Environmental Entomology, 51(4), 2022, 806–814
https://doi.org/10.1093/ee/nvac043
Advance Access Publication Date: 7 July 2022
Research

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Physiological Ecology

Effects of Dietary Iron Quantities and Sources on *Lymantria dispar* (Lepidoptera: Erebidae) Survival and Development

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Subject Editor: Man-Yeon Choi

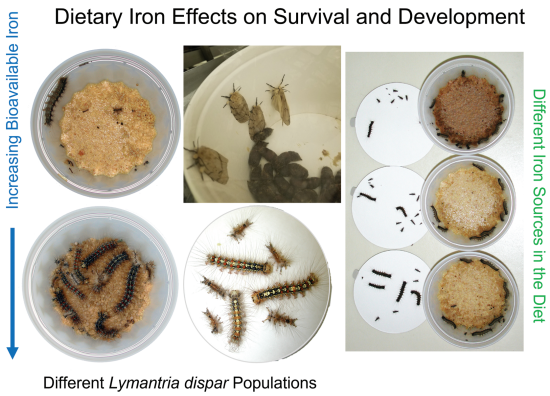
Received 15 March 2022; Editorial decision 16 May 2022.

Abstract

Lymantria dispar L. is a serious invasive defoliator of broadleaf trees in both North America and Eurasia. When rearing *L. dispar* for research, the artificial diet must have a source of iron that is bioavailable and in the quantity needed to ensure normal development and survival. The standard iron supplement in *L. dispar* diet, amorphous ferric phosphate, is no longer commercially available, which motivated studies on alternative iron sources and concentrations. The responses of three *L. dispar* populations (including an *L. dispar asiatica* Vnukovskij population) to seven different iron doses were determined over two generations. The response to eight iron compounds and no added iron was also assessed over two generations for an *L. dispar dispar* population. The optimal levels of iron were 100, 150, and 198 mg/l of available iron for the New Jersey Standard Strain, a near-wild West Virginia population, and a near-wild Russian Far East population, respectively. All three populations showed symptoms of insufficient iron when reared on the lowest dose of iron and signs of excess iron at the highest two doses. All of the alternate iron sources evaluated provided adequate available iron, although three sources had some issues that may not make them the best options. These results reveal differences in nutritional requirements among different populations of *L. dispar* and verify the existence of viable replacement sources of iron for the standard amorphous ferric phosphate long used in *L. dispar* rearing.

Key words: development, *Lymantria dispar*, survival, dietary iron

Graphical Abstract



Nearly all insect cells require iron, which is essential for deoxyribonucleic acid synthesis, cellular respiration, xenobiotic metabolism, uric acid and steroid production, melanization, immunity, and orientation to magnetic fields in insects (Locke and Nichol 1992, Nichol et al. 2002). Insect gut cytochromes are particularly sensitive to iron deficiencies and may need daily iron to function properly. However, excess free iron can be damaging to many cellular processes because, in the presence of oxygen, iron produces reactive oxygen species that cause DNA-strand breakage, lipid peroxidation, and protein denaturation (Zhou et al. 2019). Iron concentrations in insects are controlled by uptake, transport, intracellular distribution, and excretion. The insect's ability to store iron is limited by the available apoferritin and transferrin proteins, which bind and maintain the iron in a soluble, nontoxic, and available form (Locke and Nichol 1992, Nichol et al. 2002, Tang and Zhou 2013, González-Morales et al. 2015). Insects also prevent buildup of excess iron by converting ferrous ions to ferric ions and increasing excretion of iron. Excess iron is bound by holoferritin in the endoplasmic reticulum of midgut cells, then crystalized before being excreted (Tang and Zhou 2013).

When insects are reared for research, the artificial diets that are used must ensure the nutritional availability of iron while limiting the destructive effects of excess iron. Bioavailability and acceptance of iron compounds used as dietary supplements vary greatly (Lee and Clydesdale 1979), and many were originally developed for vertebrates rather than insects. Inorganic dietary iron occurs primarily as the ferric ion, but the ferrous ion has been shown to be more available in human and animal food. Bioavailability is known to vary with iron valence, solubility, and degree of chelation (Lee and Clydesdale 1979) although cooking and the addition of ascorbic acid can increase bioavailability (Sayers et al. 1974).

Some of the iron compounds in the salt mixtures used in artificial insect diets come in more than one form, and some forms are not bioavailable. For example, ferric phosphate, which comes in crystalline and amorphous forms, is used in Wesson salt mixture. The amorphous form of ferric phosphate is bioavailable for *Lymantria dispar* L. (Lepidoptera: Erebidae), but the crystalline form is not (Odell et al. 1997). When two successive generations of *L. dispar* New Jersey Standard Strain (NJSS) are reared on diet containing only the crystalline form, larvae develop slowly and asynchronously, survival to pupation is reduced, and egg hatch is poor. These effects are symptomatic of insects reared on an iron-deficient diet. The variation among individuals has both an additive genetic basis (20–40% due to response to iron dose) and a maternal effect (27–28% due to maternal egg provisioning with iron; Keena et al. 1998). Recent research has shown that in *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) ferritin enters the eggs and provides maternal iron stores that are important to early development, which could be the way that the maternal iron is provided in *L. dispar* (González-Morales et al. 2015).

Additional work with the NJSS population of *L. dispar* indicated that other iron compounds (ferric citrate and ferrous sulfate) were bioavailable (Odell et al. 1997). Suitable iron alternatives should be identified since vendors do not distinguish among the ferric phosphate form present in the salt mix and other products and change them based on cost and availability. Further work is needed to assess the bioavailability and acceptance of the many iron compounds that could be used in *L. dispar* artificial diets. Tests should be done for two successive generations because of the maternal effects seen with ferric phosphate and the likelihood that other unforeseen effects may appear with replacement.

