The Effect of Combinations of Food Insects for Continuous Rearing of the Wing Polymorphic Water Strider Limnogonus Fossarum fossarum (Hemiptera: Gerridae)

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Abstract

The water strider Limnogonus fossarum fossarum (F.) (Hemiptera: Gerridae) shows a macropterous, micropterous, and apterous polymorphism. Although a long photoperiod condition induces winged morphs, preliminary studies have revealed that crossing between winged morphs increased the proportion of macropterous individuals, suggesting that the genetic factors also affect wing-morph determination in this species. Assessing the genetic backgrounds of wing polymorphism requires constant and repeatable methods for rearing. This study attempts to establish a continuous rearing method for L. f. fossarum under constant diet conditions. Initially, we maintain the water striders with two Drosophila species as a food, but viability until adulthood is less than 20%. We then add the storage pest Plodia interpunctella (Hübner), which are readily reared in the laboratory, to the diets. As a result, nymphs fed on P. interpunctella (even only until the second instar) show significantly higher viability and shorter developmental period than nymphs fed on Drosophila alone. Moreover, feeding on D. melanogaster (Meigen) reared on cholesterol-enriched medium instead of a normal medium significantly increases viability in the next generation. This means that only the two food-insect species are enough for establishing a substantial number of individuals in segregating generations (F2 and backcross), limiting DNA and RNA contaminations from food insects with genome information. Thus, the present rearing method opens the way to elucidating the genetic backgrounds of the wing polymorphism in L. f. fossarum.

Keywords: Drosophila melanogaster, Plodia interpunctella, cholesterol

Wing morphologies have diverged greatly among the insect species. In the course of the evolution of insects, some species have lost their wings secondarily, and others show wing polymorphisms within species (Roff 1990). Wing polymorphisms in insects generally affect their flight ability, resulting in differences in dispersal abilities among individuals with different wing morphs (Harrison 1980). Thus, investigating the mechanisms controlling wing polymorphism is crucial for understanding the evolution of life history (Zera and Denno 1997).

Both environmental and genetical factors influence the determination of wing morphs in insects (Roff 1986; Simpson et al. 2011). Environmentally determined wing polymorphisms are known as wing polyphenisms in which the same genotype produces discontinuous phenotypes (Braendle et al. 2006). Much study on wing polyphenism has been done using aphids and water striders and revealed that both biotic (e.g. density [Harada et al. 1997; Harada and Spence 2000], nutrient conditions [Harada and Nishimoto 2007] and interspecific interactions [Dixon and Agarwala 1999; Sloggett and Weisser 2002; Kunert and Weisser 2003]), and abiotic (e.g. photoperiod [Harada and Taneda 1989; Harada and Numata 1993; Inoue and Harada 1997; Harada 1998a; Harada et al. 2003; Harada and Numata 2011], temperature [Harada et al. 2003], salinity [Kishi et al. 2007], and dryness [Harada 1998b; Kishi et al. 2002]) environmental cues affect wing-morph determination (Braendle et al. 2006; Simpson et al. 2011). In contrast, several studies have shown that genetic factors contribute to wing-morph determination in water striders and plant hoppers as well as aphids (Zera et al. 1983; Mori and Nakasuji 1990; Matsumura 1996; Caillaud et al. 2002). Thus the genetic basis and maintenance mechanisms of genetically determined wing polymorphisms have received ongoing scrutiny (Harrison 1980; Roff 1986; Harada and Denno 1997). However, we know little about the genetic backgrounds underlying genetically determined wing polymorphisms.
The water strider Limnogomus fossarum (Hemiptera: Gerridae) is a tropical and subtropical species in the Old World, and the subspecies L. f. fossarum is distributed in the Oriental region (Andersen 1975). L. f. fossarum shows wing polymorphism that produces macropterous, micropterous, and apterous adults (Andersen 1982), and these morphs are easily distinguished by eye. Since L. f. fossarum does not have any dormancy periods in its life history and its generation time is relatively short (ca. 50 days, see Results section), more than five generations can be continuously reared in the laboratory per year. Therefore, L. f. fossarum could be a novel model system for studying what extent genetic and environmental factors influence the determination of wing morphs.

