Specific Mining Pattern as a Result of Selective Feeding Within a Leaf by the Dipteran Leafminer *Ophiomyia maura* (Diptera: Agromyzidae)

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**ABSTRACT**

Leaf mines exhibit complicated patterns with crosses and branch structures. Mining patterns are hypothesized to be a result of selective feeding on leaf areas that are nutritionally rich or that are poor in chemical and structural defenses (the selective feeding hypothesis). The hypothesis was tested using the leafminer *Ophiomyia maura* Meigen (Diptera: Agromyzidae) by examining leaf anatomy and nutritional content of the host plant *Aster microcephalus* (Miq.) Franch. et Savat. variety *ovatus* (Franch. et Savat.) Soejima et Mot. Ito (Asteraceae). *O. maura* shows a specific mining pattern by preferentially mining the marginal part of the leaf. Cross sections of mined leaves revealed that *O. maura* consumed a layer of palisade parenchyma cells. The mining site of *O. maura* was limited to a particular area by the midrib and lateral veins, but not by minor veins, because midrib and lateral veins have well developed parenchyma cells around the vascular bundles and interrupted the palisade layer. By mining at the marginal part of the leaf, *O. maura* avoided the midrib and lateral veins, or pinnate venation of *A. microcephalus*. The nitrogen content of the marginal part of the leaf was higher than that of the inner part of the leaf, which also contributed to *O. maura* mining the marginal part of the leaf. The specific mining pattern of *O. maura* demonstrated that the leafminer could have developed an adaptive life-history strategy by responding in a most efficient manner to the arrangement of veins and the nutritional variability even at a within-leaf scale, following the selective feeding hypothesis.

**KEY WORDS**

leaf anatomy, leafminer, leaf nutrition, mining pattern, *Ophiomyia maura*
An anatomical approach using transverse sections of mined leaves should reveal the tissue arrangement within a leaf and identify particular tissues that prevent larval feeding activity. A nutritional approach has commonly been used as a nutritional assay for mined and unmined parts of the same leaf. This approach is based on the premise that if leafminer larvae remove the most nutritive tissues of a leaf during mining, the nutritional quality of the mined part of the leaf will be lower than that of comparable unmined parts of the leaf (Scheirs et al. 2001). However, mined parts of a leaf may not necessarily be nutritionally better than unmined regions. Moreover, physiological characteristics of mined leaves might have changed due to the mining activity itself. Therefore, the results of such a nutritional assay using mined leaves need to be interpreted with caution (Scheirs et al. 2001). Another problem in detecting mining patterns is that in most of the leafminer species there is intraspecific variation in mine topology.

The purpose of this study was to test the selective feeding hypothesis and explain the mining pattern of the dipteran leafminer *Ophiomyia maura* Meigen (Diptera: Agromyzidae) with respect to the anatomy and nutritional content of the leaves of the host plant *Aster microcephalus* (Miq.) Franch. et Sav. variety *ovatus* (Franch. et Savat.) Soejima et Mot. Ito (Asteraceae) and thereby test whether the leafminer can respond to a difference in local nutrition status at a within-leaf scale, following the selective feeding hypothesis. Although there is some minor pattern variation among individuals, *O. maura* has a highly species-specific and predictable mining pattern. Larvae begin mining the upper leaf surface toward the base along the midrib, proceed through the marginal part of the leaf to the inner part of the leaf, where some individuals make crossed pattern mines (Fig. 1a). Mining at the marginal part of the leaf allowed 1) a comparison of the nutritional value of the marginal part as a selective feeding site and the inner part as a nonselective feeding site of a single intact leaf, and 2) analysis of the pattern or topology of mines. Thus, the selective feeding hypothesis could be tested using *O. maura*. Here, I investigated the anatomical characteristics with transverse sections of intact leaves and specified the tissues that could affect larval feeding activity by making a careful examination of transverse sections of mined leaves. Then, I compared water and nitrogen contents, which are the critical nutritive components for insect herbivores (Scriber and Slansky 1981, Awmack and Leather 2002), of the marginal part of the leaf with those of the inner part of the leaf. Finally, the adaptive significance of feeding on the leaf margin and the specific mining pattern in *O. maura* are discussed.

