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Rearing *Theretra oldenlandiae* (Lepidoptera: Sphingidae) Larvae on an Artificial Diet

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Abstract

The hawk moth *Theretra oldenlandiae* (Fabricius) is an important insect pest because in the larval stage it feeds on agricultural crops and ornamental plants such as the eddoe and garden balsam. In this study, we established methods for rearing *T. oldenlandiae* in the laboratory using an artificial diet containing dry powder of a wild grass *Cayratia japonica* (Thunb.) Gagnep. Several artificial diets were tested with different ratios of a commercial diet, Insecta LFM, and the dry leaf powder, and including different antibiotics, and the composition of the standard diet on which larvae performed best was determined. The standard diet contains 20 g of Insecta LFM, 4 g of leaf powder, 100 ml of water, 75 mg of chloramphenicol, and 200 μl of propionic acid. Larvae reared on the standard diet became larger pupae than those reared on *C. japonica* leaves. This result suggests that the larvae have growth potential that is masked on *C. japonica* leaves, and that *C. japonica* may not be the most suitable host species for *T. oldenlandiae* larvae in terms of nutrient level.

The hawk moth *Theretra oldenlandiae* (Fabricius) is distributed in southern to eastern Asia, including Japan (Sambath 2011, Rafi et al. 2014) and Oceania (Rougerie et al. 2014), and is an important insect pest that feeds on agricultural crops and ornamental plants such as eddoe *Colocasia esculenta* (L.) Schott, grape *Vitis* spp, and garden balsam *Impatiens balsamina* (L.) in the larval stage (Okada 2003, Japanese Society of Applied Entomology and Zoology 2006). As in some other hawk moths, larvae of this species consume large amounts of host leaves and grow to a body weight of 7 g. In particular, eddoe is one of the main agricultural products in southern Kyushu, and thus effective methods for control of *T. oldenlandiae* larvae are needed. However, there is little ecophysiological information on this moth. The aim of this study was to establish methods for rearing the moth on an artificial diet.

Larvae of *T. oldenlandiae* can be reared on fresh host leaves in the laboratory, but this method requires a large number of host leaves: Even if host leaves are stuffed into a 430-ml plastic cup for one larva, the food resources are exhausted within 2 d in the midst of the final (fifth) instar when the larval body weight approaches the maximum. Thus, rearing on fresh leaves also needs much space and labor. Furthermore, we observed that rearing larvae with field-collected leaves sometimes results in parasitism by a fly *Sturmia bella* (Meigen), because female adults of this fly lay very small eggs on plant leaves, and the eggs may be swallowed by *T. oldenlandiae* larvae through feeding (Hirai and Ishii 1995). For these reasons, it is not feasible to mass-rear *T. oldenlandiae* larvae on host leaves.

In Sphingidae, rearing methods for larvae of *Manduca sexta* (L.) (Yamamoto 1969) and *Agrius convolvuli* (L.) (Kiguchi and Shimoda 1994) have been established to utilize them as common experimental insects. In the method for *A. convolvuli*, dry leaf powder of the host (the sweet potato *Ipomoea batatas* (L.) Lam.) is used as an ingredient of the artificial diet. Thus, rearing *T. oldenlandiae* larvae on an artificial diet may also be possible.

Larvae of *T. oldenlandiae* also feed on the bushkiller *Cayratia japonica* (Thunb.) Gagnep. This wild grass grows thickly from spring to autumn on vacant land, roadsides, margins of forests, and other places where land management is neglected. In this study, we established a method for rearing *T. oldenlandiae* larvae on an artificial diet, including dry powder of this easily available host plant.