Iron requirements may differ among subspecies of insects, as has been documented for species in the same genera. For example, iron concentrations in *Vespa crabro* L. (European hornet, Hymenoptera: Vespidae) are significantly higher than in *Vespa velutina* Lepeletier (Asian yellow-legged hornet, Hymenoptera: Vespidae), which is suspected to arise from differences in aerobic energy demands to sustain foraging flights (Andreani et al. 2021). Insect metabolism increases 50- to 100-fold with flight; carbohydrates have been shown to be the major fuel source in wing muscles and iron is an important cofactor for glycolytic enzymes (Beenakkers et al. 1984, Suarez et al. 2005). Since flight capability differs both within and among populations of *L. dispar* and among subspecies (*L. dispar dispar* L., *L. dispar japonica* Motschulsky, and *L. dispar asiatica* Vnukovskij (Lepidoptera: Erebidae), there may be interpopulation differences in iron requirements. If differences exist, the diets used for rearing various populations may need to differ slightly to ensure requirements are met. Research on populations of *L. dispar* from Asia is important since the females fly to lights at night and will lay their eggs on vessels or cargo bound for other countries (Wallner et al. 1995). Although *L. dispar dispar* is already established in North America, the flight-capable-female biotype present in Asian populations is not, and it is of major concern since they can spread quickly and are highly polyphagous (Keena et al. 2008, Keena and Richards 2020).

In this article, the responses of three *L. dispar* populations (one being a *L. dispar asiatica* population) to seven different $FePO_4$ iron doses were determined over two generations. The responses of the NJSS *L. dispar dispar* population to eight iron compounds, and no iron incorporated into the diet was also assessed over two generations. The significance of these results for rearing *L. dispar* is discussed.

Materials and Methods

Lymantria dispar Populations

Three populations were used in these studies: a population of *L. dispar asiatica* from the Russian Far East (RM: Mineralni, Primorski Province, 44.10°N and 133.15°E) collected August 1992, a wild North American population of *L. dispar dispar* from West Virginia (WV: Morgantown, Monongalia County, 39.37°N and 79.55°W) collected January 1993, and the New Jersey Standard Strain of *L. dispar dispar* (NJSS: Blairstown, Warren County, 40.98°N and 74.96°W) collected November 1967. The WV population is no longer in culture although the other two are still available. All populations were obtained and transported as eggs under permit to the USDA Forest Service quarantine facility in Ansonia, CT. Voucher specimens for each population were deposited at the Entomology Division, Yale Peabody Museum of Natural History, New Haven, CT.

The egg masses used in the iron dose–response studies were from the following laboratory generations: WV first and second, RM third and fourth, and NJSS forty-second and forty-third. The egg masses of NJSS used in the iron source studies were from the fifty-first and fifty-second laboratory generations. Laboratory colonies of *L. dispar* that produced the egg masses used in the studies were reared in walk-in environmental chambers maintained at 25°C, 60% RH, and a photoperiod of 16:8 (L:D) h. Larvae were reared in cohorts of 8–10 in 177 ml (NJSS) fluted opaque or 236 ml (RM and WV) clear plastic cups with waxed paper lids (Sweetheart, Chicago, IL) on a high-wheat germ artificial diet (Bell et al. 1981). Pupae of each strain were harvested and placed in groups of 25 individuals of each sex for mating in 3.8-liter rolled-paper containers (Neptune, Newark, NJ). The containers were lined with butcher paper on which the females oviposited. At each generation of the NJSS and RM populations, eggs from 100 females were scraped from the sheets and thoroughly mixed before incubation to produce random subsets of offspring and

maintain genetic diversity. Colony eggs were chilled for 140–141 d at 5°C (RM and WV) or 7–8°C (NJSS) before they were moved to 25°C for hatch.

Iron Dose–Response Study

The numbers of egg masses available for use in this study were as follows: 22 NJSS, 25 RM, and 4 WV. Ten newly hatched larvae from each egg mass from each population were set up in each of two cups (if sufficient hatch) containing 90 ml of artificial diet per iron concentration. The seven iron concentrations were achieved by adding 0, 59, 118, 177, 236, 295, or 354 mg of amorphous $FePO_4$ (ferric phosphate) per liter of diet made using a salt mixture lacking added iron. An analysis of available iron (mg/l) was performed following the protocols in Willis and Montgomery (1994), and the measured available iron concentrations (mean ± SE) for three samples of each of the diets are given in Table 1. This low-technology method of determining available iron was used and is still used because it is cheap to use, and a more sophisticated total iron analysis will not provide the needed information. There can be substantially more total iron in the diet than what is available to the insect for its use. Total hatch and embryonation of unhatched eggs were determined for each egg mass, and fecundity (total eggs) and percentage hatch of embryonated eggs were calculated. After 10 d at 25°C, 60% RH, and 16:8 (L:D) h, the surviving larvae were individually weighed (mg) and percentage survival was calculated for each cup.

Pupae were harvested from the cups at 35 and 40 d. Pupae were held by sex, iron treatment, and sibling group (from the same egg mass) in 473-ml squat unwaxed paper containers (James River Corporation, James River, VA) with clear plastic lids (Sweetheart, Chicago, IL) until adult emergence. Percentage pupation and male to female ratio were calculated for each cup. All adults that eclosed normally were mated within iron treatments either in groups of 25 pairs in the paper 3.8-liter containers lined with butcher paper or in single pairs in the 473-ml paper cups (if few adults emerged), and sibling matings were avoided. Females lay only one egg mass so group matings are possible. Time to adult (d) was recorded for each individual and the number of pupae that eclosed poorly or not at all was also recorded. After 40 d, enough time for the eggs to embryonate, egg masses were scraped from the paper cups and held individually in glassine envelopes. Thirty first-generation egg masses (or all egg masses if fewer than 30 resulted from the matings) were randomly chosen and chilled at 5°C for 135 d. To subject siblings to more than one treatment, the egg masses were split in half lengthwise. Half of each egg mass was incubated at 25°C immediately and the other half was held at 5°C for an additional 7 d before incubation to delay hatch. When most of the eggs hatched, the newly hatched larvae and the egg mass were chilled at 10°C until used. The number of egg masses available for use in the second generation is given in Table 1.