**Materials and Methods**

**Insects.** *O. maura*, ~1.5–2.5 mm in adult body length, is multivoltine and forms its mines in leaves of asteraceous plants, such as *Erigeron annuus* (L.) Pers., *Solidago virga-aurea* L. variety *asiatica*, and *Aster* *vo-

[Fig. 1. Mining pattern of *O. maura* (a) and pinnate leaf venation of its host plant *A. microcephalus* (b). (a) *O. maura* shows a specific mining pattern that exactly traces the edge of the leaf after mining toward the base of the leaf along the midrib. Pupation occurs at the end of the mine. (b) A midrib runs in the center of the lamina, and several pairs of lateral veins branch from the midrib toward the edge. Minor veins are not represented, but form a continuous mesh in the lamina. Leaf anatomy was investigated at several different locations in longitudinal direction within a leaf and the inner versus the marginal part (see text for details). In nutrient assays, sample leaves were cut into the inner and marginal (shaded area) parts (see text for details). (Online figure in color.)]
Sampling Protocol. Sample leaves were collected in Okazaki City, Aichi Prefecture, Japan (34° 57’ N, 137° 10’ W). The site was ≈8 by 10 m² and dominated by A. microcephalus, which begins growing in early May, blooms in late October, and drops lower leaves as it develops at the site (Ayabe and Shibata 2008). A. microcephalus has long oval, serrated leaves with a pinnate venation that consists of a central vein running from the stem to the tip (i.e., midrib) and lateral veins branching from the midrib (Fig. 1b). Minor veins form a continuous mesh in the remaining lamina. O. maura shows preference in resource utilization at a within-plant scale. The larvae preferentially use upper positioned leaves in which the mean vertical position is 7.85th downward from the top youngest leaf of each plant (Ayabe and Shibata 2008). Therefore, sampling was performed for leaves around seventh in a downward position from the top leaf.

Leaf and Mine Anatomy. Intact leaves and leaves with empty mines due to adult eclosion were collected in 27 June 2007 (but only three sample leaves with empty mines were also collected in 12 July 2007 due to failure in sample handling in June), avoiding repeated sampling from the same plant. Both intact and mined leaves (n = 20 for each) were cut into small pieces at the leaf base, at one fourth, one half, and three fourths of the way along the leaf, and at the leaf tip (Fig. 1b). These leaf pieces were fixed in Formaldehyde–acetic acid–alcohol, which contained 5% (vol:vol) acetic acid, 45% (vol:vol) ethanol, and 5% (vol:vol) formaldehyde. They were dehydrated in a graded ethanol series at room temperature (≈18–25°C) and then embedded with Technovit 7100 (Kulzer and Co. GmbH, Wehrheim, Germany). Sections (10 μm in thickness) were stained with 0.5% (wt:vol) safranin, which colors all sclerified tissues red.

The anatomy of the intact leaves of A. microcephalus was investigated with respect to whether anatomical characteristics varied with location within a leaf. For each section of five different locations along the leaf (Fig. 1b), I measured 1) the distance between the upper and lower epidermis (i.e., leaf thickness), 2) thickness of palisade tissues as resources consumed by miner larvae (see Results), and 3) vascular bundle diameter of veins (midribs, lateral and minor veins). For the sections one fourth, one half, and three fourths of the way along the leaf, leaf and palisade thicknesses also were measured at two latitudinally different locations: a middle point between the midrib and a adjacent lateral vein (i.e., the inner part), and a point in the vicinity of the leaf edge (i.e., the marginal part) (Fig. 1b). Because the sections of the base and the tip of the leaf corresponded to the marginal parts of the leaves, measurements were performed only at a point in the vicinity of the leaf edge (Fig. 1b). The size of lateral veins was determined for the lateral veins that branched from the nearest base of the leaf, and the size of minor veins was determined for one minor vein that was selected randomly.

For each section from the different positions within the mined leaves, I observed all of the mine views in the section, measured the horizontal width of mines as an index of miner body size, and recorded whether veins were consumed or not, and if consumption occurred, 1) the types of the consumed veins, i.e., midrib, lateral and minor veins; 2) details of the consumed parts in the vein, e.g., xylem and phloem tracts; and 3) percentages of the consumed area to the whole vein area. Thus, I evaluated how mining behavior (i.e., mining preference and avoidance for specific tissues) could be affected by both leaf anatomy and degree of each leafminer development represented with the mine size.

