

Effect of Altitudinal Variation on Phenology and Herbivory in *Trifolium repens* †

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Abstract: Phenology is an important ecological feature that can be influenced by many aspects. Mountainous regions are great sites to perform studies to help the understanding of the reproductive cycle of plants and herbivores. In this work, the phenological cycle and leaf damage rate caused by herbivores in *Trifolium repens* were observed among three different altitudes in the Itatiaia National Park from June to August 2021, and statistical analyses were performed using linear mixed-effects models. The preliminary results show that altitude affected vegetative phenophases and herbivory ($p < 0.01$). The highest altitude sampled stands out for having less open and damaged leaves and for being the only altitude without any flowering events. Nevertheless, the influence of growing season climate on phenology is often observed in transplanting experiments, in which at lower altitudes plants typically develop earlier than those in their higher altitude native sites. As for the damage caused by herbivores, it is known that environmental conditions of higher altitudes can reduce the aptitude of various insects.

Keywords: life cycle; white clover; altitude gradient



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1. Introduction

Montane environments have several biotic and abiotic variations related to elevation, which can affect plant phenology [1,2]. Climatic conditions related to the increase in elevation can influence the reproductive phenology of plants (e.g., affecting seed production and size [3–7]). Moreover, the temperature decrease is the most important environmental factor that affects the lifecycle and activity of insects and herbivores [8–12].

Trifolium repens L. (Fabaceae) is one of the most important and widely used legumes throughout the world as a forage crop and nitrogen fixative [13]. Popularly known as white clover, it is a perennial and stoloniferous herb native to Eurasia [14,15]. Commonly sown with grasses in temperate pastures, naturalized populations of the species can also be found in highland grasslands [16]. It can reproduce vegetatively through stolons and is also capable of sexual reproduction through flowering and seed production [17].

The present work aims to evaluate the phenology of white clover (*T. repens*) and damage by herbivores at different altitudes (between 1.700 and 2.400 m) in the Itatiaia National Park (PNI), Brazil.

2. Materials and Methods

2.1. Study Site

The Itatiaia National Park (PNI) was created on 14 June 1937 (and was expanded on 21 September 1982), being the first National Park established in Brazil. It is located between the states of Rio de Janeiro and Minas Gerais (22°15' and 22°30' S, 44°30' and 44°45' W) (Figure 1). It is entirely inserted in the Atlantic Forest Biome [18], with a Cwb—mesothermal climate [19], with an annual average temperature of 11.5 °C [20] and precipitation of 2600 mm [21]. The biological importance of this integral protected area is related to the extensive altitudinal gradient of more than 2.000 m in height. It allows for the occurrence of different phytophysognomies related to the Dense Ombrophilous Forest, in addition to the Campos de Altitude, which is a Brazilian tropical mountain grassland found above the forest limit (1.800–2.000 m altitude) [22]. In the PNI, *Trifolium repens* is found in the elevated region, inhabiting an altitudinal gradient, and is found on road sides (Figure 1 and Table 1).

