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Selective Feeding of the Duckweed Weevil on Three Common Species of Duckweed in Japan

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ABSTRACT

Duckweed is a floating aquatic plant that inhabits freshwater ecosystems and has attracted attention for its potential applications in animal feed and biofuel production. To facilitate such practical applications, an efficient and cost-effective cultivation system is required. Duckweed weevils are among the most common and widely distributed insect herbivores that feed on duckweed. In this study, we investigated the duckweed weevils (*Tanysphyrus brevipennis*) behaviors and feeding preferences for three common duckweed species: *Spirodela polyrhiza* (Sp), *Landoltia punctata* (Lp), and *Lemna aequinoctialis* (La). Fasted duckweed weevils were placed with individual duckweed species, and the Lp consumed significantly smaller areas than Sp and La because of fewer duckweed weevil feeding events and less damage per feeding bout. In the mixed-species settings, duckweed weevils showed a clear feeding preference for Sp and La over Lp; the preference in descending order was Sp, La, and Lp. We further examined the effect of anthocyanins on preference using stress-induced anthocyanin accumulation in Sp. Plants with anthocyanins had smaller areas of consumption than those without anthocyanins, suggesting that anthocyanins serve as a deterrent to duckweed weevil feeding. In conclusion, our findings regarding the feeding preferences of duckweed weevils and the defensive role of anthocyanins provide valuable insights for the dynamics of the community composition of coexisting duckweed species, contributing to more robust and efficient duckweed cultivation systems.

1 | Introduction

Lemnaceae, commonly known as duckweed, is a family of aquatic monocots comprising 36 species across five genera: *Spirodela*, *Landoltia*, *Lemna*, *Wolffiella*, and *Wolffia* (Sree et al. 2016; Bog et al. 2020). Duckweed is widely distributed from tropical to subarctic regions, and multiple species often coexist within the same natural habitat (Landolt 1986; Yosef et al. 2022). It forms colonies consisting of leaf-like structures termed fronds, and plants of some genera produce one or more roots per frond. Duckweed has been used as an ideal system in physiological studies since the 1950s because of its small size, rapid growth, and ease of aseptic cultivation (Acosta et al. 2021). Furthermore, its global distribution and clonal propagation make it an optimal model for investigating local adaptations and species interactions under varying environmental conditions (Acosta et al. 2021; Isoda et al. 2022; Muranaka et al. 2022). Twelve duckweed species, including one species of *Spirodela*, one of *Landoltia*, nine of *Lemna*, and one of *Wolffia*, are distributed in Japan (Landolt 1986; Kadono 2016). Among them,

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S. polyrhiza, *Landoltia punctata*, and *Lemna aequinoctialis* are commonly found in paddy fields and ponds throughout the country.

The feeding preferences of aquatic herbivores play a critical role in shaping the interactions and coexistence of duckweed species in freshwater environments. Multiple duckweed species frequently coexist in freshwater habitats, where they are consumed by various herbivores such as snails, moths, and duckweed weevils (Mariani et al. 2020; Lee et al. 2022; Malacrinò et al. 2024). Previous studies have reported various duckweed feeding preferences of aquatic herbivores, offering diverse interpretations of the underlying mechanisms. For instance, native European flies do not accept the invasive *Lemna minuta* as a host (Baars 2011). This rejection is hypothesized to stem from the small resource of *Lemna minuta*, which may increase mortality rates for endophagous species by forcing them to move frequently between individual plants. In contrast, larvae of the native moth *Cataclysta lemnata* show no significant preference between alien *Lemna minuta* and native *Lemna minor* (Mariani et al. 2020). The results may support the hypothesis that alien plants are vulnerable to novel enemies because they have not evolved specific defenses against them. Furthermore, the water-lily aphid *Rhopalosiphum nymphaeae* favors *Spirodela polyrhiza* over other species (Subramanian and Turcotte 2020). While this preference is positively correlated with plant size, the role of chemical defense remains complex. Although certain phenolics are known to deter aphids, total phenolic concentrations do not readily explain the observed hierarchy. These interspecific feeding preferences are often mediated by the chemical and physical defense traits of the host plants. To defend against herbivores, plants produce secondary metabolites, such as alkaloids, terpenoids, and anthocyanins, which can deter feeding or retard herbivore growth (War et al. 2012). However, most of the prior research has focused on comparing fixed traits between different species. In contrast, the specific role of induced physiological states in shifting these preference hierarchies among sympatric duckweed remains poorly understood. Therefore, direct experimental investigation of how both interspecific differences and intraspecific physiological variations influence herbivore decisions is essential for elucidating the mechanisms that maintain duckweed community structure.

Interspecific variation in plant traits, encompassing both growth strategies and chemical defenses, may play a critical role in determining duckweed tolerance and resistance to herbivory. For example, *L. minor*, which contains high levels of flavones, sustains less damage from duckweed weevils, whereas *Lemna perpusilla*, which suffers more damage, appears to gain resistance by increasing its growth rate (Lee et al. 2022). To investigate these complex dynamics, we utilized the Japanese duckweed weevil, *Tanysphyrus brevipennis*, which inhabits freshwater environments where it coexists with various duckweed species (Hayashi 2023). This species serves as an effective model system for investigating such interspecific feeding preferences because it is easily maintained in laboratory environments due to its minute size and flightless nature. In addition, the morphologically distinct feeding marks of adults and larvae enable a separate and detailed evaluation of their respective herbivorous impacts (Lee et al. 2022). Using this system, we aimed to understand how feeding decisions are influenced by interspecific differences in plant traits and variations in physiological states, such as the accumulation of secondary metabolites, such as anthocyanins.