Leaf Quality. Leaf quality was evaluated for water and nitrogen content from the inner and marginal parts of leaves. Intact leaves (n = 100) were transported on ice to the laboratory in August 2006. Sample leaves were severed at 2 mm inside along the leaf edge on ice, and separated into the inner part and the marginal part. The marginal part of 2 mm width corresponds to the width of O. maura mine. These severed portions of the leaves were then lyophilized at −30°C for 48 h and ground. For both the inner and marginal parts of 20 leaves, I weighed the fresh mass before lyophilization and thereafter dry mass to determine water content as follows; ([fresh mass – dry mass]/fresh mass) × 100 (%). Dry masses of both the inner and marginal parts of the leaves were ≈6–18 mg and small to be analyzed. Therefore, the severed marginal parts of two to three leaves were combined together as one sample, and the corresponding inner parts of the same leaves also were combined as one sample. For the samples of 20 inner parts and 20 marginal parts, the nitrogen content was determined with a C-N analyzer (JM1000CN, J-SCIENCE LAB Co., Ltd., Kyoto, Japan). Defensive compounds in leaves were not measured in this study because A. microcephalus did not contain tannic acid, one of the most common defensive compounds (unpublished data).

Statistical Analysis. A generalized linear mixed model (GLMM) with a normal distribution was used to investigate whether leaf anatomical characteristics varied among different locations in the leaf. The thickness of the leaf, the thickness of the palisade tissue, and the size of the veins were each treated as a dependent variable. Because of leaf segmentation, individual leaves were treated as a random effect, and different locations in a longitudinal or latitudinal (the inner versus the marginal parts) direction within a leaf were treated as fixed effects in the model. To test whether development of leafminers can affect the likelihood that they consume a vein, a generalized linear mixed model with a binomial distribution and a logit link function was used. The dependent variable was whether the vein was damaged (1) or not (0), and the independent variable was the mine width as an index of the degree of development of the miners. The random effect was miner individuals. These GLMMs were fitted using the maximum likelihood method.

In the analyses of tissue quality, water content was arcsine-transformed to meet parametric assumptions of analysis of variance (ANOVA). The water and nitrogen contents of the inner and marginal parts of a
leaf were compared using a repeated measures ANOVA to take the paired nature of the data into account. All statistical tests in the current study were performed with Stata/SE 9.0 (StataCorp 2005).

Results

Leaf and Mine Anatomy. *A. microcephalus* leaves consist of a single layer of upper epidermis, a single layer of palisade parenchyma cells, about two layers of spongy parenchyma cells, and a single layer of lower epidermis (Fig. 2a). Across different locations within a leaf, the thickness (mean ± SD) of the leaf and palisade tissues was 72.8 ± 11.8 and 29.5 ± 7.3 μm, respectively. Both thicknesses varied significantly in latitudinal direction within a leaf but not in longitudinal direction; leaf and palisade tissue of the marginal part of the leaf was significantly thinner those of the inner part (Table 1; GLMM; n of level 1 and 2 U = 160 and 20, respectively; variance and covariance of random effect 20.92 and 11.17 for leaf thickness; variance and covariance of random effect 7.74 and 4.24 for palisade thickness).

Vein architectures were different among vein types. Vascular bundles of the midrib and lateral veins were surrounded by well-developed nonphotosynthetic parenchyma cells (Fig. 2b and c). These cells around the vascular bundles extended to the upper and lower epidermis, interrupting the palisade tissue at the midrib and lateral veins. Vascular bundles of the minor veins were surrounded only by a layer of parenchyma cells (Fig. 2a). Therefore, the palisade tissue was not interrupted by minor veins. These architectural differences among vein types were consistent throughout all longitudinal positions of a leaf. The size of the vascular bundle was different among vein types and was the largest in the midrib, followed by the lateral veins and minor veins (Fig. 3). In all vein types, the size of the vascular bundles decreased significantly in longitudinal direction from the base toward the tip of a leaf (Fig. 3, GLMM; n of level 1 and 2 U = 100 and 20, respectively; variance and covariance of random effect = 37.41 and 46.54; Z = −17.58; P < 0.0001 for midribs; n of level 1 and 2 U = 61 and 20, respectively; variance and covariance of random effect = 98.48 and 52.17; Z = −10.23; P < 0.0001 for lateral veins; n of level 1 and 2 U = 67 and 15, respectively; variance and

