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Alkaloid Contents in *Epichloë* Endophyte-Infected *Elymus tangutorum* Sampled along an Elevation Gradient on the Qinghai-Tibetan Plateau

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Abstract: Alkaloids produced by endophytic fungi can have an important influence on agricultural ecology, and can often be affected by climatic factors. At present, there are no studies that have assessed the relationship between alkaloid production and elevation or climatic factors in the Qinghai-Tibetan Plateau. To address this knowledge gap, we explored ergot alkaloid and peramine production in *Epichloë*-infected (E+) *Elymus tangutorum* collected from the Qinghai-Tibetan plateau and assessed the relationship between the concentration of these alkaloids and elevation. The effects of temperature and precipitation on these relationships were also investigated. The concentrations of ergonovine, ergine, and peramine ranged from 0.47–0.84, 0.35–1.72 and 9.18–13.00 $\mu\text{g}\cdot\text{g}^{-1}$, respectively. Fitted cubic regression curves describing an arc-pattern across the elevational series were statistically significant for ergine and peramine concentrations. The elevational trend in peramine concentration was linked to mean daily temperature, while the ergine elevational trend was linked to mean precipitation. Our results provide a new understanding of the climatic factors that influence the alkaloid production of endophytic fungi at different elevations in the Qinghai-Tibetan plateau.

Keywords: elevation; ergot; peramine; temperature; precipitation

1. Introduction

Interactions between plants and endophytes are a widespread phenomenon in nature and are crucial to the establishment and maintenance of populations, influencing community dynamics and even ecosystem function, particularly under environmental stress [1–4]. Between 20–30% of cool-season grass species can form symbiotic associations with *Epichloë* endophytes [5]. Asexual endophyte species of the genus *Epichloë*, formerly known as *Neotyphodium*, usually form symbioses with cool-season grasses [6]. Asexual *Epichloë* are vertically transmitted through host plant seeds, and do not normally cause symptoms in the host grass [7–9].

The association between cool-season grasses and *Epichloë* endophytes can promote host plant growth [10,11], increase plant nutrient uptake [12,13], inhibit plant pathogen growth [14–16], increase host fitness and improve host tolerance to biotic (e.g., pests, disease, animal grazing) [15–18], and abiotic factors (e.g., waterlogging, drought, cold, soil acidity, mineral imbalance stresses) [13,19–22]. There are four major classes of alkaloids produced by this symbiosis; indole-diterpenoids (e.g., lolitrem B), pyrrolizidine (e.g., lolines), pyrrolopyrazine (e.g., peramine) and ergot alkaloids [23]. The ergot alkaloid, ergovaline is responsible for “fescue toxicosis” in livestock [24,25]. The main symptoms

of fescue toxicosis are reduced feed intake, excessive salivation, reduced reproductive performance, tissue necroses of the feet and tail, and high mortality [26–28]. The presence of peramine is a significant deterrent to feeding for the Argentine stem weevil (*Listronotus bonariensis*), and is viewed as a desirable alkaloid due to its insect-resistance activity. It also has benign effects on grazing animals [29]. Lolitrem B is responsible for “ryegrass staggers”, and symptoms range from mild neck tremors following strenuous exercise to severe tetanic spasms and collapse [30]. The activity of lolines against insects has been reported in a number of studies [31–35], and lolines also have harmful effects on mammals [34].

Elymus spp. not only provide high quality herbage for livestock grazing [36], but they are also important grass species for ecological restoration of grasslands and for reducing land desertification in western China [22,37]. They often contain the endophytic fungus *Epichloëbromicola* [38], and the *E. bromicola* associations with *Elymus* spp. are host specific [39]. Endophyte-infected *Elymus dahuricus* was found to produce peramine in 21 sites across China [40]. No cases of toxicity to livestock grazing on *Elymus* spp. have been reported, although ergot alkaloid gene sets have been identified in *E. bromicola* [41]. There are many *Elymus* spp. in China and little is known concerning their ergot alkaloid and peramine production potential, especially in the Qinghai-Tibetan plateau (QTP) where natural grasslands play an important role in the ecology and economy of the region.

The QTP covers a large area that includes complex landscapes, climatic gradients and different elevations, and contains rich biodiversity [42]. Elevation often has an influence on the ecosystems of the QTP because it alters a range of climate factors including temperature, precipitation and atmospheric pressure that shape the evolutionary adaptation of plant species. [43,44]. Prior research on the QTP has investigated the effects of elevational gradients on diversity [45], evolutionary history [44,46], underlying adaptation [47], geographical distribution [48] and the chemical composition of plants [49,50], and fungal assemblages [51,52]. However, there are no studies that assess the relationship between the alkaloid production of endophyte-infected cool-season grasses and elevation and climate factors across the QTP.