In the second generation, two concentrations of available iron were used: which were the equivalent of treatments 1 and 5 (68 and 213) in the first generation. The means (±SE) of available iron in mg/l of diet based on three samples from each of the 8–10 batches of diet of each type required for this part of the study were as follows: 49.5 ± 0.6 mg/l for larvae from the first week of hatch and 213.8 ± 1.8 mg/l for larvae in the second week of hatch. The no-iron added diet (49.5 mg/l) was used to assess whether the first-generation diet had enough iron to provide the iron maternal provisioning to the second generation because if the larvae received insufficient iron for two generations their development becomes asynchronous and survival declines (Keena et al. 1995). The high level of iron diet (213.8 mg/l) was used to assess the upper limit of acceptable available iron for each population because if the larvae receive too much

Table 1. Numbers of egg masses available from each *Lymantria dispar* source population, and numbers incubated and used in the second generation of the iron dose–response study

		Population and egg mass information								
		New Jersey Standard Strain			Russian Far East			West Virginia		
Available iron concentration (mean ± SE) (ml/l)	Treatment number	Available	Incubated	Used	Available	Incubated	Used	Available	Incubated	Used
68 ± 2.9	1	156	30	25	2	2	1	2	2	1
108 ± 2.9	2	149	30	25	99	30	25	13	13	13
150 ± 1.2	3	156	30	25	128	30	25	18	18	17
198 ± 4.6	4	161	30	25	127	30	25	13	13	13
213 ± 4.6	5	92	30	26	133	30	26	9	9	8
264 ± 3.5	6	38	25	6	126	30	24	13	13	8
277 ± 4.1	7	9	8	3	130	30	26	11	11	6

iron for two generations egg hatch decreases, development is slowed, and survival can decline. The plan was to set up two cups of 10 larvae each from 25 first-generation egg masses on diet containing each iron concentration for a total of 50 cups per treatment per population. However, some first-generation egg masses only had enough hatch to set up one cup per treatment, so an extra first-generation egg mass was chosen and one cup per treatment was added from that egg mass. Other first-generation treatments had fewer than 25 egg masses for one or more populations, so the number of cups per egg mass was increased to achieve the 50 cups per second-generation treatment. When fewer than 10 egg masses were available for a population and treatment combination, all larvae that hatched from each half egg mass were used.

Total hatch and embryonation of unhatched eggs were determined for each half of the first-generation egg masses. Total fecundity and percentage hatch of embryonated eggs were then determined for each egg mass as a whole. As in the first generation, percentage survival was calculated for each cup, and individual larvae were weighed at 10 d. Pupae were again harvested, and both percentage pupation and male to female ratio were calculated.

Iron Dietary-Source Study

One hundred NJSS egg masses were combined and mixed, and from them, 27 egg packets were made (each containing ~500 eggs) and incubated for hatch at 25°C, 60% RH, and 16:8 (L:D) h. Ten newly hatched larvae were set up in 50 cups per treatment, each containing 90 ml of artificial diet. Nine different high-wheat-germ *L. dispar* diets containing salt mixture with no added iron were prepared by incorporating the following amounts of each iron compound into 10-liter batches: 1) 0.0 g (no iron), 2) 0.6 g amorphous ferric phosphate, $FePO_4$, 3) 1.3 g ferric ammonium sulfate, $Fe(NH_4)(SO_4)_2$, 4) 0.9 g ferric citrate, $FeC_6H_3O_7$, 5) 0.5 g ferrous fumarate $C_4H_2FeO_4$, 6) 1.3 g ferric pyrophosphate, $Fe_4(P_2O_7)_3$, 7) 0.5 g ferrous sulfate, $Fe_2(SO_4)_3$, 8) 0.8 g ferric tartrate, $Fe_2(C_4H_4O_6)_3$, and 9) 1.0 g ethylenediamine tetracetic acid, $C_{10}H_{12}N_2\ O_8FeNa$. The amount of iron compound to be added was determined by dividing the molecular weight of the iron in the compound by the total molecular weight of the compound plus associated water molecules to calculate percent iron and then scaling the amount of the compound to be added to achieve an estimated available iron level of 100 mg/l (85–115 mg/l is considered optimal for this NJSS population). An analysis of available iron (mg/l) was performed following the protocols in Willis and Montgomery (1994) using three samples from each batch of diet, amounts of iron found in the diets are given in the results. After 10 d at 25°C, 60% RH, and 16:8 (L:D) h, the instar of the surviving larvae was determined, and percentage survival was calculated for each cup. Larvae that were close to molting to the next instar (new larger head capsule visible beneath the skin) were assigned the value of the instar number plus 0.75. Pupae were harvested from the cups at 34 d, and percentage mortality, pupation, and male to female ratio were calculated per cup. The pupae from each iron treatment were held in six 3.8-liter paper containers lined with butcher paper, each containing 25 males and 25 females. The adults were allowed to emerge, mate, and oviposit, and egg masses were harvested after 40 d. Thirty egg masses (50 for the no-iron treatment) were chilled at 7–8°C for 168 d. Half of each egg mass was incubated at 25°C immediately, and the other half was held in chill for an additional 7 d before incubation. Once most of the eggs hatched, the egg mass was chilled at 10°C until use.

