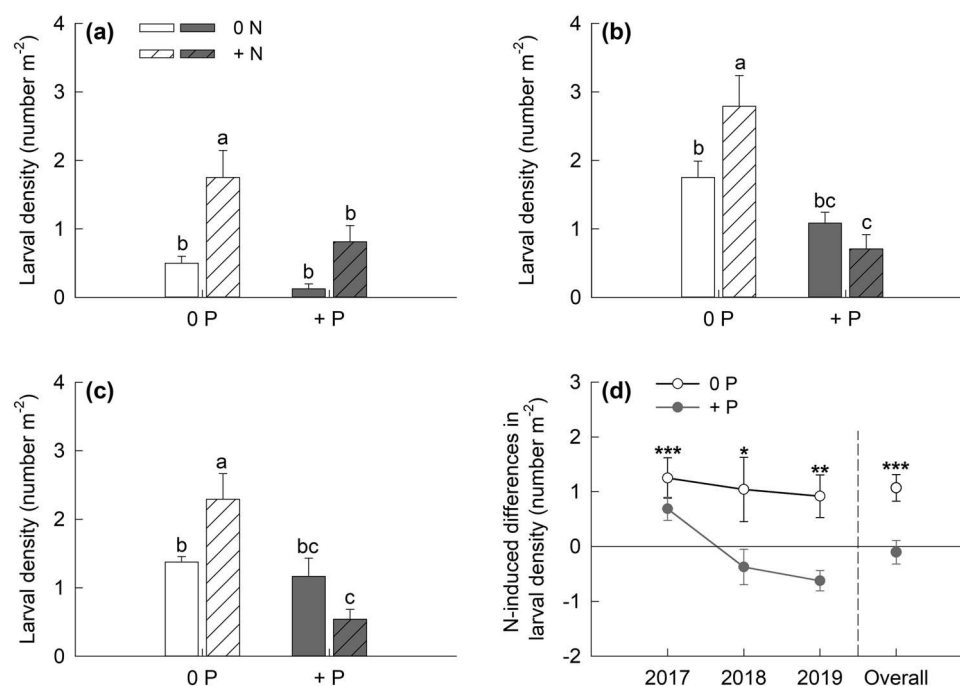

Fig. 3. Effects of nutrient additions on larval density in 2017 (a), 2018 (b) and 2019 (c) and nitrogen (N)-induced differences in larval density in the field experiment (d). 0 N (0 P), without nitrogen (phosphorus) addition; + N (+ P), with nitrogen (phosphorus) addition. Error bar represents 1 standard error. Different letters represent significant differences at $P < 0.05$ level. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
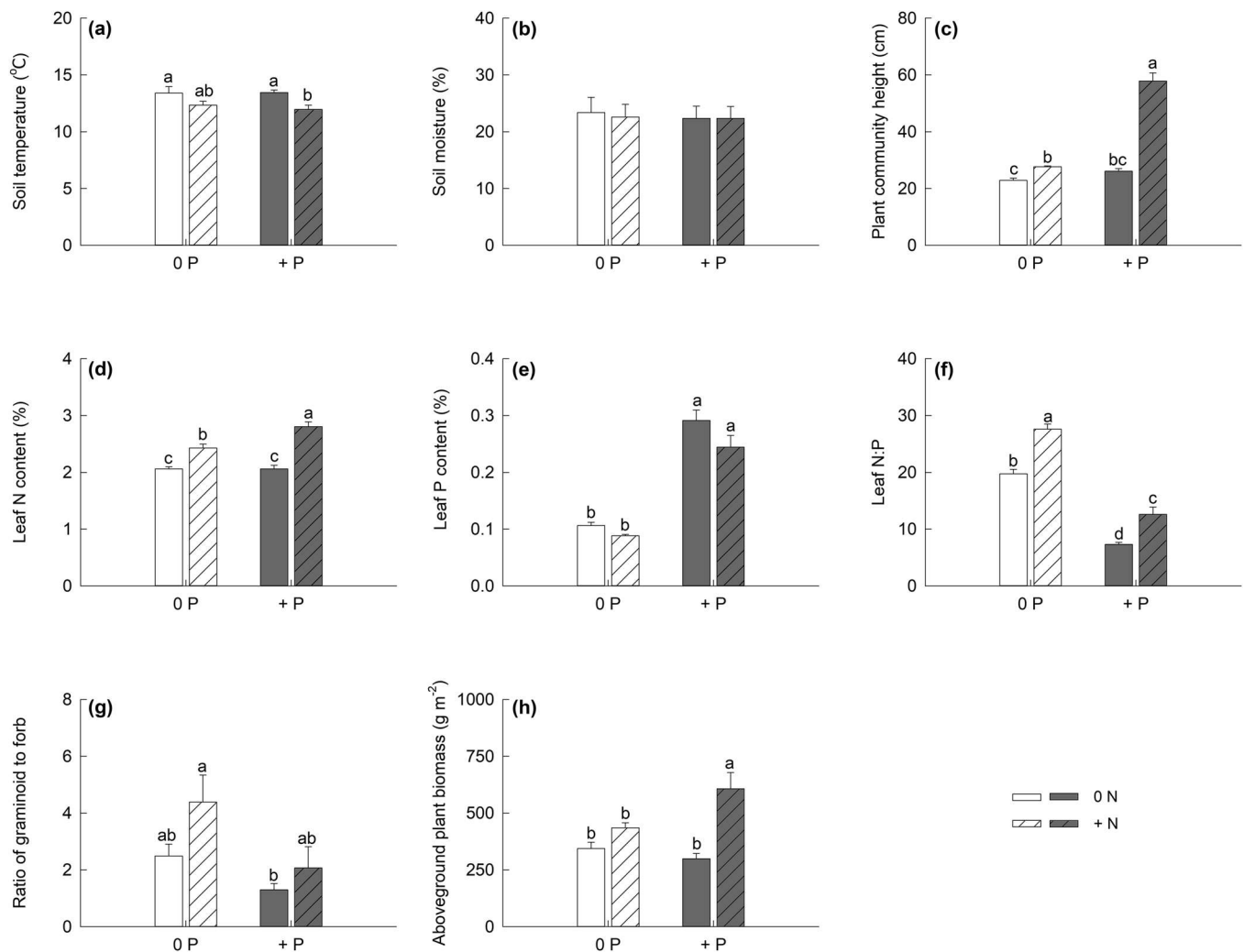
Fig. 4. Effect of nutrient additions on environmental factors of *G. menyuanensis* in the field experiment. (a-c), habitat. (d-g), food quality. (h), food quantity. 0 N (0 P), without nitrogen (phosphorus) addition; + N (+ P), with nitrogen (phosphorus) addition. Error bar represents 1 standard error. Different letters represent significant differences at *P* < 0.05 level.

dynamics of insect herbivores. Further research is required to investigate the potential mechanisms of the neutral effects of P addition on larval density.

An important finding was that P addition suppressed the positive effects of N addition on the larval density in our study. We revealed that the leaf N:P of host plants was a crucial driver for the nutrient-induced effects on larval density. It has been well proposed that herbivorous insects achieve their stoichiometric homeostasis through excretion regulation and compensatory and selective feeding when facing nutritionally imbalanced host plants (e.g., low N:P) (Berner et al., 2005; Jonas and Joern, 2008; Tao et al., 2014). In our studied grassland, the community-level leaf N:P was approximately 20, which should be below the body N:P ratio of *G. menyuanensis* given that the global average value of invertebrate terrestrial herbivores is around 26 (Elser et al., 2000a). Thus, herbivorous insects in plots with P addition may cost more time and metabolic energy to maintain the stoichiometric homeostasis after foraging, and these processes will reduce their fitness (Loaiza et al., 2008; Zhang et al., 2014).