Interestingly, previous studies have reported that climate factors (mainly temperature, precipitation and CO₂) affect fungal alkaloid concentration [53–62]. For example, McCulley et al. [56] found that ergot alkaloids of tall fescue (*Festuca arundinacea*) significantly increased at higher temperature in transition zone pastures in the U.S. Drought stress can increase the production of ergovaline in *Epichloë*-infected grass [54,59]. Ryan et al. [58] found that the alkaloid concentration of endophyte-infected tall fescue decreased under elevated CO₂. The majority of studies on the influence of climate factors on the alkaloid production of *Epichloë* endophytes have been conducted under controlled conditions, and relevant studies conducted in natural conditions are lacking.

To address this knowledge gap, we explored ergot alkaloid and peramine production in *Epichloë*-infected (E+) *E. tangutorum* collected from 25 sites across the QTP and assessed the relationship between the concentration of these alkaloids and elevation. The climate factors that affect the relationships were also investigated.

2. Materials and Methods

2.1. Characterization of the Study Area

The QTP ranges from the eastern edge of the Hengduan Mountains to the western boundary of the Pamir Mountains, and from the northern edge of the Kunlun Mountains to the southern edge of the Himalayan Range [63], with latitude 26°00′–39°47′ N and longitude 73°19′–104°47′ E. Our sampling sites were all distributed on permanent grassland of the QTP in Qinghai and Gansu Provinces; the elevation ranged from 2100 m to 3920 m, and the longitude ranged from 99°21′47″ to 102°07′19″, while the latitude ranged from 34°32′56″ to 38°57′35″ (Figure 1). A highland cold climate prevails in this region [64]. In winter, the mean daily temperature remains below 0 °C for nearly six months, while the mean daily temperature in summer typically ranges from 10–20 °C [65]. The mean annual

precipitation is about 410 mm, with approximately 140 mm in the cold months (November–January), 90 mm in early spring (February–April), and the remainder from May to October [66].

