A single application of fertilizer can affect semi-natural grassland vegetation over half a century

Michio Tsutsumi1*, Syuntaro Hiradate2*, Masashi Yokogawa3, Eri Yamakita2, Masahito Inoue4, Yoshitaka Takahashi5

1 Western Region Agricultural Research Center (Kinki, Chugoku and Shikoku Regions), National Agriculture and Food Research Organization, Shimane, Japan, 2 Faculty of Agriculture, Kyushu University, Fukuoka, Japan, 3 Osaka Museum of Natural History, Osaka, Japan, 4 The Shimane Nature Museum of Mt. Sanbe, Shimane, Japan, 5 Japan Grassland Conservation Network, Shimane, Japan

* These authors contributed equally to this work.

CASE: mcot@affrc.go.jp

Abstract

Restoration of species-rich semi-natural grassland requires not only a seed source but also appropriate soil properties. In Europe, approximately 10 years are required for the properties of fertilized soils to reach suitable conditions and be considered successfully restored. However, restoration may require additional time in Japan because heavier precipitation causes leaching of basic cations from soils, resulting in soil acidification; volcanic ejecta also forms active Al and Fe hydroxides with high phosphate sorption. Within this context, we aimed to answer the following questions: i) whether and how the impacts of fertilization remain in the soil properties after half a century in Japan; and ii) how fertilization affects the restoration of semi-natural grasslands in Japan. We investigated the vegetation and soil properties of a Zoysia japonica pasture improved half a century ago with a single application and an adjacent semi-natural grassland (native pasture) in Japan, and found the following: (1) the two pastures had similar dominance of Z. japonica, but differed in the species composition; (2) the improved pasture exhibited lower species richness than the native pasture; (3) soil nutrients, including N, P, K, Mg, and Ca, were higher in the improved pasture than in the native pasture; and (4) many chemical properties of the soils were associated with species composition; namely, the vegetation on nutrient-rich soil had more alien species and fewer native species. We conclude that a single dose of fertilization can affect soil properties in semi-natural grasslands over half a century in Japan, leading to species loss and changing the species composition. We suggest that fertilized soils under grazing in Japan may require more than half a century to restore the nutrients to suitable levels for the establishment of a species-diverse grassland.

Introduction

Grasslands are an important land cover with world-wide distribution, and these grass-dominated communities can occur both naturally and artificially [1]. Semi-natural grasslands
maintained by human activities can also have diverse plants and animals, similar to natural
grasslands. During the past century or earlier, the area of semi-natural grasslands has
decreased because of land-use changes in many countries [1], including Japan [2, 3]. Conse-
quently, many grassland plants and animals are endangered [1].

Numerous studies have been conducted on the restoration of semi-natural grasslands [4–
8]. Restoration of species-rich semi-natural grassland requires not only a seed source for the
grassland plants but also soil with favorable properties [9]. Low levels of extractable P,
exchangeable Ca, and low pH are necessary for the successful restoration of heathlands or
semi-natural grasslands in England [10]. Typical vegetation of semi-natural grassland in Japan
is found only on soils with low levels of available P (<200 mg P₂O₅ kg⁻¹ dry soil, Bray II) and
low pH (H₂O) (<5.7); alien species are seldom found under these conditions [11]. Similar
results were obtained in Germany [12], where soil pH, P, and Mg are drivers of species com-
position and richness in semi-natural grasslands. However, a recent study stated that N was more
important than P and K as a driver of species loss [13]. Therefore, eutrophication is a major
driver of species loss in semi-natural grasslands.

Most arable soils in England are predicted to require at least 12 years before nutrient levels
become suitable for the establishment of a heathland sward [10] based on experimental data
[14]. Several methods to strip the soil of nutrients and promote acidification, including burn-
ing, cutting/mowing, topsoil stripping, cropping, and grazing, are available [15]. When these
techniques are used, only several years may be required to restore the properties of fertilized
soils to the condition before fertilization in England [10]. Although more than 50 years are
required to restore semi-natural grassland vegetation on ex-arable fields in Sweden, young ex-
arable fields (<10 years) and semi-natural grasslands have similar soil properties, including
exchangeable P and pH [7].

The Japanese Archipelago lies in the northeast tip of the Asian Monsoon Zone and has
heavier precipitation than Europe [16]. The heavier precipitation causes leaching of basic cat-
ions such as Ca²⁺, K⁺, and Mg²⁺ from the soils, resulting in soil acidification. In addition, the
availability of soil P is very low in the natural Japanese ecosystem because volcanic ejecta,
which covers wide areas of the archipelago, forms active Al and Fe hydroxides with high ability
for phosphate sorption [17]. Therefore, the restoration period for limed and P-implemented
soils in Japan is likely different from European soils. Although it was suggested that fertiliza-
tion on grassland affected the vegetation over approximately 40 years in Japan [18], it has
never been assessed the long-term effects of fertilization on soil properties.

We hypothesized that fertilized soils in Japan require more time (several decades or more)
to restore species-rich semi-natural grasslands. Within this context we aimed to answer the fol-
lowing questions: i) whether and how the impacts of fertilization remain in the soil properties
after half a century in Japan; and ii) how fertilization affects the restoration of semi-natural
grasslands in Japan.

**Materials and methods**

**Study site**

The study site was located at Urumi, Chibu, Shimane, Japan (36.018° N, 133.014°E) at an alti-
tude of 280–290 m in Chiburi Island (S1 and S2 Figs). This island belongs to the Oki Archipe-
lago lying in the Sea of Japan. The area is within the temperate monsoon climate region. The
annual average temperature is 14.9°C, and the annual average precipitation is 1589.3 mm
(data are from Ama on Nakanoshima Island, near Chiburi Island [19]).