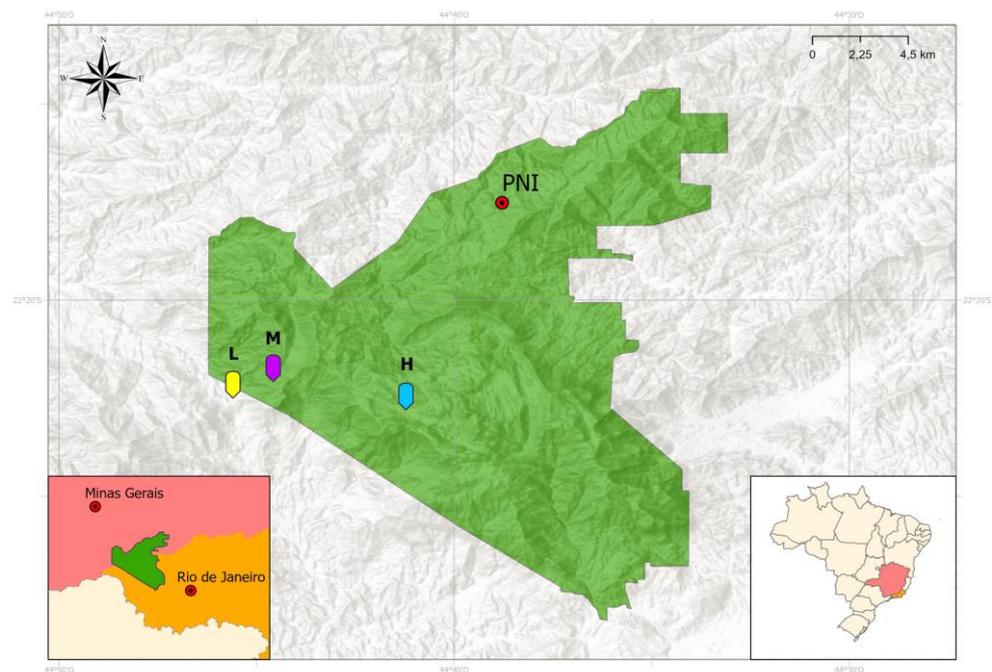


Figure 1. Illustrative map of Itatiaia National Park (in green), located between the limits of the states of Rio de Janeiro and Minas Gerais. The collection sites for the study of morphological data are in yellow (1.700 m) purple (2.000 m) and blue (2.400 m).

Table 1. Location and number of samples of *Trifolium repens* at different altitudes in the Itatiaia National Park. ID = code of the collection area; L = low; M = medium, and H = high.

| ID | Latitude | Longitude | Samples | Altitude (m) |
|----|-------------|-------------|---------|--------------|
| L | −22.375.710 | −44.760.847 | 15 | 1.721 |
| M | −22.368.405 | −44.743.229 | 15 | 2.001 |
| H | −22.380.871 | −44.687.722 | 15 | 2.406 |

2.2. Phenology and Herbivory

For the evaluation of the phenology and frequency of herbivory, 45 individuals of *Trifolium repens* (Figure 2) were selected at different altitudes of the PNI (Table 1 and Figure 1). Monthly observations were carried out from June to August 2021. For each individual, each phenophase of leaf budding, open leaves, flowering, fructification (ripe fruit), abscission (leaves in senescence and petiole without leaf), in addition to leaves burned by frost and leaves damaged by herbivory were registered and quantified.



Figure 2. *Trifolium repens* in Itatiaia National Park. (A) Inflorescence; (B) leaf composed of three leaflets (photos: Alessandra Pinto), and (C) stolon (photo: Amanda Alencar).

2.3. Data Analysis

To assess whether altitude had an effect on phenology, herbivory, and damage, linear mixed-effect models were performed. Six models were generated with different response variables (number of shoots, number of open leaves, number of leaves burned by frost, number of leaves with herbivory damage, and number of leaves in the process of abscission and inflorescence) with the function “lmer” from the “lme4” package [23]. Altitudes (three levels: 1.700 (L), 2.000 (M), and 2.400 m (H)) were used as the predictor variable in all models. For the number of shoots and number of leaves models, the individual was used as a random variable, and for the other models, the number of leaves of each individual was used as a random variable. The adequacy of the models was evaluated by visual inspection of the residues using the “qqnorm” function and the ANOVA (type II) of the models was performed using the “Anova” function of the “car” package [24]. Differences between altitudes were also calculated using the “emmeans” package [25]. All analyses were performed in R v. 3.5.3 [26].

3. Results

Altitude affected vegetative phenophases (Figure 3 and Table 2) and damage caused by herbivory (Figure 3 and Table 2). Regarding the number of leaves, the individuals of region H (2.400 m) differed from those of regions L (1.700 m) and M (2.000 m), having a smaller number of leaves. Region M had more shoots than region H, but there was no difference in shoots between region L and the others (Figure 3 and Table 2). Regarding the abscission, the M region had a greater number of abscised leaves per individual than the L region; H did not differ from L or M. Altitude H had a lower herbivory rate than the others. It was not possible to observe an influence of altitude on the percentage of leaves burned by frost. Finally, altitude H was the only one that did not present any flowering individual in the months of observation; however, due to the low intensity of this phenophase at other altitudes, it was not possible to determine whether the altitude was, in fact, affecting flowering.