In this study, we conducted a laboratory investigation of the feeding preferences of Japanese duckweed weevils for three duckweed species common in Japan: *Spirodela polyrhiza* (Sp), *Landoltia punctata* (Lp), and *Lemna aequinoctialis* (La). The primary novelty of this study lies in establishing fundamental data on weevil feeding preferences among different species and investigating how those preferences are modified by the physiological state within those species. Our findings provide new insights into the complex mechanisms of herbivore preference, highlighting the importance of considering both interspecific differences and intraspecific physiological variations to understand the maintenance of duckweed community structure.

2 | Materials and Methods

2.1 | Plant Materials and Growth Conditions

Spirodela polyrhiza 7498 was provided by the Rutgers Duckweed Stock Cooperative (Figure 1a and Sp). *Landoltia punctata* FL and *Lemna aequinoctialis* 5636 (previously termed Nd) were collected from Kyoto, Japan and isolated in the Oyama laboratory (Figure 1a; Lp and La). All plants were aseptically maintained on modified NF medium without sucrose (NF0s) and 5 mM MES [2-(N-morpholino)-ethanesulfonic acid], as previously described (Muranaka et al. 2015). Plants were cultured in 30 mL NF0s medium at 23°C ± 1°C, under continuous light, supplied by fluorescent lamps (FLR40S•W/M-X•36R; Panasonic) at approximately 30 μmol m^{-2} s^{-1}.

2.2 | Duckweed Growth Experiment

The plants were maintained under a 15:9 light/dark photoperiod before the experiment. Each culture was initiated from single colony plants, which were cultured in six-well plates containing 10 mL of NF0s medium at 25°C with continuous light for 7 days. Growth was monitored using hourly time-lapse imaging using a PENTAX WG60 camera, and wet and dry weights were measured at the end of the cultivation. The growth rate (relative growth rate) was defined as the slope of the linear regression of log_2 (A_t/A_0) over time, where A_t is the frond area at time t and A_0 is the initial area. Statistical differences in final wet weight, final dry weight, and growth rate among species were analyzed using pairwise Wilcoxon-rank sum tests with the Bonferroni correction.

2.3 | Insect Materials and Growth Conditions

Duckweed weevils, *Tanysphyrus brevipennis*, are widely distributed throughout Japan (Morimoto and Kojima 1999). The Japanese population was previously identified as *Tanysphyrus lemnae* but was reclassified as *Tanysphyrus brevipennis*. We collected duckweed weevils from Kagoshima, Japan, where they were observed feeding on Sp and La, and maintained in