In the second generation, 10 newly hatched larvae from each egg mass were set up in each of two cups containing 90 ml of artificial diet that contained no added iron (first week of hatch) and two cups that contained diet made with the same iron compound as the parental generation (second week of hatch). One exception was that larvae from parents that were reared without added iron were reared on diet containing 0.6 g $FePO_4$ as a control that would show the restorative effect of adequate available iron. The no-iron diet was used to determine whether the iron sources were available in quantities sufficient to prevent the induction of the slow, asynchronous development observed when larvae are reared on diet containing insufficient iron for two generations. The use of the same diet as the parental generation tested whether the iron source would cause deleterious effects if used for multiple generations. As in the first generation, percentage survival was calculated for each cup and the instar of each larva was determined at 10 d. Larvae on the same diet as the parental generation were maintained on the diet for 34 d, pupae were harvested, and percentage pupation and male to female ratio were calculated. Total hatch and embryonation of unhatched eggs were determined for each generation's egg mass. Percentage hatch of embryonated eggs was then calculated.

Statistical Analyses

The fit of the data for each parameter to various distributions was assessed using PROC UNIVARIATE (SAS Institute 2015), and the Shapiro–Wilk test was used to assess normality. The following dependent continuous variables were analyzed in PROC GLIMMIX (SAS Institute 2015) using a normal distribution with an identity link function: larval weight at 10 d (WV population excluded in first generation) and days to adult eclosion in the dose response study, and instar at 10 d and fecundity in the iron source study. A Beta distribution with a logit link function was used for percentage data calculated for egg masses or containers: percentage survival, percentage females, and percentage hatch of embryonated eggs. Percentage values of 0 and 1 were replaced with 0.0001 and 0.9999, respectively, before analysis, because all values must be between 0 and 1 for the Beta distribution. A completely randomized design was used to evaluate the effects of population, iron treatment (one or both generations), sex (adult parameters only), and the interactions between these on the listed variables in each model. Interactions that were not significant were dropped from the final model. In the iron source study, the egg mass was included as a random effect in the second-generation analyses of continuous variables. For each model, residuals were evaluated for normality and the homogeneity of variance was assessed using Levene's test. A conservative Tukey–Kramer grouping was used to assess differences among means determined by the least-squares means test with $\alpha = 0.05$ (SAS Institute 2015).

Results

Iron Dose–Response Study

The population ($F = 295.67$; df = 1, 1356; $P < 0.0001$), iron dose ($F = 13.65$; df = 6, 1356; $P < 0.0001$), and the interaction between the two ($F = 51.11$; df = 6, 1356; $P < 0.0001$) all had significant effects on mean larval weight at age 10 d. The NJSS larvae achieved maximum weight at 100 mg/l of available iron and, as dose increased, weight declined (Fig. 1). The RM larvae were heaviest at doses above 200 mg/l, and the weights declined as the dose declined. Maximum NJSS larval weights attained at 10 d exceeded those of the RM larvae. Percentage pupation followed a similar pattern, with pupation above 80% for the NJSS population at doses of 68–213 mg/l and for the RM population at doses of 198–277 mg/l. The WV population exceeded 60% pupation only at doses of 108, 150, 264, and 277 mg/l.

The interaction between population, iron treatment, and sex had a significant effect ($F = 2.78$; df = 14, 5000; $P = 0.0004$) on mean time from egg hatch to adult (Fig. 2). NJSS individuals completed development fastest over most of the iron dose range when compared with the other two populations, but days to adult increased with increasing iron doses >200 mg/l for both sexes. There were no significant differences between the two sexes for the WV or RM populations, but the RM males that developed on diet with only 68 mg/l of iron developed more slowly than those on diets with ≥150 mg/l of iron.

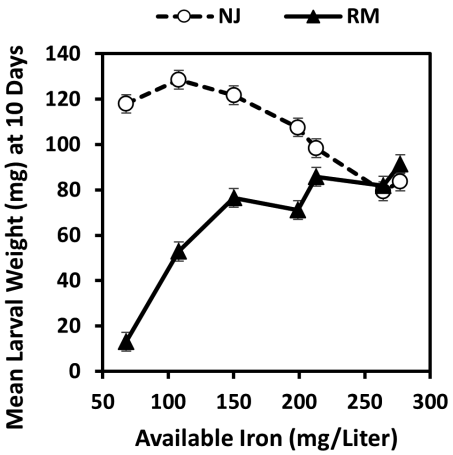


Fig. 1. Mean (± SE) larval weights (mg) at age 10 d of New Jersey Standard (NJ) and Russian Far East (RM) populations of *Lymantria dispar* reared on diets containing various amounts of available iron.

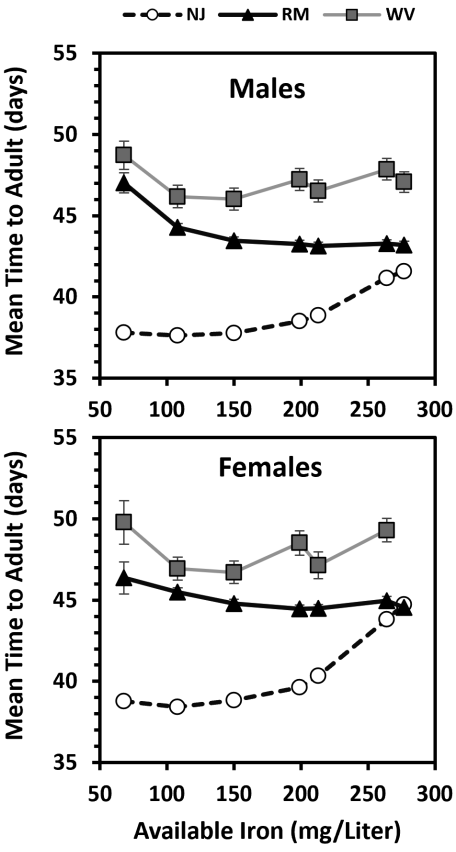


Fig. 2. Mean (± SE) time from egg hatch to adult (days) for *Lymantria dispar* males and females from three populations reared on diets containing various amounts of available iron. The populations used were the New Jersey Standard (NJ), West Virginia (WV), and Russian Far East (RM).