4.2. Increased ratio of graminoid to forb contributed less to N-induced increase in larval density

We observed an increased ratio of graminoid to forb under N addition alone, which was in line with previous findings at the same

experimental platform (Luo et al., 2019). This result has been attributed to the fact that N addition enhances the light-competition ability of graminoids (Ren et al., 2017; Borer et al., 2014). We further found that the increase in the ratio of graminoid to forb had limited effects on larval density, although our feeding experiment revealed a feeding preference of grassland caterpillars on graminoid species. This may be because the aboveground biomass of graminoids far outweighed the demand of caterpillars in the studied grassland during the observation years (Yan et al., 2005). Another explanation was that N-induced increase in ratio of graminoid to forb might be a result of increases in non-preferred graminoid species and decreases in non-preferred forbs in the studied grassland (Fig. S1).

Although we observed a limited contribution of the increased ratio of graminoid to forb, shifts in species composition may regulate population dynamics of herbivorous insects in some scenarios. For instance, our finding derived from three normal years when there were no herbivore outbreaks. As the population size of herbivorous insects becomes larger, shifts in species composition may play a more important role in regulating the population dynamics by changing host plant quality and quantity (Sassi et al., 2012; Song et al., 2018). In this case, the feeding preference of herbivorous insects may also in turn influence plant community composition (Kempel et al., 2015; Seabloom et al., 2021b). In addition, we found that the stimulation of P addition for leaf P content (175%) was far larger than the stimulation of N addition for leaf N
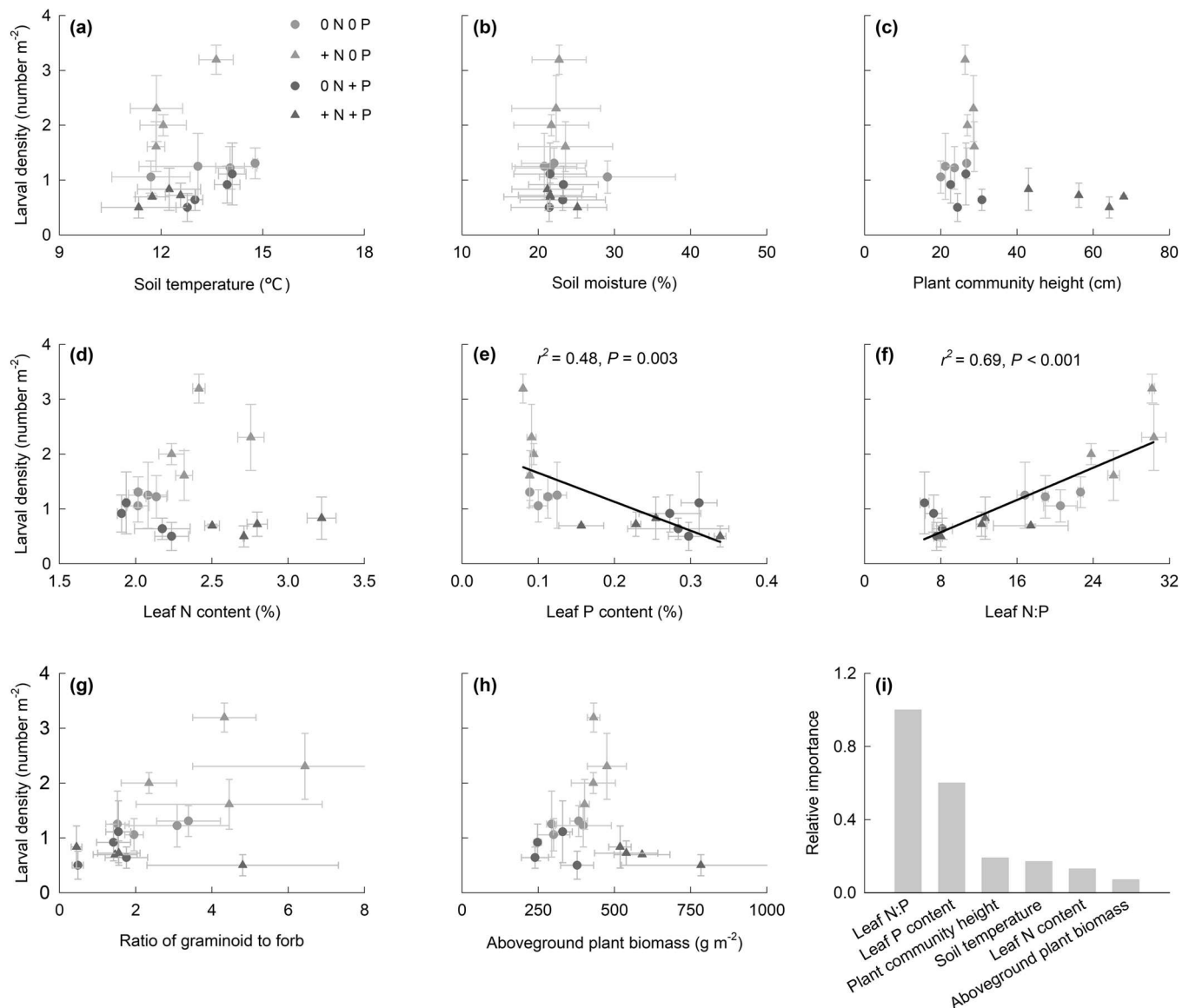
Fig. 5. Effect of environmental factors on larval density across different nutrient addition treatments. (a-c), habitat. (d-g), food quality. (h), food quantity. (i), relative importance of the examined factors in regulating larval density is estimated by the multi-model inference approach. In (a-h), solid line is shown only when $P < 0.05$. 0 N 0 P, without nutrients addition; + N 0 P, with nitrogen addition alone; 0 N + P, with phosphorus addition alone; + N + P, with nitrogen and phosphorus additions. In (i), the two variables, that is soil moisture and ratio of graminoid to ford, were not included in top models (see Table S1), suggesting that they contribute less to responses of larval density to nutrient additions.