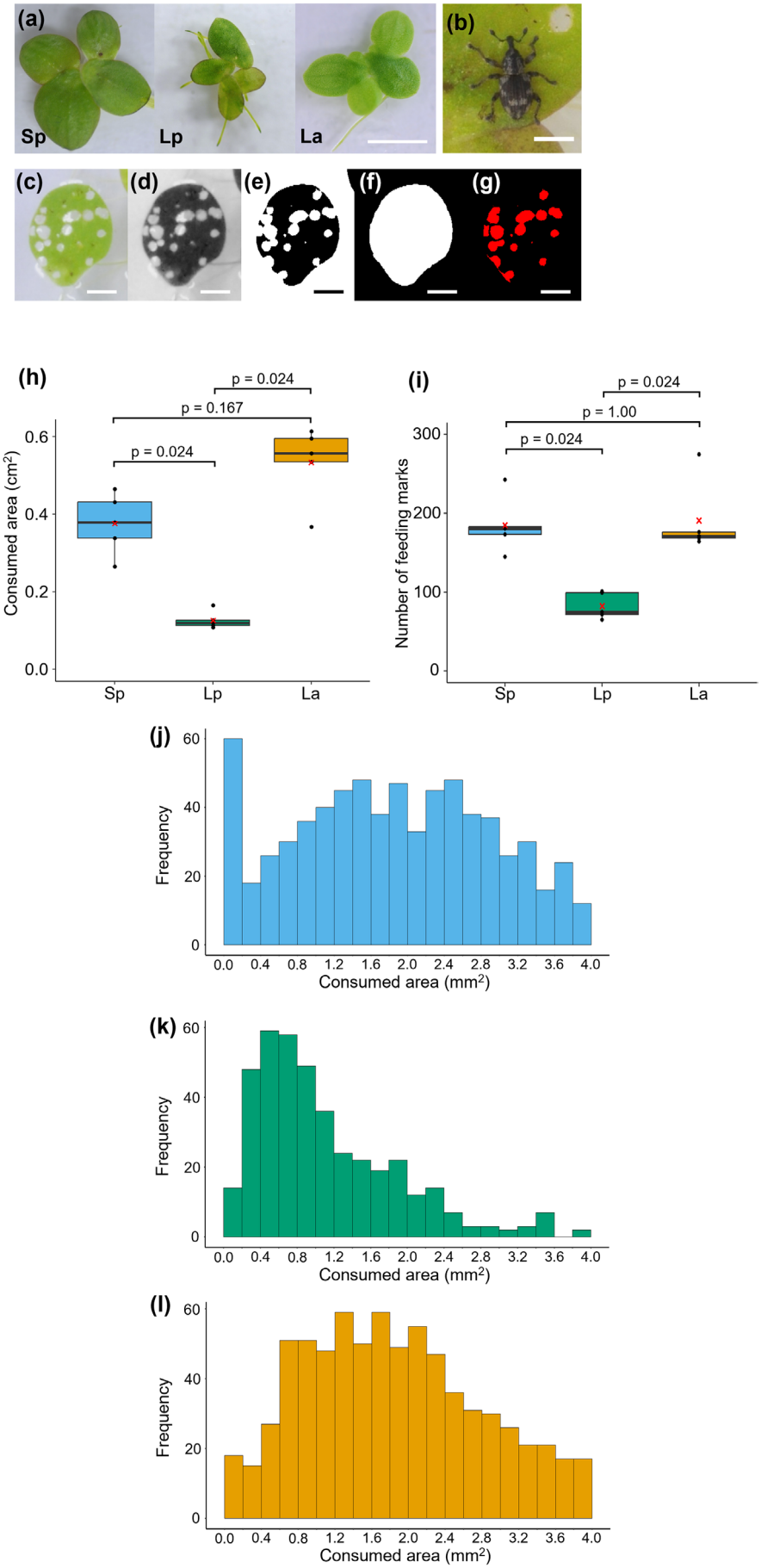


FIGURE 1 | Legend on next page.

FIGURE 1 | The feeding preference of the duckweed weevil. (a) Image of duckweed species. From left to right: Sp (*Spirodela polyrhiza*), Lp (*Landoltia punctata*), La (*Lemna aequinoctialis*). Scale bar is 5 mm. (b) Adult duckweed weevil. Scale bar is 1 mm. (c–g) Representative images used for consumption area measurement. Scale bar is 2 mm. (c) raw image, (d) blue channel image, (e) binarized image, (f) contour image, and (g) consumed area image. (h) Comparison of the consumed area using three duckweed species (Wilcoxon rank sum test with Bonferroni correct, $n = 5$ for each species). The black dots represent the values of each sample, and the red crosses indicate the average values. (i) Comparison of the number of feeding marks using three duckweed species (Wilcoxon rank sum test with Bonferroni correct, $n = 5$ for each species). (j–l) Histograms showing the consumed area per site for each duckweed species. The data represent the size of individual feeding marks observed on the fronds. (j) Sp, (k) Lp, and (l) La.

the laboratory (Figure 1b). Their broad geographical overlap of duckweed weevils and duckweed species suggests that they interact in various local environments, where both the species composition and the physiological condition of the duckweed can vary. They were placed in a plastic container with a height of 8 cm, length of 16 cm, and width of 24 cm. The container was subsequently filled with tap water and duckweed was added. The setup was maintained under constant light conditions. The lid of the container had 36 ventilation holes with a diameter of 0.6 mm, which were covered with a filter. A mixture of Sp, Lp, and La was supplied as food within the container and replaced after duckweed weevils had consumed more than 80%.

2.4 | Feeding Experiments and Measurement of the Consumed Areas

Adult duckweed weevils (within 6 months of emergence) were used for all experiments. We randomly selected individuals regardless of sex because the minute body size makes it difficult to distinguish between males and females. However, potential sex-specific effects were expected to be minimal across multiple replicates. The duckweed weevils were starved for 24 h before the feeding experiment. The experiments were conducted in 90 mm Petri dishes containing 30 mL of tap water and maintained under continuous light at a temperature of 25°C ± 1°C.

2.4.1 | Single-Species Assay

To evaluate the feeding preferences for each duckweed species, three duckweed weevils and eight colonies of a single duckweed species (Sp, Lp, or La) were placed in each dish. After 3 days, the area consumed was measured. Five independent biological replicates were used for each species.

2.4.2 | Plant Density Assay

To confirm that food availability was not a limiting factor in our experimental design, we supplied 4, 8, or 12 colonies of each duckweed species to the three duckweed weevils. The consumed area was measured after 3 days. Five independent biological replicates were used for each species at each density level.

2.4.3 | Time-Course Assay

To investigate the temporal dynamics of feeding, three duckweed weevils and eight colonies of each duckweed species were incubated for 3 days. The consumed area was measured every 24 h, and the daily feeding course was plotted. Five independent biological replicates were used for each species.

2.4.4 | Dual-Choice Assay

To determine feeding preferences, three duckweed weevils were provided with four colonies of two specific duckweed species in the same dish. The area consumed was measured after 3 days. Five independent biological replicates were analyzed for each pairwise combination.

2.4.5 | Anthocyanin Preference Assay

To evaluate the effect of anthocyanin accumulation on feeding behavior, we induced anthocyanin accumulation by culturing Sp in NF0s medium for more than 4 weeks. Both single- and dual-choice assays were performed using these plants. For the single assay, three duckweed weevils and eight fronds of either anthocyanin-accumulating or anthocyanin-free plants were placed in each dish. After 3 days, the consumed area was measured. Five independent biological replicates were analyzed for each anthocyanin condition. For the dual-choice assay, four anthocyanin-accumulating fronds and four anthocyanin-free fronds were placed in the same dish. Three duckweed weevils were introduced, and the area consumed was measured after 3 days. Eight independent biological replicates for the assay.

2.5 | Measurement of the Consumed Areas

After feeding, photographs were captured using a Nikon D300s, and the consumed area was calculated using ImageJ/Fiji software (National Institute of Health, version 1.54f). First, the blue channel was extracted from RGB images as the contrast between fronds and background was mostly clear, and the image was binarized using the auto-threshold "minimum" method (Figure 1c–e). Contours of the assumed intact fronds were obtained by manual tracing (Figure 1f). The respective feeding mark images were calculated from the binarized and contour images (Figure 1g). The consumed area was calculated from these images.