In this study, our goal is to establish a method for rearing L. f. fossarum successively in the laboratory under constant environmental conditions. Because environmental conditions influence wing-morph determination (Braendle et al. 2006; Simpson et al. 2011), establishing uniform environmental conditions for rearing is very important for distinguishing genetic contributions from environmental contributions to wing-morph determination. Of the biotic and abiotic conditions affecting wing-morph determination (Braendle et al. 2006; Simpson et al. 2011), nutrients are the most difficult condition to establish uniformly in water striders because rearing with only a single food-insect species often results in very low survival rates in their nymphal stage (for reviews, see Lefcheck et al. 2013; De Clercq et al. 2014). For this reason, we have to use multiple insect species to recover high survival rates in successive rearing of water striders, but this increases the complexity of contaminating DNA and RNA from the food-insect species and violates the trimming of the contaminating sequences in the step of genome-data analyses. Thus, food insect species should be those which genome information is available, and we should establish a method using as few food species as possible.

Here, we present a method for successive rearing of L. f. fossarum using two food insect species, the fruit fly Drosophila melanogaster (Diptera) and the Indian meal moth Plodia interpunctella (Lepidoptera), whose genome information is available (Adams et al. 2000; Harrison et al. 2012). The two food species are readily maintained in the laboratory and have short generation times. Our method uses a cholesterol-enriched medium for rearing D. melanogaster. This significantly reduces mortality of its predator, L. f. fossarum, and enables us to rear L. f. fossarum successively with only two food-insect species. Thus, our present rearing method opens the way to make L. f. fossarum a suitable system for studying the genetic mechanisms underlying wing polymorphisms.

Materials and Methods

Insects: Adults of L. f. fossarum (P generation) were collected from Ishigaki Island (24°22′-27″N, 124°9′-12″E), Okinawa Prefecture, Ryukyu Archipelago, Japan. Twenty-six females and 16 females were obtained on 3 April and 13 August 2012, respectively. Collected adult females were maintained with D. melanogaster as food and allowed to lay eggs at 25°C under a 16:8 (L:D) h photoperiod in the laboratory of Kyoto Prefectural University, and obtained eggs were used for the subsequent experiments. We also established a laboratory population from two females sampled in spring and 16 females sampled in summer 2012, and used these for the experiments.

As food insects, two species of Drosophila (D. melanogaster and D. hydei Stervetant) were maintained on a medium Jazz-mix (Fisher Scientific Inc., Tokyo, Japan) at 25°C under a 16:8 (L:D) h photoperiod in the laboratory. We maintained two wild types (Oregon-R and Canton-S) and two mutants, Curly (Cy) and vestigial (vg) of D. melanogaster, and randomly used these as food for L. f. fossarum. In addition to the two Drosophila species, P. interpunctella were collected near the campus of Kyoto Prefectural University (Shimogamo, Sakyo, Kyoto, Japan). Adult pairs of P. interpunctella were introduced to single clear plastic containers (135 × 85 × 30 mm) containing brown rice as larval food and maintained at 28°C under a 16:8 (L:D) h photoperiod in the laboratory. Emerged adults of all these food insects were killed by freezer and used as prey in experiments of food insect combinations.

General rearing method: Eggs from each collected wild female or the laboratory population were incubated on a water-soaked filter paper in plastic Petri dishes (52 mm diameter, 13 mm depth). Newly hatched nymphs were introduced to translucent plastic containers (60 mm × 60 mm × 45 mm) in groups of up to five individuals from the same parent. These nymphs were then transferred individually to other clear plastic containers (60 mm × 60 mm × 95 mm; Plant Box, #CUL-JAR300, AGC Techno Glass Co., Ltd., Tokyo, Japan) at the third or fourth instar depending on the subsequent experimental design. Each container was filled with 5 mm water that had been dechlorinated by being incubated over 24 h in the laboratory. A cut piece of extruded polystyrene plate was added on the water surface as a resting site. All food insects, polystyrene plates, and water in the rearing containers were replaced daily regardless of what food insects were provided to the water striders. Eggs and nymphs were maintained at 25°C under a 16:8 (L:D) h photoperiod in all experiments.