| Table 1. Mixed model regression analyses of the leaf anatomy in *A. microcephalus* (n = 160) |
|---------------------------------------------------------------|
| Response variable | Effect      | Coefficient ± SE | Z    | P > z |
| Leaf thickness    | Longitudinal | −1.44 ± 2.72     | −2.60| 0.598 |
|                   | Latitudinal  | −4.49 ± 1.72     | −2.60| 0.009 |
| Palisade thickness| Longitudinal | −0.85 ± 1.66     | −0.53| 0.597 |
|                   | Latitudinal  | −4.42 ± 1.05     | −4.22| 0.000 |

*a* Longitudinal locations within a leaf were treated with dummy variables; the location of the leaf base was 0, the leaf tip was 1, and the remaining locations at 1/4, 1/2, and 3/4 of the leaf length were transformed to 0.25, 0.5, and 0.75, respectively.

*b* Latitudinal locations was a dummy variable; the inner part of the leaf was 0 and the marginal part of the leaf was 1.

Fig. 2. Cross sections of leaves of *A. microcephalus*. (a) Leaf anatomy three-fourths of the way from the base along a leaf. (b) Midrib at the leaf base. (c) Mine and a lateral vein mid-way along a leaf. (d) Impaired midrib mid-way along a leaf. Scale bar = 100 μm.
covariance of random effect = 80.36 and 15.55; Z = −2.25; P = 0.024 for minor veins). The size reduction in the distal minor vein (coefficient ± SE = −6.86 ± 3.05) was small compared with that of the midribs (−111.67 ± 6.35) and lateral veins (−77.72 ± 7.60).

I examined 296 mine views for 20 mined leaves. *O. maura* consumed only palisade tissue. Among almost all of the mines viewed (n = 278), mines were frequently formed adjacent to intact midrib and lateral veins and overlaid minor veins (Fig. 2c), clearly showing that the midribs and lateral veins only were barriers to mining sites. A small fraction of the mines (n = 18) were found to impair veins. Only parenchyma cells were impaired by larvae, whereas vascular bundles (vessels and sieve tubes) were left intact (Fig. 2d). Impaired parenchyma cells accounted for 5.6 ± 2.3% (mean ± SD; n = 18) of the area of the veins. Impaired veins were five midribs, nine lateral veins, and four minor veins, indicating that there was no relationship between the types of veins, i.e., vein size (Fig. 3), and likelihood of them being consumed. By contrast, as mine size increased, veins were impaired more frequently (GLMM; n of level 1 and 2 U = 296 and 20, respectively; variance and covariance of random effect = 6.62 × 10−22 and 2.48 × 10−11; coefficient ± SE = 0.0018 ± 0.0005; Z = 3.96; P < 0.0001). In conclusion, *O. maura* larvae consumed a layer of palisade cells avoiding veins, but they rarely impaired parenchyma cells around the vascular bundles.

**Tissue Quality Between the Inner Versus the Marginal Parts of the Leaves.** Both water and nitrogen content was significantly different between the inner part and the marginal part of the leaf. Water content was higher in the inner part (mean ± SD = 73.0 ± 3.0% fresh mass) than in the marginal part (71.0 ± 3.5%) (F = 39.99; df = 1, 19; P < 0.0001), whereas nitrogen content was higher in the marginal part (mean ± SD = 31.0 ± 1.1 mg g⁻¹ dry mass) than in the inner part (30.27 ± 0.93 mg g⁻¹ dry mass) (F = 19.59; df = 1, 19; P = 0.0003).

**Discussion**

The effects of leaf anatomical and nutritional traits on mining design of leaf-miners have only been demonstrated for leafminer species that use monocotyledons as host plants (Scheirs et al. 1997, 2001). In the current study, the feeding site of *O. maura* within a leaf of the dicotyledon was also related to both anatomical characteristics and local nutritional quality of a leaf. Thus, the adaptive significance of the mining pattern as a result of selective feeding was supported in leaf-miners using not only monocotyledons but also dicotyledons.