2.6 | Statistical Analysis

All statistical analyses and data visualization were performed using R software (version 4.3.3) and RStudio (version

2024.09.0 + 375, "Cranberry Hibiscus"). For the duckweed growth experiment, single-species assay, and plant density assay, differences were analyzed using the Wilcoxon rank-sum test with Bonferroni correction. Wilcoxon rank-sum test was used for the anthocyanin single assay. For the time-course assay, dual-choice assay, and anthocyanin dual-choice assay, differences were analyzed using the Wilcoxon signed-rank sum test. Statistical significance was set at p value < 0.05.

3 | Results

3.1 | Lp was Less Preferred by Duckweed Weevils Than Sp and La

Before examining the feeding behavior of duckweed weevils, we compared the growth characteristics of the three duckweed species under controlled conditions (Figure S1). Lp showed higher values than Sp and La for both dry and wet weight per unit area, yet no significant differences were detected (Sp vs. Lp: $p = 0.86$, $W = 0$, Lp vs. La: $p = 0.86$, $W = 16$, La vs. Sp: $p = 1.00$, $W = 4$; Figure S1a, Sp vs. Lp: $p = 1.00$, $W = 4$, Lp vs. La: $p = 1.00$, $W = 11$, La vs. Sp: $p = 1.00$, $W = 6$; Figure S1b). The growth rate did not significantly differ among the species, but it tended to follow the order of La > Sp > Lp (Sp vs. Lp: $p = 0.86$, $W = 16$, Lp vs. La: $p = 0.86$, $W = 0$, La vs. Sp: $p = 0.86$, $W = 0$; Figure S1c). Thus, interspecific differences in both biomass and growth rates have been suggested.

We performed a single-species assay to investigate the feeding preferences of duckweed weevils. The average consumed areas were $0.38 \pm 0.02\,cm^2$ for Sp, $0.13 \pm 0.02\,cm^2$ for Lp, and $0.53 \pm 0.1\,cm^2$ for La. Duckweed weevils consumed 2.97 times more Sp and 4.21 times more La than Lp (Sp vs. Lp: $p = 0.024$, $W = 0$, Lp vs. La: $p = 0.024$, $W = 25$, La vs. Sp: $p = 0.167$, $W = 22$; Figure 1h). The areas of Sp and La consumed were not significantly different. The number of feeding marks was counted. The average counts were 185 ± 35.8 for Sp, 82.4 ± 16.5 for Lp, and 191 ± 47.2 for La, with Lp exhibiting significantly fewer feeding marks than the other two species (Sp vs. Lp: $p = 0.024$, $W = 0$, Lp vs. La: $p = 0.024$, $W = 25$, La vs. Sp: $p = 1.00$, $W = 10$; Figure 1i). To further analyze the feeding behavior, we measured the area of each feeding mark (Figure 1j–l). Feeding marks larger than $0.4\,mm^2$ were considered an overlap of multiple feedings and they were excluded. These histograms are presented for the exploratory visualization of feeding patterns. Statistical comparisons were not performed to prevent pseudoreplication because the same duckweed weevil likely generated individual feeding marks. Compared with Sp and La, the size of the feeding marks on Lp was smaller. Therefore, the smaller areas of Lp consumed were due to a lower frequency of duckweed weevil feeding events and less damage per feeding bout, indicating that Lp was less preferred by duckweed weevils than Sp or La.

3.2 | Duckweed Weevils Consumed a Specific Amount of Duckweed Per Day

To confirm whether the standard quantity (8 colonies) provided sufficient food for duckweed weevils across the three duckweed species, we performed a plant density assay. When 4, 8, or 12 colonies of Sp were supplied, the average consumed areas were 0.41, 0.38, and $0.29\,cm^2$, respectively (Figure 2a). When 4, 8, or 12 colonies of Lp were supplied, the average consumed areas were 0.13, 0.10, and $0.12\,cm^2$, respectively. When 4, 8, or 12 colonies of La were supplied, the average consumed areas were 0.39, 0.61, and $0.53\,cm^2$, respectively. No significant differences were observed in the areas consumed among the three colonies tested for each species.

The single-species assay revealed the total consumed area after 3 days, but it did not account for temporal variations in feeding behavior. For example, if a particular duckweed species is difficult for duckweed weevils to digest, feeding activity may decline after the initial consumption on the first day. Therefore, we performed a time-series assay to investigate whether species-specific temporal changes exist in feeding patterns. We measured the area consumed over 3 days and plotted the daily course of feeding (Figure 2b,d,f). From these data, we calculated the area consumed per day ($p = 0.063$, $V = 0$; Figure 2c, $p = 0.186$, $V = 2$; Figure 2e, $p = 0.125$, $V = 14$; Figure 2g). No significant differences were observed, indicating that the duckweed weevils had similar feeding patterns for these duckweed species.