Food insect combinations: To assess effective food insect combinations for rearing L. f. fossarum successively, we estimated viability from F1 eggs to adults, fertility of F1 adults (i.e. germination rates of F2 eggs), hatchability of F2 nymphs, and viability of F2 nymphs until the second instar or adults with the following four food insect experiments. Detailed names of food insect species and the amount of each food insect used per day in each experiment are shown in Table 1.

Experiment 1: D. melanogaster and D. hydei. F1 offspring derived from 26 females from the spring samples (collected on 3 April 2012) were used in this experiment. Hatched F1 nymphs were fed on two species of Drosophila (Dm + Dh treatment, Table 1). F1 viability was estimated as the percentage survival of individuals until adulthood of all eggs obtained. F1 offspring which successfully survived to adulthood were crossed with other adults from the same parent (i.e., full-sib mating) and F2 eggs were obtained. Eyespots emerge 4 d after oviposition when eggs have been successfully germinated. Thus, we checked for the presence of eyespots on each egg 6 d after oviposition, and the presence of eyespots was regarded as an indicator of successful egg germination.

Experiment 2: P. interpunctella for 1st and 2nd instars in addition to D. melanogaster for all instars. F1 offspring from 16 females from the summer samples (collected on 13 August 2012) were used for this experiment. To assess the effect of adding P. interpunctella as a food insect, 20 eggs each from 16 females were split 1:1 into two food treatments. In the first treatment (Dm treatment), L. f. fossarum nymphs were fed only on D. melanogaster for all stages; in the second treatment (Dm + P1 1 treatment), the nymphs were fed daily, alternating between D. melanogaster and P. interpunctella, until the end of the second instar and then fed only on D. melanogaster until adulthood (Table 1). In each treatment, we estimated F1 viability as the mean percentage of individuals surviving until each instar or adulthood of 16 sibling groups and compared the viabilities of the two treatments in each stage. We also compared F1
Experiment 1: D. melanogaster and D. hydei, and Plodia interpunctella, respectively. Numbers after abbreviations indicate daily amounts of each food-insect species per L. f. fossarum larva. Under lines indicate periods in which L. f. fossarum were reared in groups.

Table 1. Species and amount of adult food insects fed to each stage of L. f. fossarum

| Rearing methods | 1st | 2nd | 3rd | 4th | 5th | Adult |
|------------------|-----|-----|-----|-----|-----|-------|
| Experiment 1     | Dm+Dh | Dm 1| Dm 2| Dm 3| Dh 1| Dh 3 |
| Experiment 2     | Dm   | Dm 1| Dm 2| Dm 3| Dm 3| Dm 4 |
| Experiment 3     | Dm+Pi1, 2, 3 | Dm 1, Pi 1/3| Dm 2, Pi 1/2| Dm 3, Pi 1| Dm 3| Dm 4 |
| Experiment 4     | Dm+Pi1, 2, 5 | Dm 1, Pi 1/3| Dm 2, Pi 1/2| Dm 3| Dm 3| Dm 4 |

Table 2. Ingredients in the cholesterol-added medium for Drosophila melanogaster in the Experiment 4 (amounts per 100 culture bottles)

| Material          | Amount |
|-------------------|--------|
| Medium (Jazz-mix)* | 68 g   |
| Cholesterolb      | 0.36 g |
| Distilled water   | 340 ml |

*a Fisher Scientific Inc., Illinois, USA.
bWako Pure Chemical Industries, Ltd., Osaka, Japan.

dm, Dh, and Pi indicate Drosophila melanogaster, D. hydei, and Plodia interpunctella, respectively.

Results

Experiment 1: D. melanogaster and D. hydei: Of 658 F1 eggs, only 102 individuals survived until adulthood (viability 15.5%). Although 1218 F2 eggs were laid by 14 F1 females, there were no fertile eggs under these food conditions. Thus, we could not assess the hatchability and viability of the F2 generation, suggesting that we could not rear L. f. fossarum continuously using only Drosophila species as food insects.