content (27%), although the amount of P addition was a half of N addition. The larger P-induced stimulation should reduce leaf N:P and negatively influence herbivorous insect density in the studied grassland. However, the nutrient absorption efficiency of plants often differs among species (Li et al., 2015). If plant community composition under nutrient inputs shifts towards species with a higher N absorption efficiency, this shift in species composition would favour the herbivorous insects.

It should be noted that the threshold elemental ratio of host plants can influence the growth and population dynamics of insect herbivores (Boersma and Elser, 2006; Zehnder and Hunter, 2009). Previous studies have reported that the grasshopper *Schistocerca americana* maximises its growth and performance when feeding on an optimal N:P food (~15) (Cease et al., 2016). Our piecewise regression analysis also suggested a threshold (~18) of leaf N:P of host plants for the grassland caterpillar population (Fig. S2). However, due to a dearth of the direct empirical evidence, the nutrient gradient experiments are still needed to examine the threshold elemental ratio for insect herbivore population dynamics.

4.3. Implications

In summary, this study demonstrated a meaningful interactive effect of N and P additions on insect herbivore populations. These findings have several important implications. First, we revealed a more important role of N:P stoichiometry of host plants than individual nutrient content in regulating insect herbivore dynamics. The nutrient stoichiometry of plant tissues, especially N:P ratio, should be incorporated into the framework on the effect of resource on plant-herbivore interactions (e.g., resource availability hypothesis). Second, many terrestrial ecosystems are experiencing the inputs of multiple exogenous nutrients (Liu et al., 2013; Yu et al., 2019). Our results of the interaction effects of N and P additions suggested that identifying the potential interaction of exogenous nutrients should be a priority for predicting ecosystem dynamics. Lastly, simultaneous additions of N and P not only boosted grassland productivity but also reduced the risk for outbreaks of herbivorous insects in our study, suggesting that a combination of multiple resource additions may be a more reasonable approach for management

of grassland ecosystems.

CRediT authorship contribution statement

HW and JSH conceived and designed research; HJG and FR analysed data. HJG and HW wrote the manuscript. All authors read and approved the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data has been deposited in the Figshare digital repository (https://doi.org/10.6084/m9.figshare.15156012.v2).

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Declarations

Ethical approval

Not applicable.

Consent to Participate

Not applicable.

Consent for publication

Not applicable.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2022.108032.

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