Semi-natural grasslands are distributed over the island. They were managed by cattle and
horse grazing from April to June and from September to November, and by mowing in
summer in the 1970s. In recent years, they are grazed only by cattle from May to November, continually. In 1970, part of the semi-natural grassland dominated by *Zoysia japonica* was improved without plowing [20]. The grassland was fertilized with 1,000 kg ha$^{-1}$ of calcium carbonate, 500 kg ha$^{-1}$ of fused magnesium phosphate, and 500 kg ha$^{-1}$ of compound fertilizer (7% N, 12% P$_2$O$_5$, and 7% K$_2$O). Introduced species were *Dactylis glomerata* (orchardgrass), *Lolium arundinaceum* (tall fescue), *Agrostis gigantea* (redtop), and *Arrhenatherum elatius* (tall oatgrass). Seeds were sown at rates of 20 kg ha$^{-1}$ for each *D. glomerata*, *L. arundinaceum*, and *A. gigantea*, and 15 kg ha$^{-1}$ for *A. elatius*. *Trifolium repens* (white clover) was also introduced, but the sowing quantity was unknown. All of these are exotic species. There was no fence between the improved pasture and the adjacent semi-natural grassland (hereafter, native pasture). After the improvement, the improved pasture was used together with the native pasture. Additional fertilizer was not applied.

**Data during 1972–1981**

Vegetation data from each pasture during 1972–1981 were obtained as follows. Every July from 1972 to 1981, a vegetation survey was carried out in the improved pasture [20]. A systematic sampling design was used, with a 12.5 × 12 m grid resolution and 1 × 1 m quadrats. Twenty fixed quadrats were used for every survey. In each quadrat, all vascular plant species, coverage (%), and length were recorded. Plant cover and community height were also assessed. In the same period, a vegetation survey in the native pasture was also conducted [21] using the same design as the survey of the improved pasture [20], except for the grid resolution (8 × 25 m) and the number of quadrats (12). Some of the data for both the improved and native pastures were lost [22], including the raw data for 1979–1981. Species cover (%) data in each quadrat from 1972 to 1978 were classified into Penfound and Howard coverage classes [23]. However, full species lists and their mean covers (%) were available for 1972–1981 [22].

**Vegetation survey in 2019**

On July 22 and 23, 2019, we conducted vegetation surveys in both the improved and native pastures. We employed the same design as the survey used for the improved pasture during 1972–1981 [20]. To precisely compare the vegetation in the two pastures, we also used 12.5 × 12 m grid resolution and 20 quadrats in the native pasture.

**Soil sampling and analysis in 2019**

Soil sampling was conducted in both the improved and native pastures at the same time as the vegetation survey. Soil samples were collected from each quadrat used for the vegetation survey for analysis of chemical properties. The surface soil layer (0–5 cm) was sampled five times from each quadrat using a soil corer (5.0 cm height, 100 mL volume) and composited into one 500-mL sample. A soil profile survey was also carried out in the two pastures. The plot of the soil profile survey was selected to represent the typical vegetation in each of the improved and native pastures. For each soil horizon of the soil profiles, the soil mechanical impedance (soil compactness) was determined with a compactness tester (compact model Yamanaka, Fujiwara Scientific Company Co., Ltd., Tokyo, Japan). All the collected soil samples were air-dried, sieved through a 2-mm mesh, and subjected to further chemical analyses.

The values of soil pH in H$_2$O (pH(H$_2$O)) were determined using a standard pH meter with a glass electrode for a soil:water mixture of 1:2.5. The total C and N contents were determined by a combustion method. Cation exchange capacity (CEC) was determined by the Schollenberger method [24]. To determine exchangeable cations (Ca$^{2+}$, Mg$^{2+}$, K$^+$, and Na$^+$), the soil sample was extracted with ammonium acetate (pH 7.0) using the Schollenberger method, and
this extract was analyzed with atomic absorption spectrophotometry. Soil NO$_3$-N and NH$_4$-N were extracted with 10% KCl solution with a soil:solution ratio of 1:10 (w:v) for 60 min. The NO$_3$-N and NH$_4$-N concentrations in the extracts were determined with naphthylethylenediamine absorptiometry and indophenol method, respectively. Hot-water-extractable N was determined by extracting N from a soil sample with boiling water using a soil:water ratio of 1:10 (w:v) for 60 min; extracted N was acid digested with concentrated H$_2$SO$_4$ at 200°C for 20 min and to 400°C for 5 min, followed by NH$_4$-N determination using distillation method (Kjeldahl method).

Two methods were used to determine plant-available P in soil samples: the Troug method and Bray II method. In the Troug method, a 1 g portion of an air-dried soil sample was extracted with 200 mL of 1 M H$_2$SO$_4$ in 0.3% (NH$_4$)$_2$SO$_4$ solution (pH 3.0) with shaking for 30 min. In the Bray II method, a 1 g portion of an air-dried soil sample was extracted with 20 mL of 0.03 M NH$_4$F in 0.1 M HCl with hand shaking for 1 min. The inorganic phosphate concentrations in the extracts were determined with the molybdenum blue method [25]. Phosphate absorption coefficient (PAC) was determined as follows: a 10 g portion of a soil sample was reacted with 20 mL of 2.5% (NH$_4$)$_2$HPO$_4$ solution (pH 7.0, 13,440 mg P$_2$O$_5$L$^{-1}$) for 24 h at room temperature, and the phosphate concentration in the supernatant was determined by the molybdenum yellow method [26].

Data analysis
We performed statistical analysis using software R version 4.1.2 [27], including the R packages simpleboot [28], boot [29], vegan [30], and labdsv [31].