Experiment 2: P. interpunctella for 1st and 2nd instars in addition to D. melanogaster for all instars: In the Dm+Pi1, 2 treatment, over 70% of individuals successfully survived to adulthood (Fig. 1). In the Dm treatment, on the other hand, viability decreased significantly after the fourth instar mainly due to failure to molt, and in 10 of 16 sibling groups, no individuals survived until adulthood. Nymphal viability was significantly higher in the Dm+Pi1, 2 treatment than in the Dm treatment starting in the fourth instar (Fig. 1; until second instar t = 0.206, P = 0.839; until third instar t = 0.124, P = 0.902; until fourth instar t = 2.33, P = 0.0270; until fifth instar t = 6.25, P = 6.97e−7; until adulthood t = 11.8, p = 8.52e−13; df = 30 for all t-tests).

The mean developmental period was 27.6 ± 1.62 d (± SD, N = 17) in the Dm+Pi1, 2 treatment, whereas it was 29.6 ± 2.43 d (± SD, N = 7) in the Dm treatment; there was a significant difference between the two treatments (t = −2.35, P = 0.0279, df = 22, t-test).

In the Dm+Pi1, 2 treatment, although the mean fertility of F1 adults was above 90%, in the subsequent F2 generation, mean hatchability and viability until the second instar decreased to
53.9 ± 40.5% and 0.52 ± 1.04%, respectively (± SD). In the Dm treatment, no fertile eggs were obtained as in Experiment 1, although a single F1 female started oviposition.

Experiment 3: P. interpunctella for third or fifth instars in addition to the dietary conditions from Experiment 2. F1 viabilities until adulthood were high (Dm + Pi1, 2, 3 = 80.0%, Dm + Pi1, 2, 5 = 83.3%) in both treatments, and there were no significant differences between the two treatments in viabilities until each instar (until second instar \( t = -0.202, P = 0.844 \); until third instar \( t = 0.483, P = 0.639 \); until fourth instar \( t = 0.661, P = 0.524 \); until fifth instar \( t = 0.788, P = 0.449 \); df = 10 for all tests; t-test) as well as until adult (\( t = 0.479, P = 0.642, \text{df} = 10, \text{t-test} \) ) (Supp Fig. 1 [online only]). The mean developmental period was 27.6 ± 0.58 d (± SD, \( N = 6 \)) in the Dm + Pi1, 2, s treatment and 28.3 ± 0.47 d (± SD, \( N = 6 \)) in the Dm + Pi1, 2, 5 treatment; there was no significant difference between the two treatments (\( t = -2.08, P = 0.0644, \text{df} = 10, \text{t-test} \) ). There was no significant difference in fertility between the two treatments (Fig. 2; \( t = 2.01, P = 0.0721, \text{df} = 10, \text{t-test} \) ).

In F2 generation, however, a substantial number of offspring failed to hatch and almost all F2 first instar nymphs did not reach the second stadium in the Dm + Pi1, 2, 3 treatment (Fig. 2). On the contrary, in the Dm + Pi1, 2, 5 treatment, mean F2 hatchability and viability until the second instar were 89.3% and 84.7%, respectively (Fig. 2); there were significant differences in hatchability and viability between the two treatments (hatchability \( t = 3.42, P = 0.00653, \text{viability until second instar } t = 8.89, P = 4.62e-6, \text{df} = 10, \text{t-test} \) ). The mean percentile viability until adulthood in the Dm + Pi1, 2, 5 treatment was 53.0 ± 23.6 (± SD, \( N = 4 \)).

Experiment 4: D. melanogaster reared on cholesterol-added medium for all instars and P. interpunctella for first, second, and fifth instars: F1 viabilities until adulthood were not significantly differentiated between the two treatments (Dm + Pi1, 2, s = 70.0%, DmC + Pi1, 2, s = 74.0%; \( t = 0.410, P = 0.693, \text{df} = 8, \text{t-test} \) ) as in Experiment 3 (Supp Fig. 2 [online only]). The mean developmental period was 28.6 ± 0.49 d (± SD, \( N = 5 \)) in the Dm + Pi1, 2, s treatment and 28.3 ± 0.74 d (± SD, \( N = 5 \)) in the DmC + Pi1, 2, s treatment; there was no significant difference between the two treatments (\( t = -0.15, P = 0.533, \text{df} = 8, \text{t-test} \) ). There was no significant difference in fertility between the two treatments (Fig. 3; \( t = -0.7244, P = 0.490, \text{df} = 8, \text{t-test} \) ).