3.3 | Sp was the Most Consumed Among the Three Species in Dual-Choice Assays

In natural habitats, several duckweed species frequently coexist in the same location (Landolt 1986). Previous studies have shown that duckweed weevils selectively feed on certain duckweed species (Lee et al. 2022). To determine the duckweed species preferred by duckweed weevils, a dual-choice assay was performed. In the dual-choice assay using La and Sp, the area of La consumed was smaller than that of Sp for all six replicates ($p = 0.031$, $V = 0$; Figure 3a). The average consumed area of La was $0.20 \pm 0.07\,cm^2$, and that of Sp was $0.31 \pm 0.06\,cm^2$. Duckweed weevils consumed 1.56 times more frond area from Sp than La, and the histograms of the size of feeding marks showed no apparent difference (Figure 3b). In the dual-choice assay using Lp and Sp, the area of Lp consumed was smaller than that of Sp for all replicates ($p = 0.031$, $V = 0$; Figure 3c). The average consumed area of Lp was $0.05 \pm 0.04\,cm^2$, and that of Sp was $0.27 \pm 0.13\,cm^2$. The duckweed weevils consumed 5.30 times more frond area from Sp than from Lp. The feeding marks on Lp tended to be smaller than those on Sp (Figure 3d). In the dual-choice assay using La and Lp, the area of Lp consumed was smaller than that of La for all six replicates ($p = 0.031$, $V = 21$; Figure 3e). The average consumed area of La was $0.38 \pm 0.13\,cm^2$, and that of Lp was $0.06 \pm 0.02\,cm^2$. The duckweed weevils consumed 6.14 times more frond area from La than from Lp. In addition, relatively large feeding marks were more frequent for La than for Lp (Figure 3f). These dual-choice assays reflected the following descending order of duckweed weevil preference: Sp > La > Lp.

3.4 | Effects of Anthocyanin Accumulation on Feeding Preferences

Even among clonal plants, physiological traits can vary depending on environmental factors. Sp, Lp, and La do not

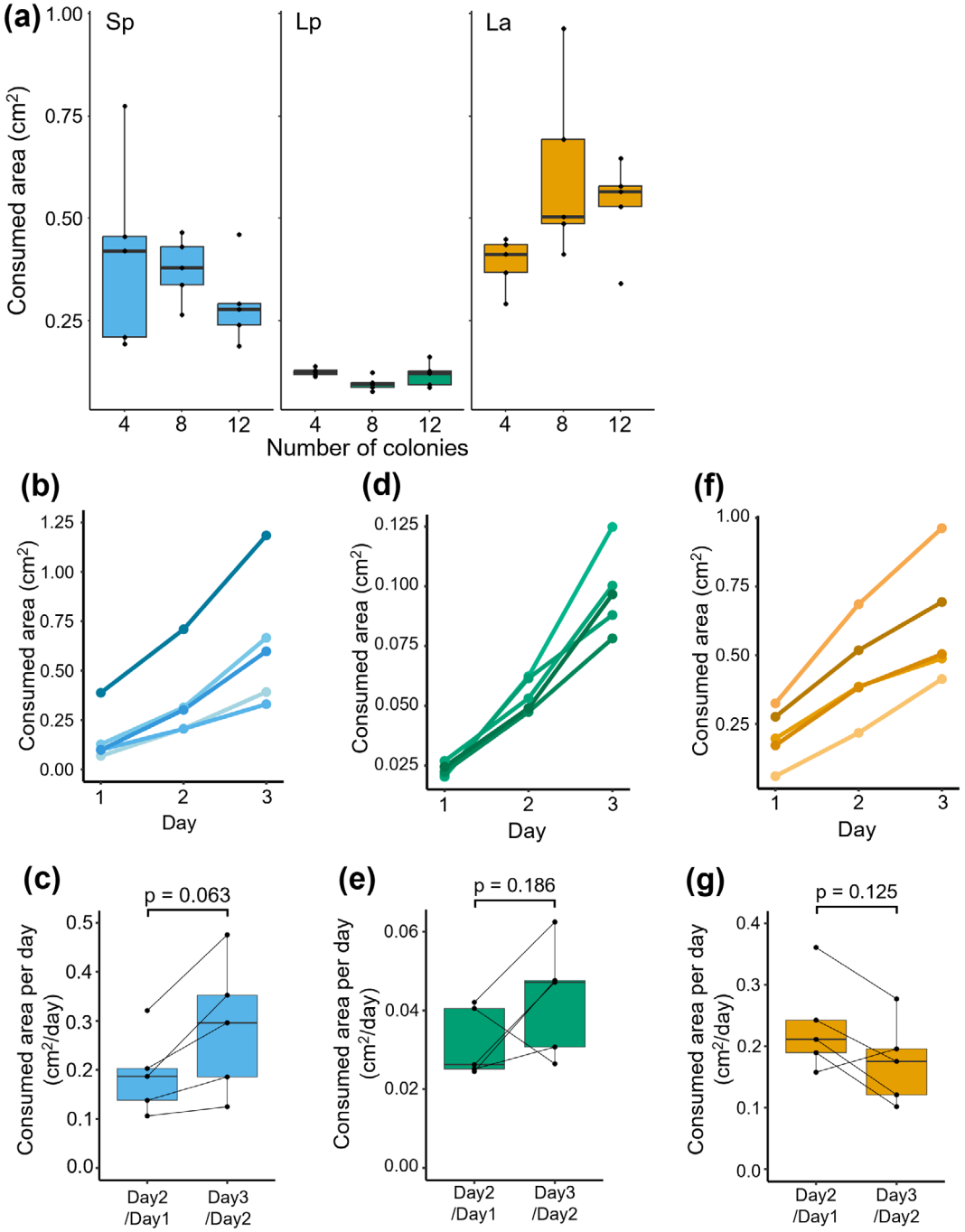


FIGURE 2 | Feeding amount and daily consumption patterns. (a) Frond area consumed by duckweed weevil (Wilcoxon rank sum test with Bonferroni correction, $n = 5$ for each species). Blue, green, and orange colors represent Sp (*Spirodela polyrhiza*), Lp (*Landoltia punctata*), and La (*Lemna aequinoctialis*), respectively. (b) Time series analysis of duckweed weevil feeding in Sp. Biological replicates ($n = 5$) are shown with different colors. (c) Comparison of daily consumption areas in Sp (Wilcoxon signed-rank sum test, $n = 5$). Lines connect paired values from the same replicate. (d) Time series analysis of duckweed weevil feeding in Lp. (e) Comparison of daily consumed area in Lp (Wilcoxon signed-rank sum test, $n = 5$). (f) Time series analysis of duckweed weevil feeding in La. (g) Comparison of daily consumed area in La (Wilcoxon signed-rank sum test, $n = 5$).