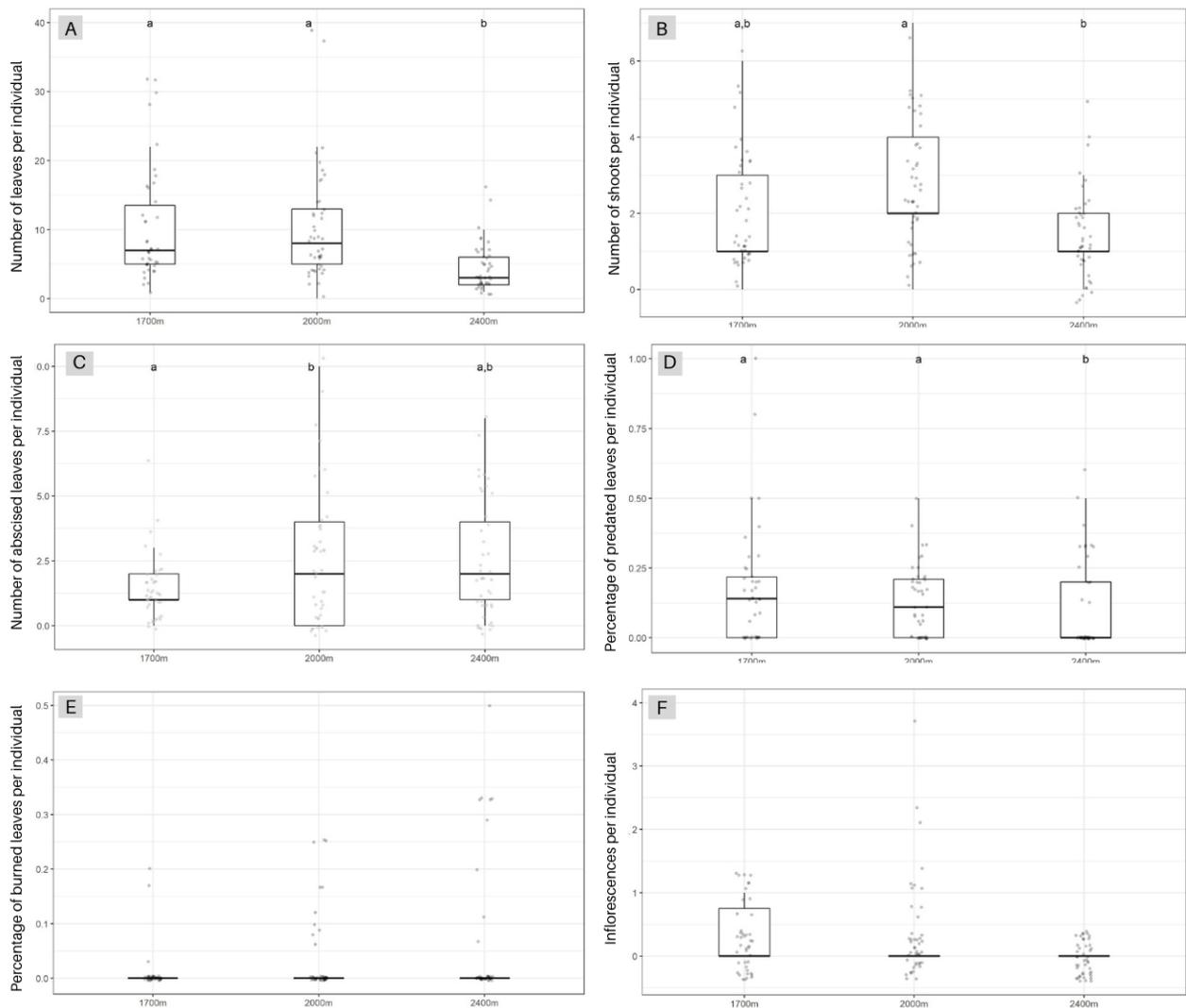


Figure 3. Effect of altitude on: (A) number of open leaves, (B) number of shoots, (C) number of abscised leaves, (D) predated leaves, (E) burnt leaves, and (F) inflorescence. Different letters (a, b) represent significant differences among the boxes.