A nonparametric bootstrap resampling approach [32] was used to determine whether there was a significant difference between parameters from the two pastures. Bootstrap bias-corrected accelerated 95%, 99%, and 99.9% confidence intervals based on 10,000 random simulations were used to determine significance. When the 95% interval did not overlap between parameters, for example, we considered that the difference was statistically significant at the $\alpha = 0.05$ level.

We used the Bray-Curtis index to quantify dissimilarity between species composition in the two pastures. The index between each quadrat in each year was calculated, and the effect of pasture on species composition was statistically tested using permutational multivariate analysis of variance (PERMANOVA [33]). Penfound and Howard’s coverage classes were used to indicate the abundance of species. The Bray-Curtis index takes a number between 0 and 1. If 0, the two quadrats share all the same species with the same coverage classes; if 1, they do not share any species.

We calculated the indicator value, IndVal [34], for each species to detect the indicator species in each pasture in 2019. In addition, a non-metric multi-dimensional scaling (NMDS) was performed to detect relationships between species composition and soil chemical properties in each quadrat in 2019. For these analyses, coverage (%) was used to indicate the abundance of species.

Results and discussion
Vegetation
In 1972, 2 years after the improvement, the total cover of the introduced grass species in the improved pasture was 4.9%, although one of the introduced species, $T$. repens, was dominant during 1972–1975 (S1 Table). This result indicates that it failed to establish pasture dominated by the sown grasses. During 1976–1981, the most dominant species in the improved pasture was $Artemisia indica$ var. maximowiczii, a well-known typical field weed [35]. Because the
A single application of fertilizer can affect semi-natural grassland vegetation over half a century.

In 1972–1981, the vegetation during this period was similar to ex-arable land [36, 37]. In 2019, Z. japonica, which is a typical grass in semi-natural grassland managed by grazing [38], was dominant in the improved pasture, and the three introduced species—T. repens, L. arundinaceum, and A. gigantea—were present with 7.2, 4.5, and 3.4% cover, respectively (S1 and S2 Tables). Z. japonica, Miscanthus sinensis, and Imperata cylindrica var. koenigii, typical grasses in semi-natural grasslands [38], were dominant in the native pasture during 1972–1981. In 2019, Z. japonica was the dominant species with 62.8% cover, which was not significantly different from the improved pasture (54.6%). The number of all species and native species per quadrat in the native pasture was consistently higher than in the improved pasture during 1972–1978 and 2019 (Fig 1). The number of alien species per quadrat was not different between the two pastures except in 1978. In 2019, both the pastures had similar vegetation in terms of dominant species, its dominance, plant cover, and community height, but differed in species richness.

The mean Bray-Curtis dissimilarity index between the quadrats in the two pastures during 1972–1978 ranged from 0.843–0.916 (Fig 2), indicating that the species composition was significantly different between the pastures. In 2019, the dissimilarity index was 0.529, which was lower than in the 1970s, but the effect of the pasture on species composition was statistically significant during both periods.

Seven species were detected as indicators for improved pasture in 2019, including two introduced species (T. repens and L. arundinaceum), two alien species (Poa pratensis subsp. pratensis and Rumex obtusifolius), and three native species (Oxalis corniculata, Aster microcephalus var. ovatusand, and Digitaria ciliaris) (Table 1). Indicators for native pasture in 2019 were M. sinensis, Lysimachia japonica f. subsessilis, Paspalum thunbergii, Luzula capitata, Viola grypoceras var. grypoceras, Imperata cylindrica var. koenigii, Adenophora triphylla var. japonica, Rosa luciae, Dianthus superbus var. longicalycinus, and Phyllanthus lepidocarpus (Table 1), all of which are native and typical species in semi-natural grasslands in Japan [22]. V. grypoceras var. grypoceras, R. luciae, and P. lepidocarpus were never or seldom recorded in the improved pasture (S1 Table). Adenophora triphylla var. japonica and Dianthus superbus var. longicalycinus were also found in the improved pasture during 1972–1981 but not in 2019.

In a United States grassland, 10 years of N addition to the grassland decreased the plant diversity, and the grassland did not recover the same diversity during the following 20 years [40]. Our studies obtained similar results only after a single application of fertilizer (Fig 1). Although the dominant species was the same, with similar percent cover, the species composition in the native and improved pastures differed even half a century after the improvement (Figs 1 and 2). The same results were observed in a pasture approximately 40 years after improvement by plowing and fertilization in the Kyushu region of Japan [18]. These results suggest that fertilization can have a long-term effect on species composition over several decades or more.

Soil properties

Soil profiles from both the improved and native pastures indicated weak soil structures with a similar horizon composition, boundary, soil color, field texture, and consistency (Table 2). Roots were more abundant in the A horizon of the soil profile in the improved pasture than in the native pasture. Both soil profiles were acidic (pH(H2O) < 5.2) with low levels of exchangeable cations, and had high phosphate adsorption ability, indicated by high PAC between 13.0 and 16.3 g P2O5 kg−1 (Table 3). A PAC value higher than 15 g P2O5 kg−1 has been used to
Fig 1. Profile of vegetation in the two pastures. Mean (a) plant cover, (b) community height, and the numbers of (c) all species, (d) native species, (e) alien species other than introduced species, and (f) introduced species per quadrat (m²) in the improved (gray) and native (white) pastures during 1972–1978 and 2019. Error bars indicate 95% bias-corrected accelerated confidence intervals. *p < 0.05, **p < 0.01, and ***p < 0.001.

https://doi.org/10.1371/journal.pone.0275808.g001
characterize volcanic ash soils with a high ability to adsorb phosphate, which could induce phosphorus deficiency for upland plants in Japan [41]. Although both profiles were not classified as Andosols because the thickness criteria were not satisfied, their ability to suppress phosphorus efficacy for plants was high, and phosphorus is a key factor controlling plant growth. From those characteristics, both soils were classified as Cambisols according to the World Reference Base for soil resources [42] and Inceptisols according to the United States Soil Taxonomy [43]. Table 3 clearly shows that available phosphates, as determined by Bray II P and Troug P, were higher through the soil profile of the improved pasture than in the native pasture.