accumulate anthocyanins when grown in nutrient-rich media, whereas Sp and Lp induce and accumulate anthocyanins on the abaxial surface of the frond when grown in nutrient-deficient media (Landolt 1986; Kumar and Sharma 1999). We observed that long-term cultivation in the NF0s medium led to nutrient depletion, which in turn induced color changes and anthocyanin accumulation in Sp fronds. Anthocyanin preference assays were performed using Sp to induce anthocyanin accumulation. In the single assay, the average consumed area was $0.12 \pm 0.07\,cm^2$ for anthocyanin-accumulation fronds and $0.46 \pm 0.15\,cm^2$ ($p = 0.008$, $W = 25$; Figure 4c) for anthocyanin-free fronds. Duckweed weevils consumed 3.98 times more area of anthocyanin-free fronds than of anthocyanin-accumulating fronds. We conducted a dual-choice assay using anthocyanin-accumulation fronds and anthocyanin-free fronds. For seven out of the eight replicates, anthocyanin-free fronds were

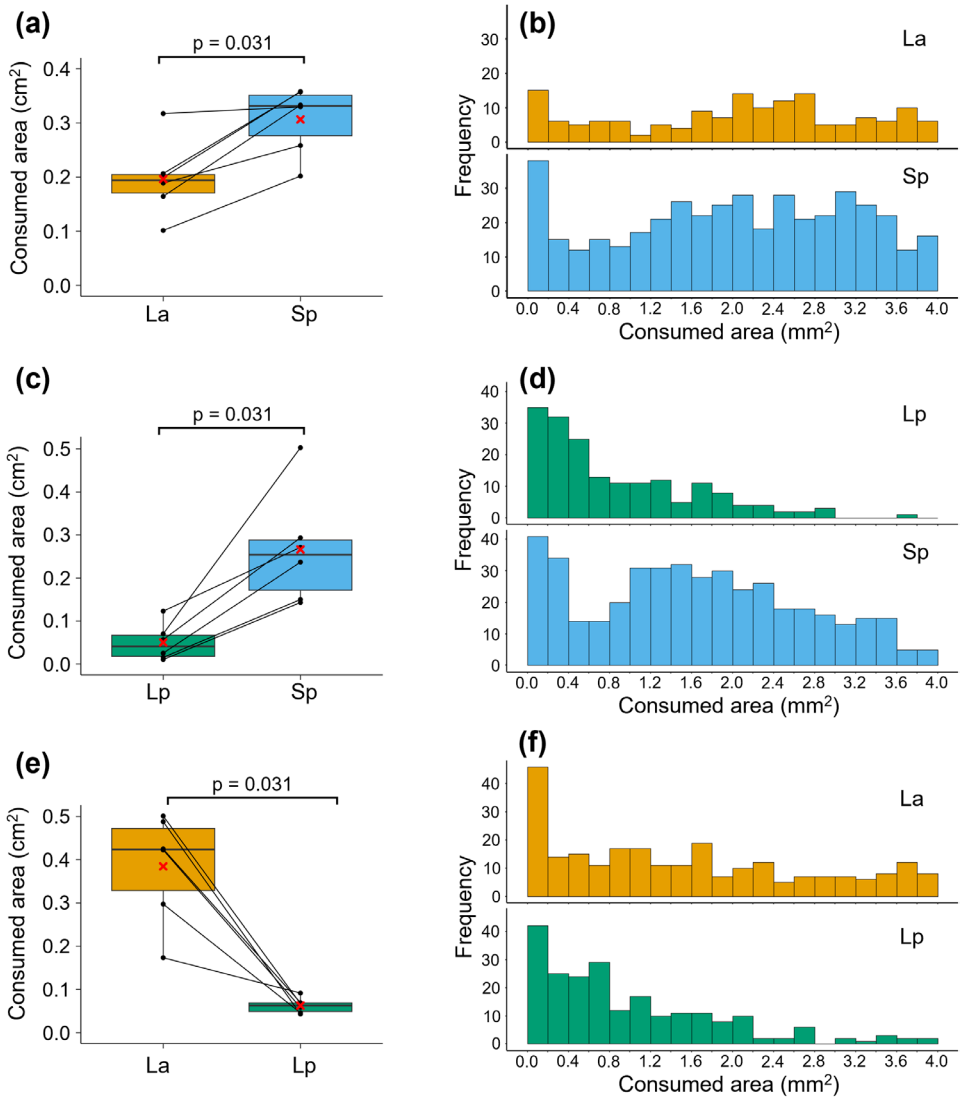


FIGURE 3 | Evaluation of the preference of the duckweed weevil by dual-choice assays. (a, c, e) Comparison of the consumed area by dual-choice assays using two different duckweed species (Wilcoxon signed-rank sum test, $n = 6$). The black dots represent the values of each sample, and the red crosses indicate the average values. Lines connect paired samples from the same Petri dish. (b, d, f) Histograms showing the consumed area per site for each duckweed species. The data represent the size of individual feeding marks observed on the fronds; locations with feeding damage areas greater than 0.4 mm^2 were removed as they were considered to be a combination of multiple feeding marks. Sp (*Spirodela polyrhiza*), Lp (*Landoltia punctata*), La (*Lemna aequinoctialis*).