Table 2. ANOVA results showing the effects of altitude on phenological data and on herbivory damage to *T. repens* leaves in Itatiaia National Park.

| Variable | <i>f</i> | <i>df</i> | <i>p</i> |
|-----------------|----------|-----------|----------|
| Leaves | 5.94 | 2 | <0.01 |
| Shoots | 5.96 | 2 | <0.01 |
| Abscission | 3.29 | 2 | <0.05 |
| Predated leaves | 4.99 | 2 | <0.01 |
| Burnt leaves | 2.20 | 2 | 0.12 |
| Inflorescence | 2.96 | 2 | 0.06 |

4. Discussion

Initial analyses of the three months of collected morphological data revealed that the number of leaves, shoots, abscission, and predated leaves per individual were affected by altitude. Altitude M had the highest number of shoots and abscissions, and altitude H had the lowest number of open leaves, this fact can be explained by the influence of climate at high altitudes. More than any other bioclimatic zone, phenological events at high altitudes are limited by a short growing season bounded by cold temperatures [27]. The influence of

climate on the growing season and, therefore, on phenology can be observed in transplant experiments. Plants transplanted to lower altitudes usually develop earlier than those left in their native high elevation locations [28]. As for the damage caused by herbivores, altitude H had a lower predation rate compared to the others. This result was already expected, ever since it is widely known that high altitudes can reduce the occurrence of various insects and herbivores [8,11,29], among them herbivores, such as mollusks, that usually feed on clover leaves [30,31].

5. Conclusions and Future Perspectives

In this preliminary study, the three-month results were satisfactory with significant indices, showing that the phenological data and the damage caused by the herbivores to the white clover were related to the altitude. However, it is necessary to increase the sampling (data collection) to achieve robustness in the study. To that end, monthly data collection fields are planned until June 2022 to include one year of morphological data in the analyses.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/IECPS2021-12048/s1>.

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References

1. Inouye, D.W.; Wielgolaski, F.E. Phenology at High Altitudes. In *Phenology: An Integrative Environmental Science*; Springer: Dordrecht, The Netherlands, 2013; pp. 249–272.
2. Prause, J.; Angeloni, P. Fenología de Espécies Forestales Nativas: Abcisión de Hojas. Universidad Nacional del Nordeste: Comunicaciones Científicas y Tecnológicas. 2000. Available online: http://www.unne.edu.ar/cyt/2000/5_agrarias/a_pdf/a_058.pdf (accessed on 20 August 2021).
3. Akhalkatsi, M.; Wagner, J. Reproductive phenology and seed development of *Gentianella caucasea* in different habitats in the Central Caucasus. *Flora* **1996**, *191*, 161–168. [[CrossRef](#)]
4. Kumar, X.J. Flowering phenology of tree rhododendron along an elevation gradient in two sites in the Eastern Himalayas. *Int. J. Biometeorol.* **2013**, *57*, 225–240. [[CrossRef](#)]
5. Matsoukis, A.; Kamoutsis, A.; Chronopoulou-Sereli, A. Air temperature effect on end of flowering of *Cirsium arvense* (L.) Scop. In mountainous region of Greece. *Anim. Plant Sci.* **2018**, *28*, 100–106.
6. Matsoukis, A.; Kamoutsis, A.P.; Chronopoulou-Sereli, A. A Note on the Flowering of *Ajuga orientalis* L. in Relation to Air Temperature in Mount Aenos (Cephalonia, Greece). *Curr. Agric.* **2018**, *6*, 261–267. [[CrossRef](#)]
7. Ziello, C.; Estrella, N.; Kostova, M.; Koch, E.; Menzel, A. Influence of altitude on phenology of selected plant species in the Alpine region (1971–2000). *Clim. Res.* **2009**, *39*, 227–234. [[CrossRef](#)]
8. Suzuki, S. Leaf phenology, seasonal changes in leaf quality and herbivory pattern of *Sanguisorba tenuifolia* at different altitudes. *Oecologia* **1998**, *117*, 169–176. [[CrossRef](#)]
9. Scheidel, U.; Röhl, S.; Bruelheide, H. Altitudinal gradients of generalist and specialist herbivory on three montane Astaceae. *Oecologia* **2003**, *24*, 275–283. [[CrossRef](#)]