The chemical properties of the surface soils between 0 and 5 cm depth are listed in Table 4 (see also S3 Table). In the improved pasture, NO$_3^-$-N, NH$_4^+$-N, and hot-water-extractable N (NH$_4^+$-N plus labile precursors of NH$_4^+$-N and NO$_3^-$-N) were significantly higher than in the native pasture. These increased N concentrations were likely derived from the fertilized N in 1970 and seemed to cause higher total N content and lower C/N ratio without influencing total C content. Available phosphates (Bray II P and Troug P) and exchangeable Ca and K were also significantly higher in the improved pasture than in the native pasture, and likely originated from fertilizers applied in 1970. These results imply that applied nutrients, such as P, N, K, and Ca, can remain as available forms for plants in surface soils for a long time (e.g., half a century). These nutrients are actively recycled and regenerated in the soil-plant ecosystem.
The NMDS plot (Fig 3) revealed that species composition in the two pastures in 2019 differed (Fig 2), while part of quadrats in the two pastures had similar species composition. The following 10 chemical properties were significantly associated with the species composition: total N, C/N, CEC, exchangeable Ca, exchangeable Mg, exchangeable K, NO$_3$-N, hot-water-extractable N, Bray II P, and Troug P (Fig 3). Soil pH (H$_2$O) was not a significant parameter, presumably because there was no difference between the pastures. All of these significant vectors, except C/N, took negative values on the NMDS axis 1 (NMDS1) and positive values on axis 2 (NMDS2). NMDS1 was negatively correlated with the number of alien species (excluding introduced species) and introduced species, and NMDS2 was negatively correlated with the number of native species (and with all species) (Table 5). The numbers of alien species and native species were not correlated. All the quadrats that took negative NMDS1 and positive NMDS2 values belonged to the improved pasture, and most of the quadrats that took positive NMDS1 and negative NMDS2 values belonged to the native pasture. These results indicate that quadrats on nutrient-rich soil had more alien species and fewer native species, and vice versa. The nutrient-rich soil resulted from the dose of fertilizer in 1970 (Tables 3 and 4). Therefore, our results suggest that a single dose of fertilizer affects the species composition in semi-natural grassland even after half a century or more.

A previous study assessed the influence of soil chemical parameters (pH, P, K, and Mg) on species composition in semi-natural grasslands, and found that P, Mg, and pH together explained 17% of the variation in species composition [12]. In our analysis, the 10 chemical properties, including not only P and Mg but also K, were significant parameters associated with species composition (Fig 3). Responses of plants to the level of chemical properties in soil vary by species [44], which may cause the differences in species composition. Although

**Table 1. Indicator species for the two pastures in 2019.**

| Pasteur | Indicator species | IndVal$^a$ | $p$  | Frequency$^b$ | Mean cover (%) |
|---------|-------------------|------------|-----|---------------|----------------|
|         |                   |            |     | Improved      | Native         |
| Improved| Oxalis corniculata| 0.7574     | 0.021| 19            | 18             | 7.3            | 1.9            |
|         | Trifolium repens  | 0.5315     | 0.021| 13            | 6              | 7.2            | 1.6            |
|         | Lolium arundinaceum| 0.4402    | 0.004| 9             | 2              | 4.5            | 0.1            |
|         | Poa pratensis subsp. pratensis| 0.4385 | 0.013| 9             | 2              | 1.9            | <0.1           |
|         | Aster microcephalus var. ovatus| 0.3857 | 0.044| 9             | 3              | 0.6            | 0.1            |
|         | Diggaria ciliaris| 0.3500     | 0.007| 7             | 0              | 0.2            | 0.0            |
|         | Rumex obtusifolius| 0.3000    | 0.026| 6             | 0              | 0.4            | 0.0            |
| Native  | Miscanthus sinensis| 0.8889   | 0.001| 5             | 19             | 0.3            | 4.7            |
|         | Lysimachia japonica f. subsessilis| 0.7763 | 0.001| 9             | 20             | 0.4            | 1.5            |
|         | Paspalum thunbergii| 0.7050   | 0.014| 16            | 20             | 3.7            | 8.9            |
|         | Lycula capitata| 0.6923     | 0.001| 6             | 18             | 0.2            | 0.5            |
|         | Viola grypoceras var. grypoceras| 0.6910 | 0.001| 1             | 14             | <0.1           | 1.9            |
|         | Imperata cylindrica var. koenigii| 0.6519 | 0.023| 12            | 20             | 2.8            | 5.2            |
|         | Adenophora triphylla var. japonica| 0.6000 | 0.002| 0             | 12             | 0.0            | 0.8            |
|         | Rosa luciae| 0.4424     | 0.009| 2             | 9              | 0.1            | 5.8            |
|         | Dianthus superbus var. longicalycinus| 0.4000 | 0.001| 0             | 8              | 0.0            | 0.3            |
|         | Phyllanthus lepidocarpus| 0.3000 | 0.024| 0             | 6              | 0.0            | 0.2            |

$^a$An indicator value.