Materials and Methods

Rearing of Insects

A laboratory population of *T. oldenlandiae* was established from ca. 100 eggs collected from *C. japonica* grown around Miyakonojo Campus of Minami Kyushu University, Miyakonojo (31°44′N, 131°06′E, ca. 160 m altitude), Miyazaki Prefecture, Japan, in August 2017, and was kept for successive generations in the laboratory. The larvae of the laboratory first generation were reared on fresh leaves of *C. japonica*. Larvae of following generations were reared on standard diet (as described below). The resulting pupae were kept at 15°C to retard pupal development until use. Newly eclosed adults

of *T. oldenlandiae* were confined in a cardboard box (45-cm width × 35-cm depth × 150-cm height) with windows of transparent plastic boards. The box was kept at 23 ± 2°C under an artificial light condition that was not precisely controlled. Pupae were transferred to the box from the 15°C stock every 3 d to keep the number of adults in the box at ca. 10–20. For provision of food, a small Petri dish (6 cm in diameter, 1.5 cm in depth) filled with honey water (ca. 20% v/v) was suspended from the ceiling of the box with a wire at a height of 130 cm. To allow the female adults to lay eggs, foliage of *C. japonica* arranged in a bottle filled with water was placed on the bottom of the box. Eggs laid on the leaves were collected carefully, placed on a moist paper towel in a large Petri dish (9 cm in diameter, 2 cm in depth), and kept at 25 ± 1°C under a photo regime of 16:8 (L:D) h until hatching. More than 200 eggs were obtained per day.

Diet Ingredients

Insecta LFM (dry powder type, Nosan Co., Yokohama, Japan) and dry leaf powder were the major solid components of the diet (Table 1). Insecta is a commercial diet used for a wide range of phytophagous insects (e.g., common cutworm, legume pod borer, carpenter moth, and yellow-spotted longicorn beetle) without admixture (Kudo et al. 2014; Nagamine et al. 2016, 2017; Nakanishi et al. 2017; Nakano et al. 2018), and is also used for oligophagous insects (e.g., fungivorous moth, swallowtail butterfly, and chrysanthemum longicorn beetle) with host powder (Kitajima 2013, Nishikawa et al. 2013, Shintani 2011). To prepare leaf powder of *C. japonica*, foliage was cut from around the campus and the leaves were dried naturally in the laboratory for at least 5 d before being pulverized with an electric blender. Chloramphenicol and propionic acid (Nacalai Tesque, Kyoto, Japan) were added as antibacterial and antifungal agents, respectively.

Preparation of a Standard Diet

A 'standard diet' (Table 1) was prepared from Insecta LFM, dry leaf powder, and water that were mixed thoroughly in a stainless steel container (20 × 14 × 7.5 cm) and steamed for 20 min. After the temperature of the mixture dropped to below 60°C, 75 mg of chloramphenicol dissolved in 750 µl of 100% ethanol, and 200 µl of propionic acid were added to the mixture. This standard diet could be stored for 30 d at 10°C.

Larval Culture

Individuals in the second and third generations of the laboratory population were used for this study. In all experiments, larvae were reared at 25 ± 1°C under 16:8 (L:D) h until pupation and all manipulations were conducted within 1–3 h after light on. Larvae that hatched within 24 h were assigned to either of two food groups: host leaf or standard diet: Leaves of the host plant (*C. japonica*) or standard diet were supplied throughout the larval stage. Thirty neonate larvae were placed in a large Petri dish and were provided with 5 g of young host leaves or 10 g of standard diet. In the standard

diet group, sliced pieces of standard diet (5 g in total) were attached to the underside of lids and the bottom of Petri dishes (Supp. Fig. S1). In *T. oldenlandiae*, ecdysis to the next instar and head capsule slippage (HCS) occur about 4–10 h after light on and about 12 h before ecdysis, respectively. Larvae that attained second head capsule slippage (2HCS) by day 6 from hatching were counted as surviving individuals. At 2HCS, larvae were weighed and transferred to new small Petri dishes individually. Larvae that did not attain 2HCS by day 6 were regarded as developmentally abnormal because almost all larvae reared on host leaves reached 2HCS by day 6. Third-instar larvae were supplied with approximately 3 g of mature host leaves or 5 g of standard diet. Each larva was individually transferred to a plastic cup (430 ml) at 3HCS and reared in this cup until pupation. A larva was supplied with 20 g of mature host leaves or 15 g of standard diet on the first day of the fourth instar, and on the first and fifth days of the fifth instar. Larvae spun cocoons in the leftover food and then pupated in the cocoons. Larval development was observed daily to record the dates and body weights at 2HCS, 3HCS, 4HCS, and pupation. The details on dietary manipulation were described in Results and Discussion.