In F2 generation, hatchability and viability until each instar and adulthood are consistently higher in the DmC + Pi1, 2, s treatment than in the Dm + Pi1, 2, s treatment, although significant differences between the two treatments are detected in the viability until adulthood alone (Fig. 3; hatchability \( t = 1.003, P = 0.346, \text{df} = 7.825, \text{viability until second instar } t = -1.727, P = 0.146, \text{df} = 4.891, \text{until third instar } t = -1.357, P = 0.243, \text{df} = 4.183; \text{until fourth instar } t = -1.836, P = 0.233, \text{df} = 4.352; \text{fifth instar } t = -2.353, P = 0.0515, \text{df} = 6.877; \text{until adulthood } t = -2.484, P = 0.0381, \text{df} = 7.935; \text{Welch's t-test} \) ). There was no significant difference in the mean larval developmental period between the DmC + Pi1, 2, s treatment (42.4 ± 2.54 d (± SD, \( N = 5 \))) and the Dm + Pi1, 2, s treatment (42.2 ± 2.36 d (± SD, \( N = 3 \))) (\( t = 1.00, P = 0.355, \text{df} = 6, \text{t-test} \) ).

Effect of food-insect combinations on wing morphs: Model comparison indicated that the model consisting of only the food-combination effect was selected as the best fit model in Experiments 2 and 4 (Supp Table S1 [online only]), whereas the best fit model contained only the blood effect of water striders in Experiment 3 (Supp Table S1 [online only]).

Discussion

The present results show that the combinations of food insects used to rear L. f. fossarum in the laboratory drastically alter viabilities in...
progeny generations. Although the origin of parental populations varied among the experiments, we compared the viability of offspring using a split design in each experiment; thus, the differences in viabilities between treatments with different food insect combinations are ascribed to the effect of the food insect combination. The developmental period is also influenced by food insects, suggesting the importance of the food insects in shortening the generation time. The present results further indicate that the combinations of food insects in larval periods indeed affect wing morph determination of adults in L. f. fossarum (Supp Table S1 [online only]) as reported in other water striders (Harada and Nishimoto 2007). They strengthen the importance of establishing a continuous rearing method under constant diet conditions for future genetic studies using L. f. fossarum.

For genetic studies such as linkage analysis and quantitative trait loci (QTL) mapping, establishing segregating generations (e.g. F2 or backcross generations) is crucial. We have demonstrated that the viability of F2 progenies depended on the F1 (i.e., parents of F2 progeny) instars that fed on P. interpunctella adults, and the majority of F2 individuals from the Dm+Pi1,2,5 treatment died before the second instar due to failure to successfully hatch or consume food insects in the first instar. In contrast, the fact that nearly 80% of nymphs successfully developed to the second stadium in the Dm+Pi1,2,5 treatment strongly indicates that not only the combination of food insects but also instars feeding on P. interpunctella in the F1 generation are very important in order to obtaining a sufficient number of individuals in the segregating generation in L. f. fossarum.

Sonoda et al. (1991) reported that the viability of the broad shouldered water strider, Microvelia douglasi Scott (Hemiptera: Velidae), which is a polyphagous predator, is also affected by the combination of food insects in the nymphal stage. Nymphs of M. douglasi that fed on both D. melanogaster and Nilaparvata lugens (Stål) (which belong to different insect orders: Diptera and Hemiptera) develop faster and recover higher viability until adulthood than do nymphs that fed only on either D. melanogaster or N. lugens (Sonoda et al. 1991). Several other studies have also demonstrated that a mixed-prey diet in nymphal stages improved the adult body size, fecundity, and the hatching success in offspring (Toft 1995; Grundy et al. 2000; Zanuncio et al. 2000; Marques et al. 2015; for reviews, Lefcheck et al. 2013 and De Clercq et al. 2014), and such advantages of mixed diets have been already explained by the toxin-dilution hypothesis (Freeland and Janzen 1974; Toft and Wise 1999) and/or the redress-nutritional imbalance hypothesis (Mayntz et al. 2005). Thus, the present results add to the growing number of cases showing the suitability of mixed-prey diets for predatory arthropods.