$^b$Per 20 quadrats.
### Table 2. Soil profiles in the two pastures.

| Pasture | Horizon | Depth (cm) | Boundary | Color | Field texture | Structure | Consistence | Root | Soil type |
|---------|---------|------------|----------|-------|---------------|-----------|-------------|------|-----------|
|         |         |            |          |       |               | Size<sup>a</sup> | Grade | Type<sup>b</sup> | Stickiness<sup>c</sup> | Plasticity<sup>d</sup> | Compactness | Size<sup>e</sup> | Abundance<sup>f</sup> |
| Improved| A       | 0–5        | Wavy     | 7.5YR | CL            | VF, F, M   | Weak         | G    | SS        | SP          | Loose       | VF, F        | C      | Cambisol [42] |
|         |         |            |          |       |               | M, C       | Weak         | SAB  |           |             |            |             |        |               |
|         | Bw1     | 5–15       | Wavy     | 7.5YR | CL            | F, M, C    | Weak         | SAB  | SS        | SP          | Loose       | VF, F        | M      | VF Inceptisol [43] |
|         | Bw2     | 15–23      | Wavy     | 7.5YR | LIC           | F, M, C    | Weak         | SAB  | S         | P           | Medium      | VF, F        | F      |               |
|         | Bw3     | 23–31      | Wavy     | 7.5YR | LIC           | F, M, C    | Weak         | SAB  | S         | P           | Medium      | VF, F        | F      |               |
|         | Bw4     | 31–50      | Wavy     | 7.5YR | LIC           | F, M, C    | Weak         | SAB  | S         | P           | Medium      | VF, F        | F      |               |
|         | Bw5     | 50–72      | Wavy     | 7.5YR | LIC           | F, M, C    | Weak         | SAB  | S         | VP          | Medium      | N            |       |               |
| Native  | A       | 0–8        | Wavy     | 7.5YR | LIC           | VF, F      | Weak         | G    | SS        | P           | Medium      | VF, F        | F      | Cambisol [42] |
|         |         |            |          |       |               | M, C       | Weak         | SAB  |           |             |            |             |        |               |
|         | Bw1     | 8–24       | Wavy     | 7.5YR | LIC           | F          | Weak         | SAB  | S         | VP          | Medium      | VF, F        | F      |               |
|         | Bw2     | 24–43      | Wavy     | 7.5YR | LIC           | M, C       | Weak         | SAB  | S         | VP          | Medium      | VF, F        | F      |               |

Both soils are derived from basaltic volcanic ejecta.

<sup>a</sup>VF: very fine, F: fine, M: medium, C: coarse.

<sup>b</sup>SAB: subangular brocky, G: granular.

<sup>c</sup>SS: slightly sticky, S: sticky.

<sup>d</sup>SP: slightly plastic, P: plastic, VP: very plastic.

<sup>e</sup>VF: very fine, F: fine, M: medium.

<sup>f</sup>N: none, VF: very few, F: few, C: common.

https://doi.org/10.1371/journal.pone.0275808.t002

### Table 3. Soil chemical properties of horizons in the two pastures in 2019.

| Pasture | Horizon | pH(H<sub>2</sub>O) | Total content (%) | Exchangeable cations (cmol·kg<sup>-1</sup>) | Available phosphate (mg P<sub>2</sub>O<sub>5</sub> kg<sup>-1</sup>) |
|---------|---------|-------------------|-------------------|---------------------------------------------|--------------------------------------------------|
|         |         |                   | C     | N     | C/N   | PAC (g P<sub>2</sub>O<sub>5</sub> kg<sup>-1</sup>) | CEC | Ca | Mg | K | Na | NO<sub>3</sub>-N | NH<sub>4</sub>-N | HN | Bray II P | Truong P |
| Improved| A       | 4.84              | 8.38  | 0.85 | 9.9  | 13.3 | 38.5 | 6.1 | 5.7 | 2.1 | 0.3 | 100          | 151          | 452 | 273       | 86       |
|         | Bw1     | 4.68              | 3.24  | 0.34 | 9.5  | 15.0 | 30.7 | 1.8 | 1.1 | 0.6 | 0.3 | 15           | 55           | 79  | 188       | 41       |
|         | Bw2     | 4.72              | 2.52  | 0.25 | 10.1 | 14.7 | 28.5 | 1.6 | 0.9 | 0.6 | 0.3 | 6            | 64           | 55  | 223       | 53       |
|         | Bw3     | 4.74              | 2.10  | 0.24 | 8.8  | 16.3 | 26.1 | 1.4 | 0.7 | 0.6 | 0.2 | 2            | 34           | 47  | 219       | 57       |
|         | Bw4     | 4.75              | 1.92  | 0.21 | 9.0  | 14.7 | 25.6 | 1.3 | 0.6 | 0.6 | 0.2 | 3            | 36           | 41  | 214       | 58       |
|         | Bw5     | 4.82              | 1.67  | 0.19 | 8.8  | 13.9 | 23.2 | 1.2 | 0.4 | 0.6 | 0.2 | 1            | 27           | 31  | 148       | 39       |
| Native  | A       | 5.15              | 6.56  | 0.59 | 11.1 | 13.0 | 30.9 | 5.1 | 4.5 | 0.9 | 0.5 | 11           | 158          | 290 | 148       | 45       |
|         | Bw1     | 5.20              | 2.51  | 0.27 | 9.2  | 14.0 | 25.0 | 2.7 | 2.3 | 0.2 | 0.5 | 5            | 58           | 62  | 43        | 9        |
|         | Bw2     | 5.19              | 1.70  | 0.18 | 9.3  | 13.7 | 21.8 | 2.1 | 1.5 | 0.2 | 0.5 | 2            | 49           | 31  | 24        | 5        |

Abbreviations: PAC—phosphate absorption coefficient, CEC—Cation exchange capacity. HN—Hot-water-extractable N. All data were calculated on the basis of oven-dried (105˚C) soil.

https://doi.org/10.1371/journal.pone.0275808.t003
Table 4. Chemical properties of surface (0–5 cm depth) soil in the two pastures in 2019.