Pupal Culture

Pupae were sexed by the morphology of the abdomen tip and transferred individually to small Petri dishes in which a moist paper towel was placed. Pupae were kept at 25 ± 1°C under 16:8 (L:D) h for observation of pupal development. The dates of adult eclosion were recorded.

Statistical Analysis

R was used for statistical analyses, mostly using R commander (Fox 2005, R core team 2017) and the means and proportions were compared by *t*-test and Fisher exact test, respectively.

Results and Discussion

Composition of the Standard Diet

The major solid components of the standard diet were Insecta LFM and leaf powder of *C. japonica* (Table 1), with the leaf powder probably providing the feeding stimulant. We tested other artificial diets with several different ratios of Insecta LFM and leaf powder, and different antibiotics. The selected standard diet gave the highest survival rate and growth increment among these test diets (Supp. Fig. S2).

Table 1. Components of the standard diet

Ingredients	Amount
Insecta (g)	20
Leaf powder (g)	4
Water (ml)	100
Chloramphenicol (mg)	75
Propionic acid (µl)	200

Table 2. Effects of food resources on the performance of early-instar larvae of *Theretra oldenlandiae*

Food resources	Survival rate (%)[a,b]	Weight at 2HCS (mg)[c,d]
Host leaf	70.0 ($n = 90$)	45.4 ± 1.2 ($n = 58$)[e]
Standard diet	56.7 ($n = 90$)	55.9 ± 1.6*** ($n = 51$)

Larvae were reared on *Cayratia japonica* leaves or standard diet from hatching.

[a]Fisher exact test.

[b]Survival rate = [(number of larvae that attained 2HCS by day 6 from hatching) / (number of larvae that hatched)] × 100.

[c]*t*-test.

[d]Mean ± SE.

[e]Data were not available for five individuals.

***$P < 0.001$: significant difference between food resources.

Survival Rate and Growth in Early-Instar Larvae

To examine the suitability of the standard diet as food for early-instar (first- and second-instar) larvae, the survival rate and growth were compared between the host leaf and standard diet groups (Table 2). The survival rate in early-instar larvae did not differ significantly between the food groups (Fisher exact test, $P > 0.05$). The larval weight at 2HCS was significantly higher in the standard diet group than in the host leaf group (*t*-test, $P < 0.001$). These results indicate that the standard diet provided the nutrients, water, and feeding stimulant required for normal development.

Survival Rate in Late-Instar Larvae

To examine the suitability of the standard diet as food for late-instar (third- to fifth-instar) larvae, survival rates were compared between the food groups (Table 3). In this experiment, larvae were fed host leaves until 2HCS, and then assigned to either of the two food groups. The survival rate through late instars was lower in the standard diet group than in the host leaf group (Fisher exact test, $P < 0.01$). There was a significant difference in the survival rate between the food groups for the third instar (Fisher exact test, $P < 0.001$), but not for the fourth or fifth instar. These results suggest that some third-instar larvae are not able to adjust to the food change from host leaves to standard diet. Thus, rearing on a standard diet throughout the larval stage may improve the survival rate in late instars.