consumed more than anthocyanin-accumulating fronds. In the remaining experiments, the anthocyanin-accumulating fronds were consumed at almost the same rate as the anthocyanin-free fronds ($p = 0.016$, $V = 35$; Figure 4d). The average consumed area was $0.11 \pm 0.06\,cm^2$ for anthocyanin-accumulation fronds and $0.32 \pm 0.16\,cm^2$ for anthocyanin-free fronds (Figure 4d). Duckweed weevils consumed 3.01 times more area from anthocyanin-free fronds than from anthocyanin-accumulating fronds. These results indicated that duckweed weevils prefer anthocyanin-free Sp fronds.

4 | Discussion

We investigated the feeding preferences of duckweed weevils for three duckweed species, and our results confirmed a clear preference for Sp and La over Lp. In addition, anthocyanin-free Sp fronds were consumed significantly more than anthocyanin-accumulated Sp fronds. These findings indicate that feeding preferences in duckweed weevils are shaped by both interspecific differences and physiological conditions of the host plants. Such selective herbivory may significantly influence the relative abundance and community composition of coexisting duckweed species, as has been shown in other aquatic herbivore–plant systems (Lee et al. 2022; Malacrinò et al. 2024).

The histograms showed that Lp had fewer large feeding marks and a lower total number of marks compared to Sp and La, suggesting that the duckweed weevils rejected this species after initial feeding. This avoidance behavior toward Lp may be explained by interspecific differences in physical or chemical resistance traits. Lp contains higher amounts of dietary fiber than Sp, although this was not directly quantified in this study (Muller et al. 2025). High fiber content, such as lignin and cellulose, generally enhances

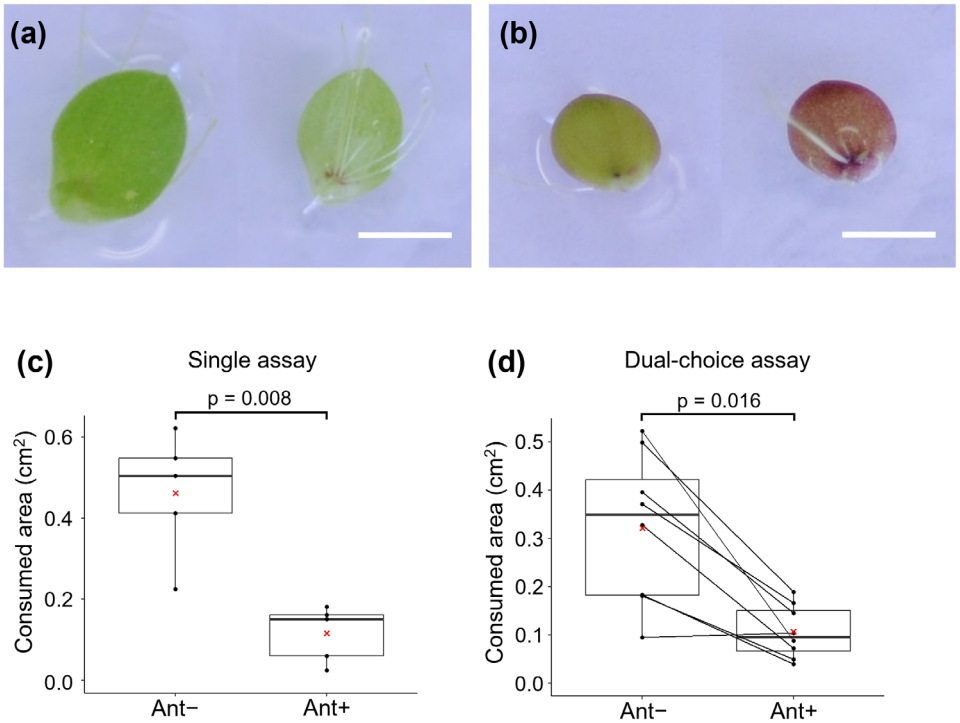


FIGURE 4 | The effect of anthocyanin accumulation on consumption by duckweed weevils. (a) Anthocyanin-free fronds of Sp (*Spirodela polyrhiza*). The left shows the adaxial side, and the right shows the abaxial side of the frond. (b) Anthocyanin-accumulating fronds of Sp. The left shows the adaxial side, and the right shows the abaxial side of the frond. (c) Comparison of the consumed area of the anthocyanin-accumulating fronds (Ant+) and anthocyanin-free fronds (Ant−) (Wilcoxon rank sum test, $n = 5$). The black dots represent the values of each sample, and the red crosses indicate the average values. (d) Comparison of the consumed area of anthocyanin-accumulating fronds (Ant+) and anthocyanin-free fronds (Ant−) by dual-choice assays (Wilcoxon signed-rank sum test, $n = 8$). The black dots represent the values of each sample, and the red crosses indicate the average values. Lines connect paired samples from the same Petri dish.