| Pasture     | pH(H$_2$O) | Total content-mean | Exchangeable cations | Available phosphate-mean |
|-------------|------------|--------------------|----------------------|--------------------------|
|             | (%)        | C/N                | PAC (g P$_2$O$_5$ kg$^{-1}$) | CEC (cmol. kg$^{-1}$) | Ca (mg kg$^{-1}$) | Mg (mg kg$^{-1}$) | K (mg P$_2$O$_5$ kg$^{-1}$) | NO$_3$-N (mg kg$^{-1}$) | NH$_4$-N (mg kg$^{-1}$) | Bray II P (mg kg$^{-1}$) | Troug P (mg kg$^{-1}$) |
| Improved    | 5.07       | 0.76               | 10.4                 | 15.2                     | 40.1                    | 7.4             | 5.5              | 2.3               | 0.4                      | 41                        | 142                       | 397                    | 299                    |
| Upper limit | 5.14       | 0.80               | 10.7                 | 15.7                     | 41.8                    | 8.3             | 6.0              | 2.5               | 0.5                      | 56                        | 161                       | 438                    | 355                    |
| Lower limit | 5.00       | 0.72               | 10.2                 | 14.8                     | 38.7                    | 6.7             | 5.0              | 2.0               | 0.4                      | 30                        | 122                       | 362                    | 258                    |
| Native      | 5.08       | 0.65               | 11.7                 | 13.4                     | 34.4                    | 5.2             | 4.5              | 1.4               | 0.4                      | 12                        | 109                       | 320                    | 127                    |
| Upper limit | 5.12       | 0.68               | 11.2                 | 13.9                     | 35.3                    | 5.5             | 4.7              | 1.6               | 0.5                      | 14                        | 115                       | 346                    | 152                    |
| Lower limit | 5.04       | 0.62               | 11.5                 | 13.0                     | 33.6                    | 4.9             | 4.2              | 1.3               | 0.4                      | 10                        | 104                       | 301                    | 109                    |
| Statistical significance | NS | NS | ** | *** | *** | ** | *** | NS | *** | * | * | *** | *** |

Abbreviations: PAC—phosphate absorption coefficient, CEC—cation exchange capacity, HN—hot-water-extractable N.

aUpper limit of 95% confidence interval.
bLower limit of 95% confidence interval.

*p < 0.05
**p < 0.01
***p < 0.001; NS: not significant. All data were calculated on the basis of oven-dried (105°C) soil.

https://doi.org/10.1371/journal.pone.0275808.t004

Fig 3. Relationship between species composition in the two pastures and soil chemical properties in 2019. Non-metric multi-dimensional scaling (NMDS) ordination of plant species composition in the improved (filled circle) and native (open circle) pastures in 2019. The vectors represent the significant (p < 0.05) chemical properties associated with the species composition. Abbreviations: NMDS1 and NMDS2, axes 1 and 2 for the NMDS, respectively; TN, total N, HN, hot-water-extractable N; BP, Bray II P; TP, Troug P; CEC, cation exchange capacity; Ca, exchangeable Ca; Mg, exchangeable Mg; and K, exchangeable K.

https://doi.org/10.1371/journal.pone.0275808.g003
responses of wild plant species to edaphic mineral variations were recently assessed, the available data are still limited [44]. A database of plant species responses to edaphic mineral variations will accelerate the understanding of how mineral content drives species composition.

Conclusions

We investigated the vegetation and soil properties of a Z. japonica pasture that was improved half a century ago with a single dose of fertilizer and an adjacent semi-natural grassland in Japan. We found the following: (1) the two pastures were dominated by Z. japonica with similar percent cover, but differed in the species composition; (2) the improved pasture exhibited lower species richness than the native pasture; (3) soil nutrients, including N, P, K, Mg, and Ca, were higher in the improved pasture than in the native pasture; and (4) many soil chemical properties were associated with the species composition, namely the vegetation on nutrient-rich soil had more alien species and fewer native species. Therefore, we conclude that fertilization, even a single dose, can affect soil properties in semi-natural grasslands after half a century in Japan, leading to species loss and affecting species composition. In addition, we suggest that fertilized soil under grazing in Japan may require more than half a century for the nutrients to be restored to suitable levels for the establishment of a species-diverse semi-natural grassland.

Supporting information

S1 Fig. The location of Chiburi Island. The study site was located in Chiburi Island, belonging to the Oki Archipelago. The original maps were created by Esri Japan Corporation, Tokyo, Japan (available at https://www.esrij.com/products/japan-shp/). (TIFF)

S2 Fig. The study site. The improved and native pastures were located within and outside of the blue polygon, respectively. The sky-blue and red squares indicated the plots for survey at the improved and native pastures, respectively. The aerial image was copyrighted by Nariyasu Watanabe (Western Region Agricultural Research Center, NARO). (TIFF)

S1 Table. Plant species composition in the two pastures. Plant species recorded in the two pastures during 1972–1981 and 2019, and their mean covers (%). (XLSX)
S2 Table. Vegetation data in the two pastures in 2019. Plant species recorded in each quadrat in 2019, and their covers (%). (XLSX)

S3 Table. Chemical properties of surface (0–5 cm depth) soil in each quadrat in 2019. (XLSX)

Acknowledgments
We thank the Certified Union of Chibu Village for Wagyu Improvement and the government of Chibu Village for supporting investigation. We thank Yoko Ohta (The Liaison Conference of Green and Water) for help with investigation and producing S1 Fig. We thank Katsunobu Shirakawa (Natural Museum of Geihoku) and Sayaka Hiradate for help with investigation. We thank Nariyasu Watanabe (Western Region Agricultural Research Center, NARO) for providing an aerial photograph for S2 Fig. We thank Tara Penner, MSc, from Edanz (https://jp.edanz.com/ac) for editing a draft of this manuscript.