*O. maura* consumed only a single layer of palisade cells, which is very similar to that of the dipteran leafminer *Liriomyza trifolii* Burgess in *chrysanthemum. Chrysanthemum indicum* L., leaves (Parrella et al. 1985). Although the likelihood of veins being impaired increased with larval body size, the frequency and area of veins impaired by larvae to the whole vein area were small. These results clearly indicate the unsuitability of veins as a food resource for miner larvae, which is consistent with the results of those previous studies showing that many other species of leaf-miners avoid veins (Kimmerer and Potter 1987 Stiling et al. 1987, Trier and Mattson 1997, Scheirs et al. 1997, 2001). In general, veins are highly sclerotized by lignin, which is a structural defense compound (Choong 1996) that causes a reduction of the feeding rate of larvae (Scheirs et al. 2001). In addition, the nutritional contents of the vein are lower than those of other leaf tissues (Kimmerer and Potter 1987, Scheirs et al. 2001). It is most likely that such characteristics of veins are involved in the avoidance of vein consumption by *O. maura*. When *O. maura* mines ran to the midribs or lateral veins of *A. microcephalus*, only parenchyma cells were damaged by the feeding activity. Parenchyma cells of *A. microcephalus* are not sclerotized, but their nutritional content is low and may be consumed by larvae only when they have to mine across veins to move toward the leaf base in early larval stages and to feed on in the inner part in later larval stages (Fig. 1b). Contrary to parenchyma cells, vascular bundles such as xylem and phloem were not impaired. Leaves with damaged vascular bundles may be disadvantageous as a food resource for *O. maura* larvae and in addition be an unsuitable habitat for pupae to reside in. Damage to midribs by insect herbivores can cause physiological changes such as gas exchange parameters and photosynthetic rates to leaves (Delaney and Higley 2006, Sack et al. 2008), while deactivating the chemical defenses of the host plant (Helmus and Dussourd 2005, Delaney and Higley 2006). Partial feeding on parenchyma cells in veins by *O. maura* larvae may have developed to avoid such physiological changes in the leaves, rather than to avoid inducing plant defense. *A. microcephalus* does not have tannic acid that is one of major defensive chemicals (unpublished data). Further studies on other defensive chemicals are required to clarify how *A. microcephalus* physiologically responds to serious vein damage.

The present results suggest that the three types of vein, midribs, lateral and minor veins would have had different effects on the formation pattern of *O. maura* mines. Larval feeding site was confined by midrib and...
lateral veins but not by minor veins. This is probably because parenchyma cells around the midribs and lateral veins developed to the upper and the lower epidermis to interrupt the palisade mesophyll, whereas parenchyma cells around minor veins developed only a single layer and did not interrupt the palisade mesophyll. Thus, the mining pattern of O. maura is influenced by the arrangement of the midrib and lateral veins (i.e., pinnate venation of A. microcephalus) only. Because lateral veins bend toward the leaf tip and do not reach the edge of the leaf (Fig. 1b), feeding on the marginal part of the leaf may enable O. maura larvae to avoid the midribs and lateral veins. Feeding on the leaf margin was not associated with the demand for space because both leaf and palisade thicknesses of A. microcephalus were less at the marginal part of the leaf when compared with the inner part of the leaf.

Feeding on the leaf margin by O. maura also could be explained in the context of nutritional advantage. A. microcephalus has higher nitrogen content in the leaf margin than in the inner part of the lamina, indicating that selective feeding on the margin of the leaf is nutritionally advantageous as several leafminer species improve the survival and growth rate during the immature stages with higher nitrogen content (Minkenberg and Ottenheim 1990, Facknath and Lalljee 2005). However, it is still unclear why O. maura prefers the marginal part of lower water content than the inner part. In some leafminer species, ovipositional and feeding preferences and larval performance depend on the nutritional variability even at a within-leaf scale, following the selective feeding hypothesis.

Many previous studies have demonstrated that the variations of resource quality such as nutrition and defensive components at host plant- and unit leaf scales could affect abundance and distribution of insect herbivores, including leafminers (Daugherty et al. 2007, Cornelissen and Stiling 2008, Van Hezewijk et al. 2008). The specific mining pattern of O. maura in this study demonstrated that the leafminer might have developed its life-history strategy to increase fitness by responding in a most efficient manner to the arrangement of veins and nutritional variability even at a within-leaf scale, following the selective feeding hypothesis.

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