resistance to herbivores, suggesting that Lp may mitigate feeding damage through these structural deterrents (Santiago et al. 2013; Perez-Alonso et al. 2025). This defense strategy is often associated with a physiological trade-off between growth and defense (Albrecht and Argueso 2017; Züst and Agrawal 2017). Our analysis of plant growth characteristics supports this hypothesis (Figure S1). When cultured from a single colony for 7 days, Lp had higher dry and wet biomass per unit area than Sp and La, and the growth rates measured based on frond area were ordered as follows: La > Sp > Lp. However, the difference was not statistically significant under our experimental conditions. Such growth characteristics of duckweeds can be described by the well-known C-S-R triangle theory, which suggests that Lp may allocate more resources to defense mechanisms at the expense of rapid growth, whereas Sp and La prioritize growth over defense (Grime 1977; Ishizawa et al. 2021). While the growth-defense trade-off hypothesis helps explain the low preference for Lp, it does not fully account for the preference between Sp and La. Sp had a slower growth rate than La, but it was preferred by duckweed weevils in the dual-choice assays (Figures S1 and 3a). This suggests that factors other than the growth rate or simple nutritional intake influence duckweed weevil feeding preferences. This preference may be driven by reproductive requirements. In many herbivorous insects, females preferentially feed and oviposit on host plants to maximize offspring performance (Jones 2022). For example, in experiments using *Lemna minor* and *Lemna perpusilla*, adult female duckweed weevils preferred species associated with higher larval survival rates (Lee et al. 2022). Considering such life-history linkages, the larger and thicker fronds of Sp may provide a more suitable substrate for oviposition and larval development than the thinner fronds of La. Similarly, the low preference for Lp might be linked to potential reductions in larval fitness, in addition to physical or chemical resistance traits. Although this study did not directly assess oviposition behavior or larval performance, the preference hierarchy observed likely reflects a complex decision-making process involving both nutritional quality for adults and habitat suitability for larvae.

In single-species and dual-choice assays (Figures 1 and 3), we used plants grown under nutrient-rich conditions to minimize anthocyanin accumulation, thereby establishing a baseline preference hierarchy independent of this chemical defense. In contrast, the experiments presented in Figure 4 utilized an extended culture period of more than 4 weeks to specifically induce anthocyanin and evaluate its functional role as a deterrent. Regarding the physiological state of plants, our results suggested that anthocyanin accumulation functions as a deterrent to feeding. Sp fronds containing anthocyanins suffered less damage than the anthocyanin-free fronds (Figure 4c,d). Anthocyanins are water-soluble natural plant pigments that protect plants from various environmental stresses such as high light intensity, ultraviolet rays, and drought (Li and Ahammed 2023). Visually, anthocyanin accumulation (red coloration) may make the leaves less discernible to herbivores or increase the visibility of insects to predators. In *Quercus coccifera*, green leaves suffer more herbivory damage than red leaves (Karageorgou and Manetas 2006). Chemically,

the biosynthetic pathway of anthocyanins is frequently associated with that of defense-related compounds such as tannins and phenols (Schaefer and Rolshausen 2006). Since anthocyanin accumulation in our study was induced under nutrient-deficient conditions, the observed deterrent effect may have resulted from a combination of visual cues and an altered metabolic profile, including increased phenolic compounds.

Environmental conditions, such as light intensity and nutrient availability, can substantially influence duckweed physiology and subsequent herbivore feeding behavior by altering traits such as growth rate, biomass, and secondary metabolite levels (Pagliuso et al. 2018; Paolacci et al. 2018; Ishizawa et al. 2021; Petersen et al. 2022; Smith et al. 2024). Our experiments were conducted under controlled laboratory conditions; thus, natural variations in light and nutrient regimes may have led to different feeding outcomes in the field. Future work should therefore evaluate feeding behavior under more realistic outdoor conditions, integrating physiological and metabolomic analyses to elucidate how environmental factors regulate plant–herbivore interactions in duckweed communities.

Recently, duckweed has attracted considerable attention as a high-quality protein source for animal feed (Sońta et al. 2019; Minich and Michael 2024). Furthermore, duckweed is considered a promising feedstock for biofuel production because of its rapid starch accumulation and low fiber content (Cui and Cheng 2015; Chen et al. 2022). Therefore, more cost-effective and efficient duckweed cultivation systems are required. To this end, it is important to understand the environmental factors that affect duckweed growth in semi-outdoor environments. However, herbivory is a major limiting factor in both open and semi-outdoor cultivation systems. Selective feeding, as observed in the current study, may suppress the population growth of preferred species, such as Sp and La, thereby providing a competitive advantage to less-preferred species, such as Lp. Such consumer-driven filtering could influence species coexistence and succession within natural duckweed communities, similar to the herbivore-mediated community dynamics reported in other aquatic systems (Mariani et al. 2020). Therefore, the selection or engineering of duckweed species that combine moderate growth rates with strong herbivore resistance is crucial to achieve sustainable biomass production. Further integration of ecological and biotechnological approaches will help to optimize duckweed-based systems for environmental and industrial applications.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Growth characteristics of three duckweed species. Plants were cultured in 6-well plates containing 10 mL NF0s medium at 25°C under continuous light conditions (LL) for 7 days ($n = 4$). Images were taken every hour. (a) Dry weight per area, (b) wet weight per area, and (c) growth rate based on frond area. Bars in (a–c) represent mean values ± SD. Sp (*Spirodela polyrhiza*), Lp (*Landoltia punctata*), La (*Lemna aequinoctialis*).