10. Dingle, H.; Mousseau, T.A.; Scott, S.M. Altitudinal variation in life cycle syndromes of California populations of the grass-hopper, *Melanoplus sanguinipes*. *Oecologia* **1990**, *84*, 199–206. [[CrossRef](#)]
11. Galen, C. Limits to the distributions of alpine tundra plants: Herbivores and the alpine skypilot, *Polemonium viscosum*. *Oikos* **1990**, *59*, 355–358. [[CrossRef](#)]
12. Scheidel, U.; Bruelheide, H. Altitudinal differences in herbivory on montane Compositae species. *Oecologia* **2001**, *129*, 75–86. [[CrossRef](#)]
13. Zohary, M.; Heller, D. *The Genus Trifolium*; The Israel Academy of Sciences and Humanities: Jerusalem, Israel, 1984.
14. Burdon, J.J. *Trifolium repens* L. *Ecology* **1983**, *1*, 307–330. [[CrossRef](#)]
15. Pederson, G.A.; Barnes, R.F.; Miller, D.A.; Nelson, C.J. White clover and others perennial clovers. *Forages* **1995**, *1*, 227–236.
16. Pola, N.S.D.; Gobetti, S.T.C. Forrageira de Clima Temperado—Trevo Branco (*Trifolium repens* L.). *UniFil* **2018**, *1*. Available online: <http://periodicos.unifil.br/index.php/revista-vet/article/view/45> (accessed on 15 June 2020).
17. Pesticides, APVMA Australian. The Biology of *Trifolium repens* L. (White Clover) Australian Government Department of Health and Ageing Office of the Gene Technology Regulator. 2008. Available online: <http://www.ogtr.gov.au> (accessed on 23 May 2020).
18. ICMBIO. Plano de Manejo do Parque Nacional do Itatiaia 2. Instituto Chico Mendes de Conservação da Biodiversidade 2013. Available online: http://www.icmbio.gov.br/portal/images/stories/docs-planos-demanejo/pm_parna_itatiaia_enc4.pdf (accessed on 28 August 2021).
19. Köppen, W. Climatologia tradicional. *Traduzido Para O Esp. Por Pedro Henchiehs Pérez* **1948**, *1*, 479.
20. ICMBIO. Plano de Manejo do Parque Nacional do Itatiaia 1. Instituto Chico Mendes de Conservação da Biodiversidade 2013. Available online: https://www.icmbio.gov.br/portal/images/stories/docs-planos-de-manejo/pm_parna_itatiaia_enc1.pdf (accessed on 28 August 2021).
21. ICMBIO. Plano de Manejo do Parque Nacional do Itatiaia 3. Instituto Chico Mendes de Conservação da Biodiversidade. 2013. Available online: https://www.icmbio.gov.br/portal/images/stories/docs-planos-de-manejo/pm_parna_itatiaia_enc3.pdf (accessed on 20 August 2021).
22. IBGE. *Manual Técnico da Vegetação Brasileira*; Instituto Brasileiro de Geografia e Estatística: Rio de Janeiro, Brazil, 2012.
23. Bates, D.; Mächler, M.; Bolker, B.; Walker, S. Fitting linear mixed-effects models using lme4. *Stat. Softw.* **2015**, *67*, 1–48.
24. Fox, J.; Weisberg, S. An {R} Companion to Applied Regression. *Oaks* **2011**, *2*. Available online: <http://socserv.socsci.mcmaster.ca/jfox/Books/Companion> (accessed on 5 September 2021).
25. Lenth, R. Emmeans: Estimated Marginal Means, aka Least-Squares Means. R Package Version 1.3.3. 2019. Available online: <https://CRAN.R-project.org/package=emmeans> (accessed on 3 September 2021).
26. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2019. Available online: <https://www.R-project.org/> (accessed on 29 August 2021).
27. Wagner, J.; Reichegger, B. Phenology and seed development of the alpine sedges *Carex curvula* and *Carex firma* in response to contrasting topoclimates. *Artic* **1997**, *29*, 291–299. [[CrossRef](#)]
28. Molau, U.; Eriksen, B.; Knudsen, J.T. Predispersal seed predation in *Bartsia alpina*. *Oecologia* **1989**, *81*, 181–185. [[CrossRef](#)]
29. Baur, B.; Raboud, C. Life history of the land snail *Arianta arbustorum* along an altitudinal gradient. *J. Anim. Ecol.* **1988**, *57*, 71–87. [[CrossRef](#)]
30. Dirzo, R.; Harper, J.L. Experimental studies on slug-plant interactions: III. Differences in the acceptability of individual plants of *Trifolium repens* to slugs and snails. *Ecology* **1982**, *70*, 101–117. [[CrossRef](#)]
31. Thompson, K.A.; Johnson, M.T.J. Antiherbivore defenses alter natural selection on plant reproductive traits. *Evolution* **2016**, *70*, 796–810. [[CrossRef](#)] [[PubMed](#)]