Crickets (e.g., Acheta domestica (L.) or Gryllus firmus Scudder) are often used as food insects for water striders in addition to dipteran species (e.g., Fairbairn and King 2009). Our present rearing method using P. interpunctella with D. melanogaster recovered the same viability until adulthood when compared to the use of A. domestica with D. melanogaster in the same split design, although we conducted only one replicate in F1 generation (60% in both combinations, data not shown). P. interpunctella is readily reared in the laboratory using brown rice as a food. We usually introduce two adult pairs of P. interpunctella into a plastic container (135 x 85 x 30 mm) filled with ca. 10 mm brown rice to generate offspring and generally obtain 50–120 adult offspring within 1 month without any subsequent care after introduction (Hirooka pers. obs.). P. interpunctella is widely used as factitious prey with other pyralid moths (e.g., Corcyra cephalonica (Stainton) and Ephestia kuehniella Zeller) to maintain predatory heteropteran insects for biological control purposes (for a review, see De Clercq et al. 2014). This study further shows that P. interpunctella could also be a useful food insect for rearing water striders like crickets.

The present results clearly indicate that adding nutrients to a Drosophila medium improves the viability of water striders. When L. f. fossarum is reared by our present method with D. melanogaster reared on the normal medium, one of the most frequent causes of death in nymphal stages is failed molting. Because cholesterol is the precursor to ecdysone (an insect molting hormone) (Lang et al. 2012; Niwa and Niwa 2014), adding cholesterol to the medium for Drosophila could aid the synthesis of ecdysone in water striders, resulting in successful molting. Although the viability until adulthood in the DmC+Pi1,2,5 treatment in the Experiment 4 was about 20% in the F2 generation, this low viability could be due to the use of laboratory lines showing inbreeding depression. Indeed, results of Experiment 3 that used less inbred individuals recovered above 50% viability until adulthood in the F2 generation even in the Dm+Pi1,2,5 treatment. Thus, the more than 50% viability in Experiment 3 coupled with the consistently higher viabilities in the DmC+Pi1,2,5 treatment than in the Dm+Pi1,2,5 treatment in Experiment 4 suggest that adding cholesterol to Drosophila medium is one of the effective ways to obtain sufficient number of individuals in segregating generations.

Mayntz and Toft (2001) have also reported that the nutrient composition of rearing medium for a food insect (D. melanogaster) can affect predator viability in the wolf spider Paradosa amentata (Clerck) (Araneae: Lycosidae). Therefore, adding nutrients to Drosophila medium could be a universal method for rearing predacious insects.

This study does not intend to assess the genetic backgrounds of wing polymorphisms in L. f. fossarum, but we preliminarily infer the contribution of genetic components (i.e., broad sense heritability, \(H^2\)) using the present rearing method (Dm+Pi1,2,5). We calculated the broad sense heritability of wing polymorphism as \(H^2=2a^2\) (among sibling groups)\(+e^2\) (among sibling groups) + \(a^2\) (within sibling groups) [Ueno et al. 2001; Fukunaga and Akimoto 2007]. We used the four inbred lines established by one-time sib-mating and we split the offspring from each sibling group fifty-fifty into two photo-periods (16:8 (LD) h and 10:14 (LD) h). The mean square of variance obtained between sibling groups (163.4) is higher than that within sibling groups (102.7), although we do not have enough sibling group replications to test statistical significance. The estimated broad sense heritability is 46%, and this indicates that a substantial amount of variation of wing polymorphism is determined by genetic factors in L. f. fossarum. Thus, further improvement of the present rearing method, especially for the nutrients to be included in the Drosophila medium, will contribute to elucidating the genetic mechanisms determining wing polymorphisms and make L. f. fossarum a model organism for studying the genetic background of wing polymorphism.

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**Supplementary Data**

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

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