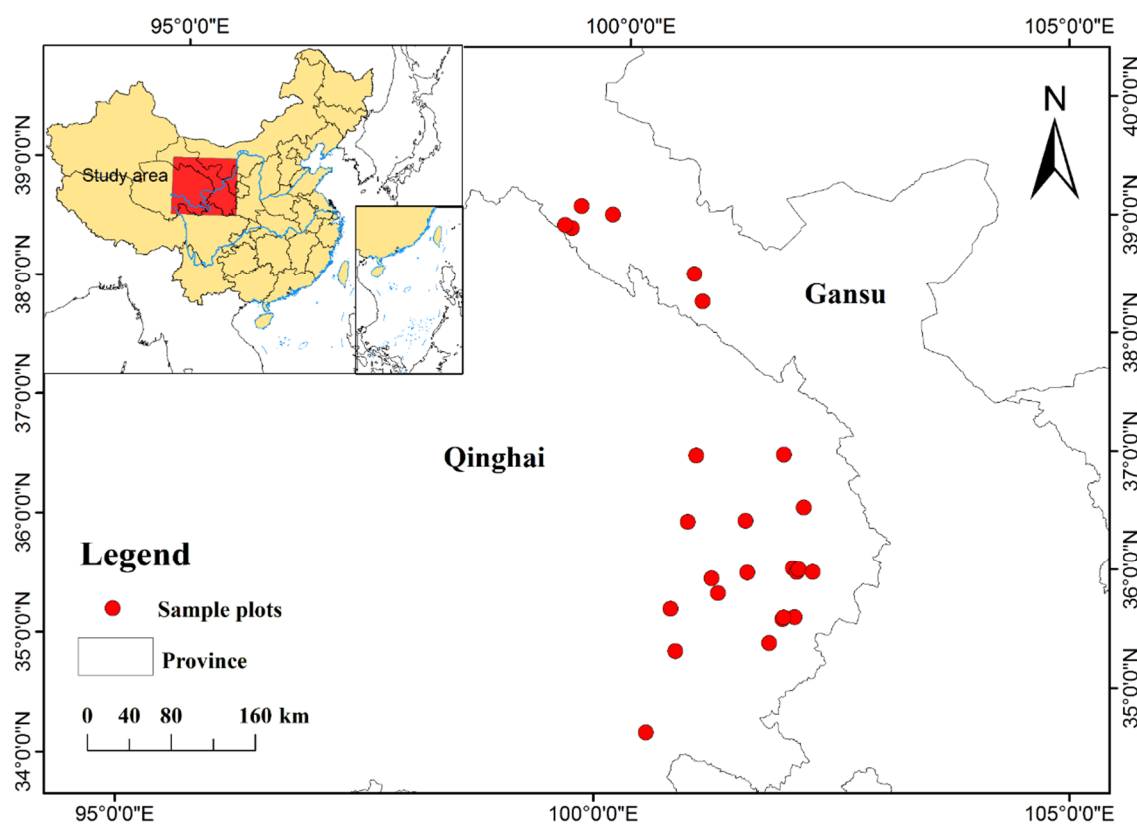


Figure 1. Geographic distribution of the sampling sites ($n = 25$) in Qinghai and Gansu Provinces.

2.2. Plant Material

From late August to early October in 2017, mature individual plants of *E. tangutorum* with fully ripened seed-heads were collected from the 25 different sites distributed on permanent grassland of the QTP in Qinghai and Gansu Provinces (Figure 1). For sites located at lower altitude (below ca. 3800 m), 20–40 plants were sampled at each site. Fewer plants (7–12) were sampled at the remaining sites due to low *E. tangutorum* abundance. For each site, tillers of individual plants were cut approximately 3 cm from the soil surface. Each plant sample was packed separately into envelopes and returned to the laboratory for analysis. The geographic coordinates and altitude of each site were taken during sampling. The endophyte-infected (E+) *E. tangutorum* was identified from populations by using the aniline blue staining method of Cheplick [67] on culms of each individual plant. E+ *E. tangutorum* plants were identified in each of the 25 sites.

2.3. Determination of Ergot Alkaloid and Peramine Concentration

Standards of ergonovine, ergine and peramine were provided by Dr Wade Mace, AgResearch Limited, Grasslands Research Centre in New Zealand. Aboveground parts of individual plants were used to determine the alkaloid concentration.

A 200 mg subsample of dried plant material collected from each site was used to measure the ergot alkaloid concentration. Ergot alkaloid concentrations were determined by using a HPLC method adapted from Zhang et al. [68]. An Agilent 1100 HPLC (Agilent, Santa Clara, CA 95051, USA), fitted with a C₁₈ column (Eclipse XDB-C₁₈, 250 mm × 4.6 mm, 5 μm) was used to quantify the ergot alkaloids. The mobile phases used were (A) 0.1 M NH₄OAc, and (B) CH₃CN: 0.1 M NH₄OAc, 3:1. The flow rate was 1 mL·min⁻¹. Detection was performed with an ultraviolet wavelength spectrophotometric

detector (Agilent G1314A, Santa Clara, CA, USA) set to 312 nm. The quantity of extracted sample injected into the injection port was 20 μL . Ergot alkaloid concentration was quantified using external standard curves.

For peramine analysis, a 50 mg subsample of freeze-dried plant material was used. Peramine was extracted following the methods of Zhang and Nan [40]. The HPLC machine, column, flow rate and detector used to quantify the peramine concentration were as described above for the determination of ergot alkaloids. For peramine detection, the mobile phases were (A) 1.8 g L⁻¹ guanidine carbonate and (B) acetonitrile, and the spectrophotometric detector was set to 280 nm. The quantity of extracted sample was 25 μL . Peramine concentration was quantified using an external standard curve.

2.4. Collection of Climate Data

Climatic data was collected from the National Meteorological Data Center (<http://data.cma.cn>). According to the latitude, longitude, and elevation of the sampling sites, we used the thin-plate smoothing spline algorithm implemented in the Anusplin package (Version 4.4, Canberra, Australia; <http://fennerschool.anu.edu.au/files/anusplin44.pdf>) for interpolation to obtain the mean daily temperature (MDT) and mean precipitation (PCP) in the growing season of each site for the period 2006–2015.

2.5. Statistical Analysis

Concentrations (average \pm standard error) of the *Epichloë* alkaloids ergonovine, ergine and peramine were calculated using SPSS (Version 24.0, Chicago, IL, USA). Regression analysis was employed to assess the relationships between the elevation and ergot alkaloid and peramine concentrations. The Pearson correlation coefficients were determined as a first assessment of the relationship between elevation, MDT and PCP. Canonical correlation analysis (CCA) using SPSS 24.0 was used to further explore the relationship between these three measures of plant environment and to examine their association with the observed concentrations of *Epichloë* alkaloids in the foliage of the *E. tangutorum* host. CCA explores the relationship between two groups of variables, in this case the concentration of ergonovine, ergine and peramine on the one hand, and the site environment factors, elevation, mean daily temperature and mean precipitation, on the other hand. With three input variables in each group, three canonical correlations with progressively decreasing information content are available. Each of these canonical correlations is for a pair of canonical variates that are linear functions of the alkaloid and environment variables, respectively, formed from canonical coefficients identified in the CCA. Here we refer to the three canonical variates derived from the alkaloid data by CCA as v_1 , v_2 , and v_3 ; and the three canonical variates derived from the environment data as u_1 , u_2 , and u_3 . Data were standardized prior to performing CCA.