Fecundity of the females was not significantly affected by iron dose with the exception that the NJSS females on 277 mg/l laid fewer eggs than those on ≤213 mg/l (Table 2). However, NJSS females tended to lay more eggs at lower iron doses (68–150 mg/l), whereas RM and WV females tended to lay more eggs at 100–200 mg/l of iron. Fewer NJSS females laid egg masses at 264 and 277 mg/l, and fewer RM and WV females laid egg masses at 68 mg/l. Percentage hatch of embryonated eggs was also affected by the interaction between population and iron dose ($F = 9.53$; df = 12, 410; $P < 0.0001$). Significantly more eggs produced by females from the NJSS population that fed on diet containing iron doses of 68–150 mg/l hatched than did those fed on iron doses of 213–277 mg/l (Fig. 3). Eggs laid by RM females fed 108 mg/l of iron hatched at higher rates than those laid by females fed on diets containing 68, 264, or 277 mg/l of iron. Egg hatch of WV females fed 108 or 198 mg/l iron was higher than for those fed 264 or 277 mg/l iron.

In the second generation, mean larval weights were significantly affected by the interaction between population, parental iron dose, and second-generation iron dose ($F = 17.61$; df = 31, 3436; $P < 0.0001$). When the second-generation larvae were fed diet containing 214 mg/l of iron, the NJSS individuals had weights similar to those of parents fed 68–213 mg/l of iron but significantly lower weights when the parents had been fed diets with 264–277 mg/l (Fig. 4). For both the RM and WV populations, the larval weights tended to be higher when the parental diet contained 108–213 mg/l of iron and lower at iron doses above and below that. When the larvae fed on the 50 mg/l iron diet, NJSS larvae weighed similar amounts over most of the parental dose range, weighing less only at the highest dose. RM larvae fed the 50 mg/l dose increased in weight up to 213 mg/l and WV up to 150 mg/l of iron in the parental diet and then weighed about the same for the higher doses of parental iron.

Mean development time from egg hatch to adult in the second generation was significantly affected by the interaction between population and iron in the parental diet ($F = 4.8$; df = 11, 707.2; $P < 0.0001$), but this did not translate into any significant differences between the means. A trend was apparent for slower development by the NJSS individuals whose parents had been fed higher doses of iron, whereas, in contrast, RM and WV individuals whose parents were fed the lowest doses of iron tended to develop more slowly (Fig. 5).

Iron Dietary-Source Study

The iron source incorporated into the NJSS larval diet had a significant effect on the mean larval instar at age 10 d ($F = 14.09$; df = 8, 4393; $P < 0.0001$), but the range of mean instar among the treatments was 2.829–3.042, so, although statistically significant, the result might not be biologically significant. Larvae reared on diets containing no iron or $FePO_4$ achieved a higher mean instar than those reared on diet containing $Fe(NH_4)(SO_4)_2$ or $C_{10}H_{12}N_2O_8FeNa$ (this diet also darkened more quickly than the other diets). Both percentage pupation ($F = 5.37$; df = 8, 436; $P < 0.0001$; Fig. 6) and percentage females ($F = 2.1$; df = 8, 436; $P = 0.0348$; Fig. 6) were affected by the diet-iron source. Individuals fed on diet containing no iron had lower percentage pupation and females than individuals fed on diet containing the other iron sources.

Iron source in the diet had a significant effect on the percentage hatch of embryonated eggs ($F = 58.97$; df = 8, 281; $P < 0.0001$) and the mean fecundity of NJSS females ($F = 5.62$; df = 8, 281; $P < 0.0001$). Females produced a lower percentage of hatched eggs

Table 2. Mean (± SE) percentage pupation (by cup) and fecundity for the parental generation of *Lymantria dispar* from three populations reared on diet containing different amounts of available iron (mean ± SE) from added $FePO_4$

	Percentage pupation (number cups)			Fecundity (number females)		
Parental available Fe (mg/l)	New Jersey Standard Strain	Russian Far East	West Virginia	New Jersey Standard Strain	Russian Far East	West Virginia
68 ± 2.9	91.4 ± 1.3 ab (44)	8.3 ± 2.3 h (50)	18.9 ± 6.7 gh (6)	760 ± 41 a (30)	587 ± 159 ab (2)	458 ± 160 ab (2)
108 ± 2.9	92.7 ± 1.1 a (44)	70.3 ± 3.2 bcde (50)	76.8 ± 7.8 abcdef (6)	697 ± 41 a (30)	809 ± 41 a (30)	796 ± 63 a (13)
150 ± 1.2	92.1 ± 1.2 a (44)	77.6 ± 2.7 abcde (50)	67.5 ± 9.6 bcdefg (6)	675 ± 41 a (30)	755 ± 41 a (30)	609 ± 53 a (18)
198 ± 4.6	89.1 ± 1.6 abc (44)	83.6 ± 2.1 abcde (50)	59.4 ± 10.5 defg (6)	598 ± 41 a (30)	750 ± 41 a (30)	702 ± 63 a (13)
213 ± 4.6	83.6 ± 2.3 abcde (44)	84.4 ± 2.1 abcde (50)	53.3 ± 10.8 efg (6)	523 ± 41 a (30)	758 ± 41 a (30)	575 ± 75 ab (9)
264 ± 3.5	51.3 ± 4.0 gf (44)	87.4 ± 1.7 abcd (50)	64.1 ± 10.0 cdefg (6)	281 ± 45 ab (25)	685 ± 41 a (30)	545 ± 63 ab (13)
277 ± 4.1	45.0 ± 4.0 gf (44)	83.9 ± 2.1 abcde (50)	62.5 ± 10.2 cdefg (6)	245 ± 80 b (8)	687 ± 41 a (30)	598 ± 68 ab (11)
Population by diet treatment	$F = 43.7$; df 12, 677; $P < 0.0001$			$F = 3.47$; df 12, 423; $P < 0.0001$		

Means in the same population and diet treatment followed by the same letter are not significantly different when analyzed by PROC GLIMMIX (SAS Institute 2015) followed by Tukey–Kramer least-squares mean test with $\alpha = 0.05$.