Author Contributions
Conceptualization: Michio Tsutsumi, Syuntaro Hiradate, Masashi Yokogawa.
Data curation: Michio Tsutsumi, Syuntaro Hiradate, Eri Yamakita.
Formal analysis: Michio Tsutsumi.
Funding acquisition: Masashi Yokogawa.
Investigation: Michio Tsutsumi, Syuntaro Hiradate, Masashi Yokogawa, Eri Yamakita, Masahito Inoue, Yoshitaka Takahashi.
Methodology: Michio Tsutsumi, Syuntaro Hiradate, Masahito Inoue, Yoshitaka Takahashi.
Writing – original draft: Michio Tsutsumi, Syuntaro Hiradate, Masashi Yokogawa.
Writing – review & editing: Michio Tsutsumi, Syuntaro Hiradate, Masashi Yokogawa, Eri Yamakita, Masahito Inoue, Yoshitaka Takahashi.

References
1. Squires VR, Dengler J, Hua L, Feng H. Grasslands of the world: diversity, management and conservation. Boca Raton, USA: CRC Press; 2018.
2. Ogura J. The transition of grassland area in Japan. J Kyoto Seika Univ. 2006; 30: 159–172.
3. Ohta Y, Tsutsumi M, Watanabe S, Inoue M, Shirakawa K, Yokogawa M, et al. Changes in the distribution of grassland in the twentieth century in the Chugoku region of western Japan. Landsc Ecol Eng. 2022; 18(1): 125–130. https://doi.org/10.1007/s11355-021-00477-4
4. Auestad I, Austad I, Rydgren K. Nature will have its way: local vegetation trumps restoration treatments in semi-natural grasslands. Appl Veg Sci. 2015; 18(2): 190–196. http://dx.doi.org/10.1111/avsc.12138
5. Critchley CNR, Burke MJW, Stevens DP. Conservation of lowland semi-natural grasslands in the UK: a review of botanical monitoring results from agri-environment schemes. Biol Conserv. 2003; 115(2): 263–278. http://dx.doi.org/10.1016/S0006-3207(03)00146-0
6. Helsen K, Hermyn M, Honnay O. Trait but not species convergence during plant community assembly in restored semi-natural grasslands. Oikos. 2012; 121(12): 2121–2130. http://dx.doi.org/10.1111/j.1600-0706.2012.20499.x
7. Öster M, Ask K, Cousins SAO, Eriksson O. Dispersal and establishment limitation reduces the potential for successful restoration of semi-natural grassland communities on former arable fields. J Appl Ecol. 2009; 46(6): 1266–1274. https://doi.org/10.1111/j.1365-2664.2009.01721.x
8. Steiner M, Ockinger E, Karrer G, Winsa M, Jonsell M. Restoration of semi-natural grasslands, a success for phytophagous beetles (Curculionidae). Biodivers Conserv. 2016; 25(14): 3005–3022. http://dx.doi.org/10.1007/s10531-016-1217-4

9. Berendse F, Oomes MJM, Altena HJ, Elberse WT. Experiments on the restoration of species-rich meadows in the Netherlands. Biol Conserv. 1992; 62(1): 59–65. http://dx.doi.org/10.1016/0006-3207(92)91152-I

10. Clarke CT. Role of soils in determining sites for lowland heathland reconstruction in England. Restor Ecol. 1997; 5(3): 256–264. https://doi.org/10.1046/j.1526-100X.1997.09730.x

11. Hiradate S, Morita S, Kusumoto Y. Effects of soil chemical properties on the habitats of alien and endemic plants. Nougyougijutsu. 2008; 63(10): 469–474.

12. Riesch F, Stroh HG, Tonn B, Isselstein J. Soil pH and phosphorus drive species composition and richness in semi-natural heathlands and grasslands unaffected by twentieth-century agricultural intensification. Plant Ecol Divers. 2018; 11(2): 239–253. https://doi.org/10.1080/17550874.2018.1471627

13. Band N, Kadmon R, Mandel M, DeMalach N. Assessing the roles of nitrogen, biomass, and niche dimensionality as drivers of species loss in grassland communities. PNAS. 2022; 119(10): e2112010119. https://doi.org/10.1073/pnas.2112010119 PMID: 35235460

14. Gough MW, Marrs RH. Trends in soil chemistry and floristics associated with the establishment of a low-input meadow system on an arable clay soil in Essex, England. Biol Conserv. 1990; 52(2): 135–146. http://dx.doi.org/10.1016/0006-3207(90)90122-6

15. Marrs RH. Techniques for reducing soil fertility for nature conservation purposes: a review in relation to research at Roper’s Heath, Suffolk, England. Biol Conserv. 1985; 34(4): 307–332. http://dx.doi.org/10.1016/0006-3207(85)90038-2

16. Japan Meteorological Agency. World weather chart [Internet]. Tokyo: Japan Meteorological Agency; 2021. https://www.data.jma.go.jp/gmd/cpd/monitor/climfig/?tm=monthly&el=TannMtn

17. Shoji S, Nanzyo M, Dahlgren R. Volcanic ash soils: genesis, properties and utilization. Amsterdam: Elsevier; 1994.

18. Yokogawa M, Sato C, Takahashi Y. Effects of past grassland improvement on grassland vegetation: a case study of Aso Region, Japan. Bull Kansai Organ Nat Conserv. 2013; 39(2): 113–119.

19. Japan Meteorological Agency. The meteorological data in Ama in the past [Internet]. Tokyo: Japan Meteorological Agency; 2021. https://www.data.jma.go.jp/obd/stats/etrn/view/annually_a.php?prec_no=68&block_no=1307&year=&month=&day=&view=

20. Ono S, Yoden Y, Takahashi Y, Uozumi S, Nakamura K, Matsumoto S, et al. Pasture in Minenoto ko, Oki (Chugoku region). In: National Grassland Research Institute, editor. Grassland dynamics in Japan. 2-II Sown pasture. Nishinasuno, Tochigi, Japan: National Grassland Research Institute; 1985. p. 151–164.