3. Results

3.1. Climate Factors and Relationships with Elevation

The latitude, longitude and elevation of the 25 sampling sites, and the interpolated mean daily temperature and mean precipitation are presented in Table 1. In general, across the sampling sites, precipitation was lower in the east, while altitude was higher to the south. Within the elevation range of the study (2100–3920 m), the mean daily temperature and mean precipitation ranged from 9.1–15.6 °C and 182–471 mm, respectively. Not unexpectedly, mean daily temperature was significantly negatively correlated with elevation ($r = -0.715$, $p < 0.001$), while precipitation displayed a marginally significant positive correlation with elevation ($r = 0.392$, $p = 0.053$) (Table 2).

Table 1. Co-ordinates of longitude and latitude, elevation and the interpolated growing season mean daily temperature and mean precipitation (average \pm standard error (SE)).

Site	Longitude (E)	Latitude (N)	Elevation (m)	Mean Daily Temperature ($^{\circ}$ C)	Mean Precipitation (mm)
Gansu Minle	100 $^{\circ}$ 49'58"	38 $^{\circ}$ 25'50"	2313	13.9 \pm 0.79	247 \pm 40.70
Gansu Minle	100 $^{\circ}$ 56'09"	38 $^{\circ}$ 12'02"	2922	13.0 \pm 0.95	256 \pm 46.93
Gansu Sunan	99 $^{\circ}$ 26'27"	38 $^{\circ}$ 46'12"	2546	10.0 \pm 0.38	225 \pm 23.94
Gansu Sunan	99 $^{\circ}$ 21'47"	38 $^{\circ}$ 47'32"	2740	9.4 \pm 0.32	269 \pm 23.69
Gansu Sunan	99 $^{\circ}$ 32'15"	38 $^{\circ}$ 57'35"	2892	11.8 \pm 0.97	186 \pm 19.41
Gansu Sunan	99 $^{\circ}$ 53'27"	38 $^{\circ}$ 54'05"	2240	14.5 \pm 0.77	182 \pm 25.28
Qinghai Gonghe	100 $^{\circ}$ 52'18"	36 $^{\circ}$ 20'17"	3230	10.4 \pm 0.49	339 \pm 32.47
Qinghai Guide	101 $^{\circ}$ 29'42"	36 $^{\circ}$ 21'56"	3351	9.8 \pm 0.51	342 \pm 45.18
Qinghai Guinan	100 $^{\circ}$ 56'03"	36 $^{\circ}$ 53'58"	2842	11.0 \pm 0.45	405 \pm 37.88
Qinghai Guinan	101 $^{\circ}$ 09'12"	35 $^{\circ}$ 51'53"	3383	11.1 \pm 0.56	362 \pm 40.94
Qinghai Guinan	101 $^{\circ}$ 13'43"	35 $^{\circ}$ 44'36"	3392	10.9 \pm 0.76	350 \pm 46.79
Qinghai Guinan	101 $^{\circ}$ 47'41"	35 $^{\circ}$ 20'17"	3920	9.9 \pm 0.40	387 \pm 51.98
Qinghai Huangzhong	101 $^{\circ}$ 53'17"	36 $^{\circ}$ 56'01"	2384	10.8 \pm 0.76	418 \pm 43.16
Qinghai Maqin	100 $^{\circ}$ 31'20"	34 $^{\circ}$ 32'56"	3620	9.1 \pm 0.35	471 \pm 55.35
Qinghai Ping'an	102 $^{\circ}$ 07'19"	36 $^{\circ}$ 29'36"	2100	15.0 \pm 0.85	366 \pm 52.01
Qinghai Tongde	100 $^{\circ}$ 43'38"	35 $^{\circ}$ 35'42"	3125	10.7 \pm 0.58	404 \pm 46.04
Qinghai Tongren	102 $^{\circ}$ 01'25"	35 $^{\circ}$ 58'25"	2230	14.7 \pm 0.83	350 \pm 58.55
Qinghai Tongren	102 $^{\circ}$ 04'01"	35 $^{\circ}$ 56'31"	2416	15.3 \pm 0.76	347 \pm 56.09
Qinghai Tongren	102 $^{\circ}$ 05'07"	35 $^{\circ}$ 57'58"	2438	15.3 \pm 0.76	347 \pm 56.09
Qinghai Tongren	102 $^{\circ}$ 03'28"	35 $^{\circ}$ 33'47"	2462	14.6 \pm 0.70	375 \pm 50.30
Qinghai Tongren	102 $^{\circ}$ 04'12"	35 $^{\circ}$ 56'50"	2707	15.6 \pm 0.99	278 \pm 49.85
Qinghai Xinghai	101 $^{\circ}$ 32'05"	35 $^{\circ}$ 55'32"	2765	13.7 \pm 0.50	354 \pm 45.97
Qinghai Xinghai	100 $^{\circ}$ 47'51"	35 $^{\circ}$ 14'31"	3321	11.2 \pm 0.59	418 \pm 49.32
Qinghai Zeku	101 $^{\circ}$ 55'44"	35 $^{\circ}$ 32'33"	2876	11.0 \pm 0.55	378 \pm 52.14
Qinghai Zeku	101 $^{\circ}$ 56'23"	35 $^{\circ}$ 33'24"	3012	11.0 \pm 0.55	378 \pm 52.14