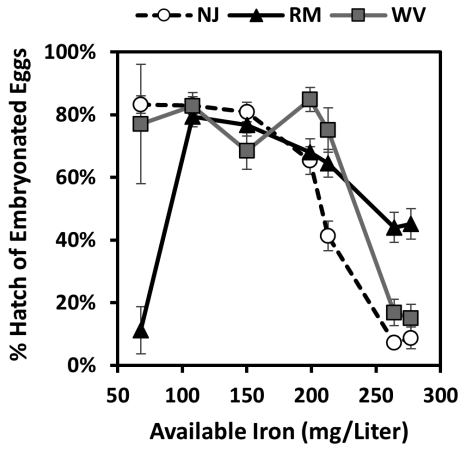


Fig. 3. Comparison of the percentage (± SE) hatch of eggs laid by females from three populations of *Lymantria dispar* reared on diets containing different amounts of available iron. The populations used were the New Jersey Standard (NJ), West Virginia (WV), and Russian Far East (RM).

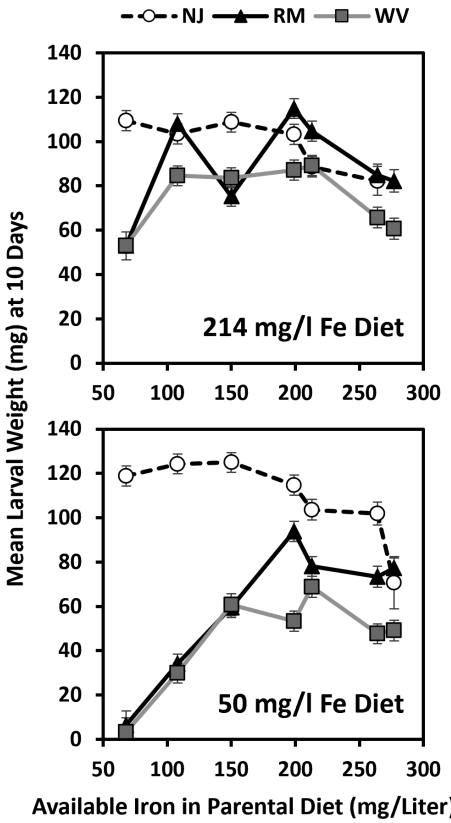


Fig. 4. Mean (± SE) larval weights (mg) at age 10 d of three populations of *Lymantria dispar* reared on two diets and whose parents were reared on diets containing various amounts of available iron. The populations used were the New Jersey Standard (NJ), West Virginia (WV), and Russian Far East (RM).

when fed on no-iron diet than females fed on the other diets (Fig. 7). The fecundity of females fed on $Fe_2(SO_4)_3$ diet was significantly lower than that of females fed on $FeC_6H_5O_7$ and $C_4H_2FeO_4$ (Fig. 8). Females fed on no-iron diet also laid fewer eggs than females fed on all the other diets except those containing $Fe_2(SO_4)_3$ and $Fe_4(P_2O_7)_3$.

In the second generation, the interaction between the iron source in the parental and progeny diets had a significant effect on the mean progeny larval instar at age 10 d ($F = 334.24$; df = 8, 8725; $P < 0.0001$). When both the NJSS parents and progeny were fed diet containing no iron, the mean instar of the progeny was lower at 10 d than for progeny of any parent–progeny combination fed on any other diet-iron sources (Fig. 9). Percentage pupation of progeny fed for the second generation on diets containing different iron sources was also affected by the interaction between parental and progeny diet treatments (Table 3). When both the parent and progeny were fed diet containing no iron, percentage pupation was lower than for all other dietary iron sources.

Discussion

The three populations of *L. dispar* differed in their response to different levels of available iron in the diet. The NJSS reached maximum larval weight, more than 100% of the larvae pupated, and egg hatch was more than 80% for two generations at 100 mg/l of available ferric phosphate. The WV and RM populations reached optimal weight (then leveled off or declined at higher iron levels) in the second generation on diet containing 50 mg/l of available iron when the parents were reared on 150 and 198 mg/l, respectively. All other parameters for WV and RM were at or near maximal levels when both generations were reared at the latter levels. All three populations showed symptoms of insufficient iron when both parents and offspring were reared on the lowest dose of iron and the RM showed signs at the lower doses in the parent generation. The

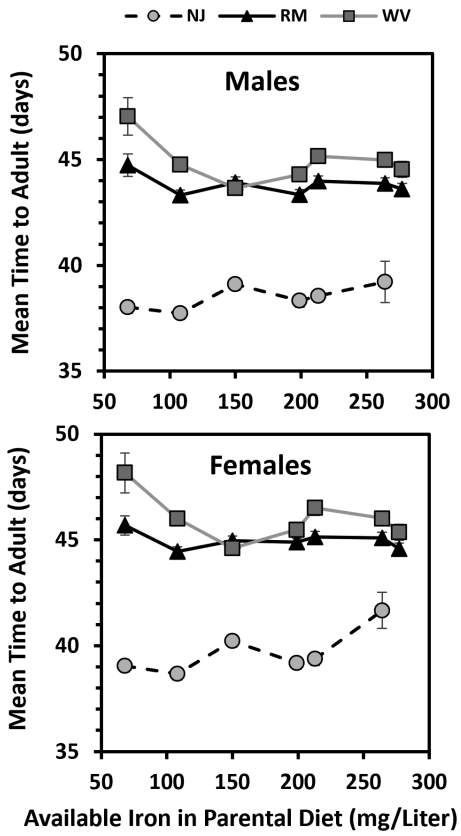


Fig. 5. Mean (± SE) time from egg hatch to adult (days) for *Lymantria dispar* males and females from three populations reared on diet containing 214 mg/l of iron and whose parents were reared on diets contained various amounts of available iron. The populations used were the New Jersey Standard (NJ), West Virginia (WV), and Russian Far East (RM).