21. Kitahara N, Yoden Y, Ono S, Takahashi Y, Uozumi S, Nakamura K, et al. Grassland in Minenoto k o, Oki (Chugoku region). In: National Grassland Research Institute, editor. Grassland dynamics in Japan. 2-I Semi-natural grassland. Nishinasuno, Tochigi, Japan: National Grassland Research Institute; 1983. p. 95–108.

22. Shimoda K, Tsutsumi M, Higashiyama M, Nakagami K. Fact database of grassland vegetation in Japan. Ecol Res. 2020; 35(6): 1057–1061. https://doi.org/10.1111/1440-1703.12166

23. Penfound WT, Howard JA. A phytosociological study of an evergreen oak forest in the vicinity of New Orleans, Louisiana. Am Midl Nat. 1940; 12(1): 165–174. https://doi.org/10.2307/2485260

24. Schollenberger C, Simon R. Determination of exchange capacity and exchangeable bases in soil: ammonium acetate method. Soil Sci. 1945; 59(1): 13–24. http://dx.doi.org/10.1097/00010694-194501000-00004

25. Murphy J, Riley JP. A modified single solution method for the determination of phosphate in natural waters. Anal Chim Acta. 1962; 27: 31–36. https://doi.org/10.1016/S0003-2670(00)88444-5

26. Pansu M, Gauthreyrou J. Handbook of soil analysis: mineralogical, organic and inorganic methods. Berlin: Springer; 2006. https://doi.org/10.1007/3-540-31211-6

27. R Core Team. R: A language and environment for statistical computing. Vienna: R Foundation for Statistical Computing; 2021. https://www.R-project.org/

28. Peng RD. simpleboot: simple bootstrap routines. 2019. https://cran.r-project.org/web/packages/simpleboot/

29. Canty A, Ripley B. boot: bootstrap R (S-plus) functions. 2021. https://cran.r-project.org/web/packages/boot/

30. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, et al. vegan: community ecology package. 2020. https://cran.r-project.org/web/packages/vegan/
31. Roberts DW. labdsv: ordination and multivariate analysis for ecology. 2019. https://cran.r-project.org/web/packages/labdsv/
32. Efron B. Bootstrap methods: another look at the jackknife. Ann Stat. 1979; 7(1): 1–26. https://doi.org/10.1214/aos/1176344552
33. Anderson MJ. A new method for non-parametric multivariate analysis of variance. Austral Ecol. 2001; 26(1): 32–46.
34. Dufrene M, Legendre P. Species assemblages and indicator species: the need for a flexible asymmetrical approach. Ecol Monogr. 1997; 67(3): 345–366. https://doi.org/10.1890/0012-9615(1997)067[0345: SAAIST]2.0.CO;2
35. Kasahara Y. Studies on the weeds of arable land in Japan, with special reference to kinds of harmful weeds, their geographic distribution, abundance, life-length, origin and history. Berichte des Ohara Institutes für landwirtschaftliche Biologie, Okayama Universität. 1954; 10(2): 72–109.
36. Hanano Y, Fujii Y, Sato K, Osozawa S, Fujihara S. Weed control by hairy vetch (Vicia villosa Roth) in Shikoku area: vegetation test and field survey in 1993 to 1997. Bull Shikoku Agric Exp Stn. 1998; 62: 45–70.
37. Tsutsumi M, Fukasawa M, Emoto S, Shinde S, Kumagai S, Takahashi Y. Dynamics of dominant wild plants after introduction of cattle grazing onto abandoned cultivated lands: a questionnaire-based study. Jpn J Grassl Sci. 2011; 56(4):267–270. https://doi.org/10.14941/grass.56.267
38. Numata M. Progressive and retrogressive gradient of grassland vegetation measured by degree of succession: ecological judgement of grassland condition and trend IV. Vegetatio. 1969; 19(1): 96–127. https://doi.org/10.1007/BF00259006
39. Nashiki M, Nomoto T, Meguro R. Studies on weed management in pastures. 1. effect of weed infestation on the population density and spatial distribution of orchardgrass in newly established swards. Weed Res Jpn. 1987; 32(1):25–29. https://doi.org/10.3719/weed.32.25
40. Isbell F, Tilman D, Polasky S, Binder S, Hawthorne P. Low biodiversity state persists two decades after cessation of nutrient enrichment. Ecol Lett. 2013; 16(4): 454–460. https://doi.org/10.1111/eol.12066 PMID: 23301631
41. The Fifth Committee for Soil Classification and Nomenclature. Soil classification system of Japan. Tsukuba, Ibaraki, Japan: The Japanese Society of Pedology; 2017. Available from: http://pedology.jp/img/Soil%20Classification%20System%20of%20Japan.pdf
42. IUSS Working Group WRB. World reference base for soil resources 2014, update 2015 international soil classification system for naming soils and creating legends for soil maps. Rome: FAO; 2015. Available from: https://www.fao.org/3/i3794en/I3794en.pdf
43. Soil Survey Staff. Soil taxonomy: a basic system of soil classification for making and interpreting soil surveys. Washington, DC: USDA, Natural Resources Conservation Service; 1999. Available from: https://www.nrcs.usda.gov/Internet/FSE_DOCUMENTS/nccs142p2_051232.pdf
44. Zhang C, Hiradate S, Kusumoto Y, Morita S, Koyanagi TF, Chu Q, et al. Ionomic responses of local plant species to natural edaphic mineral variations. Front Plant Sci. 2021; 12: 614613. https://doi.org/10.3389/fpls.2021.614613 PMID: 33854517