Table 2. Pearson correlation (R) between elevation and mean temperature and rainfall during the growing season across the 25 sites in Figure 1.

Correlation of Elevation with	R	Significance, <i>p</i>
Mean daily temperature	−0.715	<0.001
Mean precipitation	0.392	0.053

p denotes statistical significance of the correlations.

3.2. Alkaloid Concentration of E+ *E. tangutorum*

Ergonovine, ergine and peramine concentrations were determined for E+ *E. tangutorum* for the 25 sampling sites and arranged in order of ascending elevation (Figure 2; Table S1). The concentrations of ergonovine, ergine and peramine alkaloid ranged from 0.47–0.84, 0.35–1.72, 9.18–13.00 $\mu\text{g}\cdot\text{g}^{-1}$, respectively. The concentration of peramine was much higher than that of ergonovine and ergine alkaloids (Figure 2), and peramine accounted for more than 84% of the total *Epichloë* alkaloid detected (Table 1).

3.3. The Relationship between Alkaloid Concentration and Elevation

Ergine concentrations were highest at mid altitudes (Figure 2b), while peramine concentration was observed to decline with increasing elevation above 3000 m (Figure 2c). Both of these patterns were found to be statistically significant on fitting of cubic regression curves across the elevational series ($r^2_{\text{ergine}} = 0.482$, $p = 0.003$; $r^2_{\text{peramine}} = 0.726$, $p < 0.001$). The respective equations were:

$$\text{Ergine concentration} = (3.57281E - 10) \times E^3 - (4.03444E - 6) \times E^2 + 0.01407 \times E - 14.29452 \quad (1)$$

$$\text{Peramine concentration} = (-5.40925E - 10) \times E^3 + (2.78539E - 6) \times E^2 - 0.00334 \times E + 11.67137 \quad (2)$$

where E is elevation.

In contrast, ergonovine concentration displayed no elevational trends ($r^2_{\text{ergonovine}} = 0.018$, $p = 0.817$) (Figure 2a).