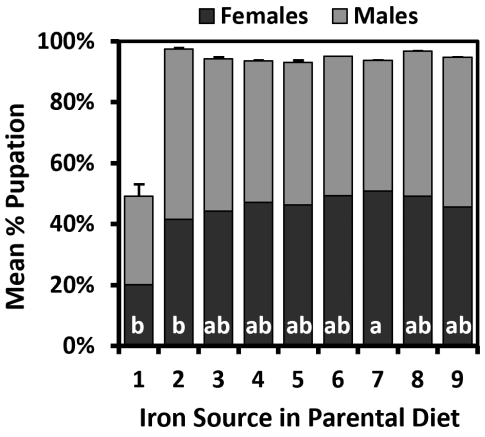


Fig. 6. Comparison of the percentage (± SE) pupation split by sex of the New Jersey Standard Strain of *Lymantria dispar* larvae reared on diet containing one of nine different iron sources: (1) 0.0 g, no iron; (2) 0.6 g amorphous ferric phosphate, $FePO_4$; (3) 1.3 g ferric ammonium sulfate, $Fe(NH_4)(SO_4)_2$; (4) 0.9 g ferric citrate, $FeC_6H_5O_7$; (5) 0.5 g ferrous fumarate, $C_4H_2FeO_4$; (6) 1.3 g ferric pyrophosphate, $Fe_4(P_2O_7)_3$; (7) 0.5 g ferrous sulfate, $Fe_2(SO_4)_3$; (8) 0.8 g ferric tartrate, $Fe_2(C_4H_4O_6)_3$; or (9) 1.0 g ethylenediamine tetracetic acid, $C_{10}H_{12}N_2O_8FeNa$. Different letters in the female part of the graph indicate significant differences in percentage female pupae.

NJSS had the most pronounced signs of excess iron at the higher iron doses, and the WV and RM populations had fewer signs of excess iron (reduced 10-d weights and % hatch of embryonated eggs) at the highest two doses.

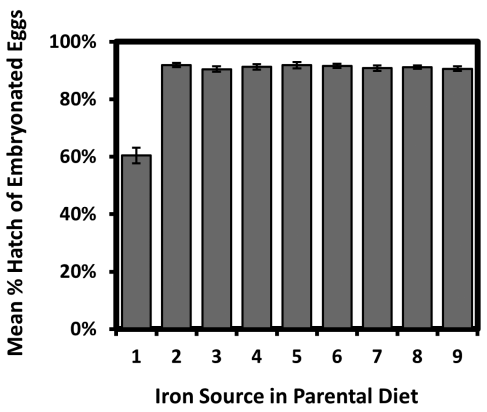


Fig. 7. Comparison of the percentage (± SE) hatch of embryonated eggs laid by the New Jersey Standard Strain of *Lymantria dispar* females reared on diet containing one of nine different iron sources (iron sources described in Fig. 6).

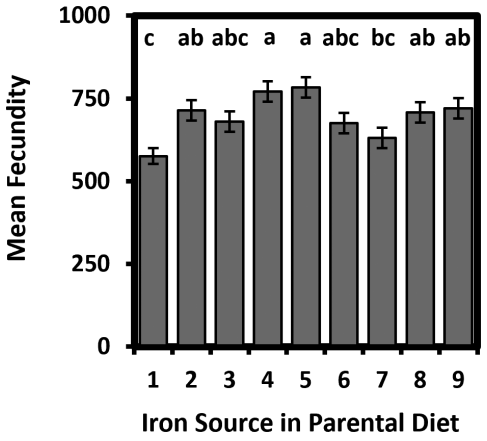


Fig. 8. Mean (± SE) fecundity of the New Jersey Standard Strain of *Lymantria dispar* females reared on diet containing one of nine different iron sources (iron sources described in Fig. 6).

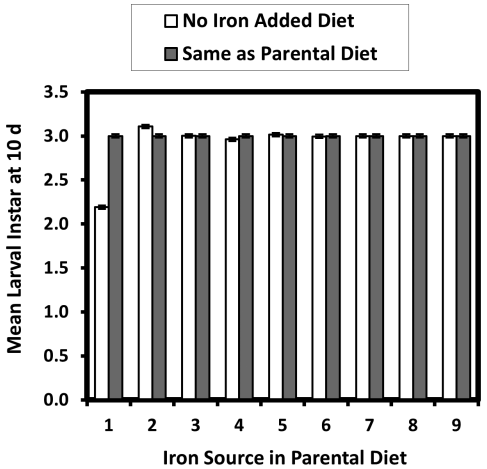


Fig. 9. Mean (± SE) instar at age 10 d for the New Jersey Standard Strain of *Lymantria dispar* larvae whose parents were reared on diets containing one of nine different iron sources. The progeny were either reared on a diet containing no added iron or on diets with the same iron sources as the parents (Diets 1–9, as described in Fig. 6).