Growth in Late-Instar Larvae

Comparison of larval growth trajectories between food groups revealed different patterns of growth (Fig. 1). The weight at 3HCS was significantly heavier in the host leaf group than in the standard diet group (*t*-test, $P < 0.001$), while there was no significant difference in weight at 2HCS (*t*-test, $P = 0.53$). The duration of the third instar (days from 2HCS to 3HCS) was significantly prolonged in the standard diet group (*t*-test, $P < 0.001$). The pupal weight in the standard diet group was significantly heavier than that in the host leaf group (*t*-test, $P < 0.001$), even though the weight at 4HCS was significantly lower in the standard diet group (*t*-test, $P < 0.01$). In contrast, the duration of the fifth instar did not differ significantly between the two groups (*t*-test, $P = 0.84$).

In *M. sexta*, larvae grown on an artificial diet ('naive larvae') are less sensitive to host-plant preference because of increased sensitivity to certain chemicals in non-host plants (Schoonhoven 1967). Moreover, naive larvae become less sensitive to deterrent chemicals through exposure to the chemical during larval development (Städler and Hanson 1978, Glendinning et al. 2001). In *T. oldenlandiae*, the growth in the third instar suggested that larvae transferred from the host leaf to a standard diet need time to increase sensitivity to certain chemicals and/or decrease sensitivity to deterrents. The growth trajectories in the fourth-instar larvae of *T. oldenlandiae* suggest probable loss of the food preference in the third instar. The fifth instar had a markedly higher performance with the standard diet, with a higher growth rate rather than longer growth period. The data distribution of pupal weight with the standard diet (3.0–4.1 g) did not

Table 3. Survival rates (%) in late instars of *Theretra oldenlandiae* larvae

Food resources	From third to fifth instars	Instar		
		Third	Fourth	Fifth
Host leaf	78.1 (*n* = 64)	93.8 (*n* = 64)	93.3 (*n* = 60)	87.5 (*n* = 58)
Standard diet	52.7** (*n* = 55)	70.9*** (*n* = 55)	89.7 (*n* = 39)	82.9 (*n* = 35)

**$P < 0.01$, ***$P < 0.001$: significant difference between food groups (Fisher exact test).
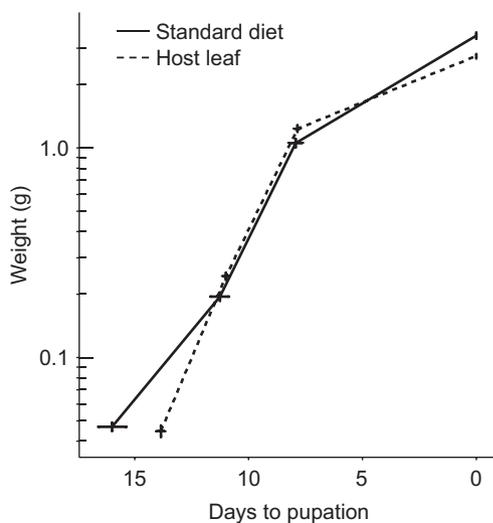

Fig. 1. Larval growth trajectories from the third instar in *Theretra oldenlandiae.* Weights at 2HCS, 3HCS, 4HCS, and pupation (within 24 h after pupation) are plotted as a function of development days and food resources. Insects were reared on *Cayratia japonica* leaves from hatching to 2HCS and then assigned to the host leaf or standard diet group. Each point is an average of 15 insects. Bars indicate the standard error.
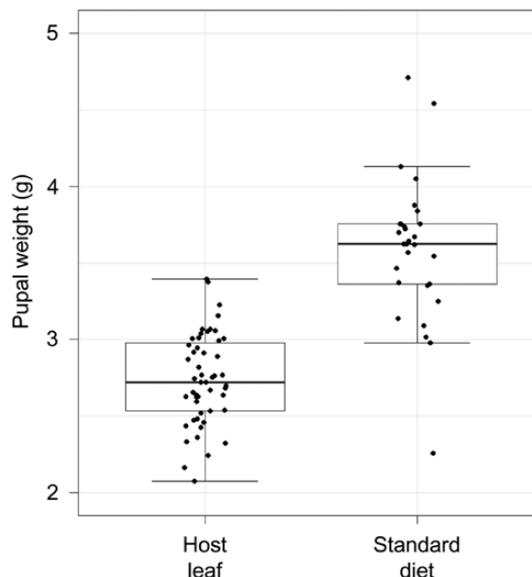