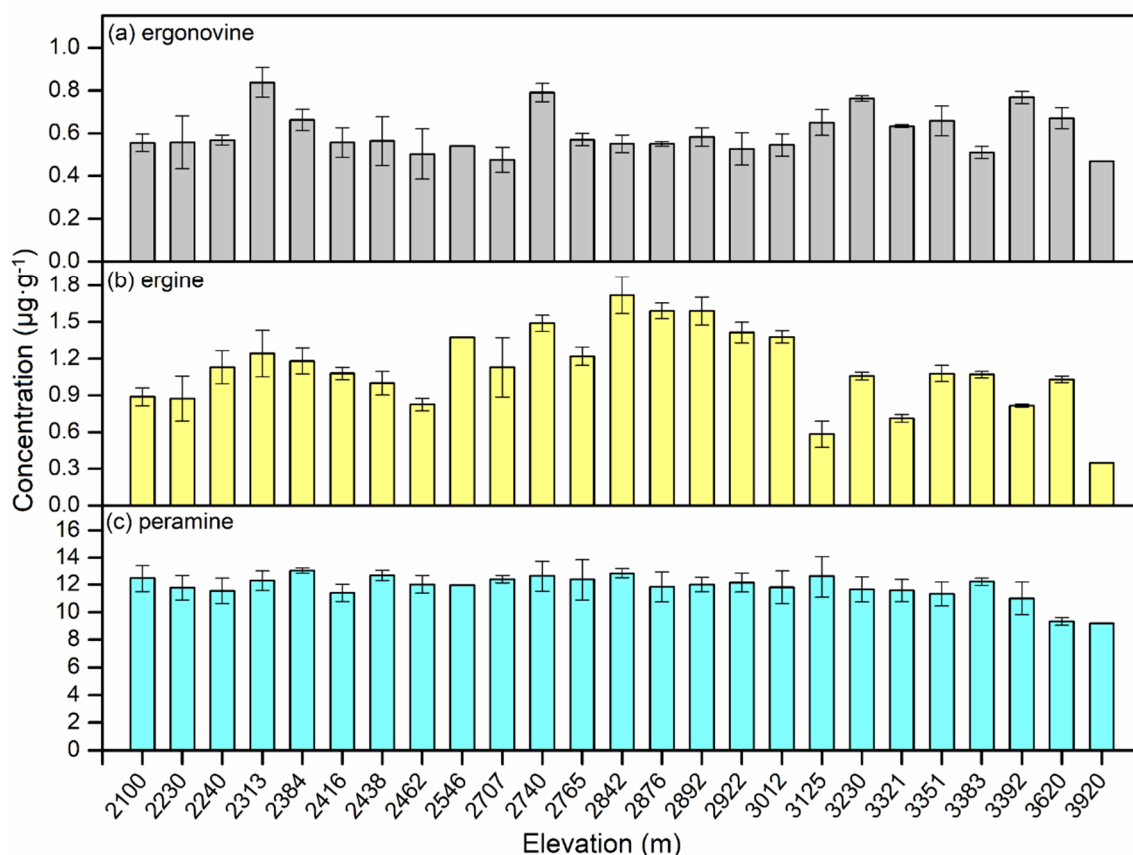


Figure 2. Elevational trends in the *Epichloë* alkaloid concentrations (average \pm SE; the average number of samples per site was 6) of *Elymus tangutorum* foliage collected from 25 different sites in Qinghai and Gansu provinces: (a) ergonovine; (b) ergine; (c) peramine.

3.4. The Relationship between Alkaloid Concentration and Climatic Factors

When the data were submitted to CCA, the first two of the three available pairs of canonical variates (designated here as Canonical 1 and Canonical 2) displayed statistically significant canonical correlations ($r = 0.690$, $p = 0.006$; $r = 0.603$, $p = 0.038$, respectively), and between them explained 97.2% of the data variation (Table 3). Hence, these first two canonical correlations are reported in the results that follow and the third canonical correlation was discarded.

Table 3. Overview of canonical correlations between ergine, ergonovine and peramine concentrations and climate variables.

Canonical	Canonical Correlation	Eigenvalue	% Variance Explained #	F (d.f.) *	p
1	0.690	0.908	59.6/22.7	3.08 (9,46)	0.006
2	0.603	0.573	37.6/8.7	2.91 (4,40)	0.038
3	0.203	0.043	2.8/1.2	0.90 (1,21)	0.354

The number on the left is the % of canonical variance as determined from the canonical eigenvalue; the number on the right is the % of standardized variance of alkaloid variables explained by the canonical variates of the environmental data. * d.f. denotes the numerator and denominator degrees of freedom, respectively, in brackets. p denotes statistical probability of the canonical correlations.

Inspection of the canonical coefficients (Table 4) and the correlations between the original variables and their canonical variates (Table 5) shows that the first pair of canonical variates identifies a tendency for all three alkaloids (but especially ergine and peramine) to be present at higher concentrations at sites with lower elevation and precipitation (with correlations between the original data and canonical variate of -0.696 and -0.527 , respectively). Temperature is not involved in this relationship as the correlation between mean daily temperature and the first climate canonical variate (designated u1 in Table 5) is only 0.034 . Meanwhile, the second pair of canonical variates identifies a tendency for peramine in particular ($r = 0.604$) to be present at higher concentrations at sites of lower elevation ($r = -0.710$) with a higher mean daily temperature ($r = 0.912$).

Table 4. Canonical coefficients for derivation of canonical variates from standardized data.

	Canonical 1	Canonical 2
	v1	v2
Ergonovine	0.329	-0.539
Ergine	0.562	-0.637
Peramine	0.529	0.909
	u1	u2
Elevation	-1.256	-0.291
Mean daily temperature	-0.948	0.825
Mean precipitation	-0.300	0.437

The first two canonical variates of the alkaloid data are designated v1 and v2; the first two canonical variates of the climate data are designated u1 and u2.

Table 5. Correlation coefficients between the original variables and their canonical variates (canonical loadings).

	Canonical 1	Canonical 2
	v1	v2
Ergonovine	0.375	-0.534
Ergine	0.814	-0.256
Peramine	0.793	0.604
	u1	u2
Elevation	-0.696	-0.710
Mean daily temperature	0.034	0.912
Mean precipitation	-0.527	0.093

The first two canonical variates of the alkaloid data are designated v1 and v2; canonical variates of the climate data are designated u1 and u2.

4. Discussion

The production of ergot alkaloids in a number of *Epichloë*-infected grass species, throughout the world, is responsible for mammalian toxicoses [26]. In China, endophytic fungus-infected drunken horse grass (*Achnatherum inebrians*) can produce ergonovine and ergine, which can lead to livestock toxicity. The ergonovine and ergine concentrations in drunken horse grass can be as high as $120\text{--}280 \mu\text{g}\cdot\text{g}^{-1}$ and $45\text{--}170 \mu\text{g}\cdot\text{g}^{-1}$, respectively [68]. In this study, the total ergonovine and ergine alkaloid concentration was less than $2.30 \mu\text{g}\cdot\text{g}^{-1}$ (Figure 2). In the QTP, the absence of toxicity to livestock grazing on *E. tangutorum* may be due to the low level of ergot alkaloid production in the grass, although the toxicity threshold of ergine and ergonovine remains undefined in the literature. In this study, ergonovine, ergine and peramine were detected in *Epichloë*-infected *E. tangutorum* collected from different elevations. Peramine concentration in endophyte-infected *E. tangutorum* was much higher than that of ergonovine and ergine alkaloids (Figure 2, Table 1).

Superficially, ergine and peramine concentrations displayed a statistically significant arc-shaped trajectory along the elevational gradient with the highest concentrations in *E. tangutorum* foliage observed at mid-elevation and the lowest concentrations at higher elevation ($p < 0.05$) (Figure 2). An elevation gradient involves associated changes in various climatic factors, especially temperature

and moisture [69]. A highland cold climate prevails [64] in our sampling sites on the QTP, and the mean daily temperature during the growing season ranged from 9.1–15.6, while the mean precipitation ranged from 182–471 mm in the growing season (Table 1). These two climate factors were significantly correlated to elevation (Table 2). Canonical correlation analysis was able to resolve the superficial relationship between alkaloid concentration and elevation into two independent linear components that together explain 97.2% of the canonical data variation, with 31.4% of the standardized variance in the alkaloid data explained by the environment canonical variates (Tables 3–5). The first pair of canonical variates linked the increased concentration of all three alkaloids to sites at lower elevation with lower precipitation, independently of temperature. The second pair of canonical variates linked the increased peramine concentration to sites at lower elevation with a higher mean daily temperature, independently of precipitation. Many previous studies have examined the association between temperature and precipitation (an index of plant water supply) and alkaloid production (for example, [53–62,70,71]). In particular, the studies conducted by Repussard et al. [71] and Żurek et al. [62] were also carried out in natural grassland areas. Repussard et al. [71] found that ergovaline concentration of endophyte-infected *Festuca arundinacea* was positively correlated to cumulative temperature in the south of France. A similar conclusion was found in our study, in that peramine concentration was positively correlated to mean daily temperature. However, among the studies to date, there is no consensus as to the effects of the environment on *Epichloë* alkaloid concentration in the host grass. Zhou et al. [61] analyzed the relationship between temperature and the ergot alkaloid concentrations of *Festuca sinensis* and showed that ergot alkaloids significantly increased as temperature decreased. Interestingly, McCulley et al. [56] found ergot alkaloid concentration increased (by 30–40%) in *Epichloë*-infected tall fescue (*F. arundinacea*) under higher temperature in transition zone pastures of the U.S., but loline alkaloid concentration was not affected. Many studies have indicated that sufficiency of water is not conducive to the production of alkaloids [54,59,60,62]. Żurek et al. [62] found that higher amounts of ergovaline produced by endophyte-infected tall fescue were much more frequent in regions of lower summer precipitation. Similar conclusions with regard to precipitation were also reached by Vazquez-de-Aldana et al. [60], who suggested that lower ergovaline production can be linked to higher precipitation. However, McCulley et al. [56] and Bourguignon et al. [53] concluded that precipitation had no effect on alkaloid levels.