Fig. 2. Comparison of pupal weight ranges between food resources in the late-larval stage in *Theretra oldenlandiae*. Larvae were reared on *Cayratia japonica* leaves from hatching to 2HCS and then assigned to the host leaf or standard diet group.

Table 4. Effect of food resources in the larval stage on pupal development in *Theretra oldenlandiae*

Food resources	Pupal duration (days)[a,b]	Eclosion rate (%)[b,c,d]	Female rate (%)[d]
Host leaf	15.0 ± 0.4 (*n* = 45)	88.2 (*n* = 51)	58.8 (*n* = 51)
Standard diet	14.5 ± 0.2 (*n* = 26)	89.7 (*n* = 29)	58.6 (*n* = 29)

No significant difference between food groups in any column at $P = 0.05$ ([a]*t*-test, [d]Fisher exact test).
[b]Mean ± SE.
[c]Eclosion rate = [(number of adults that eclosed) / (number of pupae)] × 100.

fall within that in the host leaf (2.2–3.4 g) (Fig. 2) and the rages were similar. This suggests that it is unlikely that only the individuals that would grow relatively better in a population on host leaves survived to the pupal stage on the standard diet. That is, the standard diet brings generally better growth in the late-instar larvae. Taken together, the results show that *T. oldenlandiae* larvae have growth potential that is masked when the larvae are fed *C. japonica* leaves.

Cayratia japonica may not be the most nutritious host plant for *T. oldenlandiae* because larvae reared on the standard diet grew better than on *C. japonica* in the early- and late-larval stages. Many factors influence the suitability of a host plant for a herbivore insect: nutritional quality of the host plant (Dethier 1954, Ehrlich and Raven 1964, Levins and MacArthur 1969, Bernays and Chapman 1994), predation risk associated with a particular plant (Bernays and Graham 1988, Mira and Bernays 2002), availability and reliability throughout the season (Futuyma and Moreno 1988), and competition with other herbivorous species (Fritz 1992). In *M. sexta*, for instance, there is a trade-off in host use between nutritional quality and predation. Larvae have high growth with high risks of predation and parasitism on a typical solanaceous host, *Datura wrightii* (Regel), and low growth with low risk on a novel non-solanaceous host, *Proboscidea parviflora* (Woot.) Woot. & Standl. (Mira and Bernays 2002). Thus, it is possible that *C. japonica* is chosen by *T. oldenlandiae* as a result of such a trade-off, despite this host plant being less nutritious than some other host plants.

Development of Pupae and Fecundity of Adults

There were no significant differences in eclosion rate (Fisher exact test, $P > 0.05$), pupal duration (*t*-test, $P > 0.05$), and sex ratio (Fisher exact test, $P > 0.05$) between the food groups (Table 4). The eclosed adults were able to lay fertilized eggs (K.N., personal observation). These results suggest that provision of standard diet for the late-instar larval stage does not negatively affect pupal development and adult fecundity.

Future Problems

The growth increment and survival rate differed between experiments, even if larvae were fed the same diet (Standard diet in Table 1, Pro in Supp. Fig. S1f, and L4 in Supp. Fig. S1h). Considering that larvae from different generations and/or parents were used for these experiments, the results may be affected by genetic variation altering compatibility with the artificial diet. This hypothesis is supported by the gradual rise in survival rate in early-instar larvae of two further generations of the laboratory population (K.N., personal observation). Selection of such larvae will lead to establishment of a strain with stable growth and survival rates on an artificial diet.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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