To resolve the conflicting conclusions of various studies cited above, we hypothesize that the *Epichloë*-host relationship has evolved alkaloid concentration responses that maximize protection to the host from biotic stressors such as insect predation and attack by pathogenic fungi, while minimizing the metabolic cost of alkaloid production by limiting alkaloid synthesis when the plant is less exposed to biotic stress. Under this hypothesis, the findings from the present study that more ergine and peramine were produced at lower elevation sites with lower mean precipitation, and more peramine was produced at lower elevation sites with higher mean daily temperature are intuitively sensible and align with a number of the studies cited above. The results also indicate that future studies could include the collection of data on fungal pathogen and insect predation loads with a view to clarifying whether such factors are mechanisms by which climate factors influence *Epichloë* alkaloid concentrations in the host grass. Also, factors such as the effect of winter cold at higher elevations on the biotic stress load of the plant population could be assessed. Interestingly, a recent global analysis found that insect herbivory is reduced with increasing elevation [72,73], which could, in turn, select for reduced need for defense in high elevation plants [73]. Reduced insect herbivory might be a factor in the decreased concentration of peramine in host plants at high elevation sites.

Previous studies have indicated that in addition to temperature and precipitation, CO₂ concentration is also an important factor affecting alkaloid concentration [58,74,75]. CO₂ affects alkaloid production by producing carbohydrates for plant growth and the synthesis of alkaloids [75]. Elevated CO₂ reduces the concentrations of ergot alkaloid and loline produced by E+ tall fescue [74]. A similar result was found by Ryan et al. [58], where the alkaloid concentration of E+ tall fescue decreased under elevated CO₂. However, Hunt et al. [75] found that elevated CO₂ had only a marginally

positive effect on peramine and ergovaline production under high N conditions. Low air pressure is one of the plant environment characteristics of the QTP [63], with the air pressure at 3500 m elevation being approximately 70% of that at sea level. Carbon may be preferentially allocated to plant growth due to the carbon limited biosynthesis (under ambient CO₂) [75]. Therefore, reduced atmospheric partial pressure of CO₂ may be a possible explanation for the lower ergot alkaloid and peramine concentrations at higher elevation in this study. The impact of CO₂ concentration on ergot alkaloid and peramine concentration at high elevation could be assessed in the future in studies specifically designed for that purpose.

Beyond climate factors, soil nutrient status and symbiont genotype also have an influence on alkaloid production, and regional genetic variation in the host grass or *Epichloë* endophyte may also be responsible for alkaloid variation [75–78]. Further possible explanations for the different responses of the three measured alkaloids to different combinations of elevation, mean daily temperature and mean precipitation include differences in soil nutrient status, and genotype or genetic variation in the *Epichloë* symbiont or the *E. tangutorum* host. While further exploration of these factors is obviously highly relevant to building an improved understanding of the factors driving alkaloid production in the *Epichloë-Elymus* symbiosis, the present study did not collect relevant data so these must remain as points for future study.

To our knowledge, this is the first study to evaluate the elevational trends in alkaloid production in the native cool-season grass, *E. tangutorum*, on the QTP. There are a number of factors that co-vary with elevation in the QTP and cannot be easily disentangled. In this study, our main findings were that ergine alkaloid and peramine concentrations in endophyte-infected *E. tangutorum* were highest at mid-elevation and lowest at high elevation and that the peramine elevational trend in the QTP was driven by mean daily temperature while the ergine elevational trend was driven by mean precipitation. These results suggest that the different alkaloid profiles relate to different climate factors at the different sites, and increased peramine at warmer sites may reflect greater insect challenge. Our study not only addresses the knowledge gap relating to climate and elevation effects on alkaloid production by *Elymus-Epichloë* in the QTP, but also provides a new understanding of the alkaloid production of endophytic fungi under varying climatic conditions with different elevations in the QTP. Further research to explore how other environment factors such as soil nutrient status impact on alkaloid production by *Epichloë* symbionts could be worthwhile.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/10/11/1812/s1>, Table S1: Ergonovine, ergine and peramine concentrations within endophyte-infected *Elymus tangutorum* from 25 sites, expressed as a proportion (%) of the total *Epichloë* alkaloid detected. The sites are listed in order of increasing elevation.

Author Contributions: Q.S. and Z.N. conceived and designed the experiments; Q.S. and W.L. performed the experiments; Q.S. and C.M. analyzed the data; Q.S. and C.M. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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