Different populations of *L. dispar* exhibited different requirements for available iron. The NJSS required the least amount of iron (100 mg/l) in the diet to grow and develop normally. This laboratory

Table 3. Mean (± SE) percentage pupation (by cup) for *Lymantria dispar* from the New Jersey Standard Strain reared for two generations on diet containing iron from different sources

Iron source added	Percentage pupation (number cups)	Available iron (mg/l of diet)	Cost for 500 g of iron compound	Product name (molecular weight)
No iron	49.1 ± 3.5 b (44)	50.2 ± 1.4		
$FePO_4$	97.4 ± 1.0 a (44)	110.4 ± 1.8	Not available	Amorphous ferric phosphate (150.8)
$Fe(NH_4)(SO_4)_2$	94.3 ± 1.3 a (44)	108.6 ± 4.4	$176	Ferric ammonium sulfate (482.2)
$FeC_6H_5O_7$	93.5 ± 1.4 a (44)	103.2 ± 0.8	$158	Ferric citrate (244.9)
$C_4H_2FeO_4$	93.1 ± 1.4 a (44)	104.9 ± 4.8	$134	Ferrous fumarate (169.9)
$Fe_4(P_2O_7)_3$	95.1 ± 1.1 a (44)	92.2 ± 0.8	$173	Ferric pyrophosphate (745.2)
$Fe_2(SO_4)_3$	93.8 ± 1.3 a (44)	81.3 ± 3.6	$122	Ferrous sulfate (278.0)
$Fe_2(C_4H_4O_6)_3$	96.7 ± 1.0 a (44)	83.3 ± 6.6	$220	Ferric tartrate (555.9)
$C_{10}H_{12}N_2O_8FeNa$	94.7 ± 1.2 a (44)	63.4 ± 2.1	$276	Ethylenediamine tetracetic acid (292.3)

Bioavailable iron amounts for each diet (mean ± SE), cost of 500 g of compound, and the product name and molecular weight are also given. PROC GLIMMIX (SAS Institute 2015) results indicated a significant diet treatment effect ($F = 47.9$; df 8, 417.9; $P < 0.0001$) and a subsequent Tukey–Kramer least-squares mean test with $\alpha = 0.05$ compared means. Means followed by the same letter are not significantly different. Cost estimated based on web search conducted May 2022.

population has been in culture for decades and during that time has been exposed to periods of reduced iron in the diet which may have selected for individuals that can function with less iron. It has already been documented that this strain is different from wild North American *L. dispar dispar* in several other ways (Keena and ODell 1994). Both the NJSS and WV populations of *L. dispar dispar* have flightless females, whereas >90% of the RM population of *L. dispar asiatica* females exhibit strong directed flight. The RM population's increased needs for glucose to produce energy for flight, which depends on the glycolytic enzyme pathway that, in turn, depends on an iron cofactor, may be one reason for the higher iron requirement (200 mg/l available iron compared with 150 mg/l for the WV) of this population as was suggested for the *Vespa* sp. discussed in the introduction (Andreani et al. 2021). Other possible reasons may be associated with the larger wings in the flighted females that would require more iron in the melanization process, or differences in host plant utilization requiring different detoxification enzyme pathways that utilize iron cofactors. Also, many *L. dispar asiatica* larvae are slower to develop and grow, which may influence larval requirements for iron (Limbu et al. 2017). Effects on larval weight gain and survival in response to iron doses in the diet have been seen in *Bombyx mori* L. (Lepidoptera: Bombycidae) when different doses of iron were sprayed on its host leaves (Zhou et al. 2019). Larvae that fed on leaves with no added iron weighed less than those with added iron, and when higher doses of iron were added, survival to pupation was reduced compared with lower doses. Analyses of which enzymes were up- or downregulated suggested that immune-related, protease, and cuticular genes were most affected by the iron dose the *B. mori* larvae fed on (Zhou et al. 2019). The reasons for the differences in *L. dispar* population response to available iron in the diet should be explored further since hypotheses for them have been untested. Also, care should be taken since only three populations of *L. dispar* were used and other populations may have different iron requirements. However, the levels that were best for the WV (*L. dispar dispar*) and RM (*L. dispar asiatica*) have been in use with other laboratory colonies from the same subspecies (the *L. dispar asiatica* levels have been also used for *L. dispar japonica*) for many generations without deleterious effects.

As with most essential nutrients, a range of iron doses is tolerated by *L. dispar* populations, above and below which deleterious effects appear. The effects of iron deficiency seen in this study were slower and more asynchronous development, reduced weight gain at 10 d, reduced ability to pupate, fewer egg masses produced, and lower percentage hatch of embryonated eggs. These effects are consistent with earlier documented iron-related problems with rearing the NJSS (North American origin, laboratory-adapted *L. dispar*) discussed in the introduction and in Keena et al. (1995, 1998). These effects are expected when the functions of important enzymes are limited because of insufficient quantities of the iron cofactor. This study verifies that the same effects are observed in populations only a few generations removed from the wild in both *L. dispar dispar* and *L. dispar asiatica*, and is the first to document the effects of excess iron on *L. dispar*. Excess iron resulted in reduced weight gain in the larvae at 10 d, slowed development, reduced pupation and oviposition, and lower percentage hatch of embryonated eggs. It was also noted that, starting at the fourth instar, larvae (NJSS in particular) had runny frass at the higher doses. This deleterious effect is consistent with damage to cellular processes caused by excess unbound iron reacting with oxygen and the insects' attempts to excrete the excess iron.

All of the alternate iron sources evaluated provided adequate available iron, but three sources had minor issues: larvae reared on $Fe(NH_4)(SO_4)_2$ and $C_{10}H_{12}N_2O_8FeNa$ grew slightly slower, females reared on $Fe_2(SO_4)_3$ laid fewer eggs, and diet made with $C_{10}H_{12}N_2O_8FeNa$ turned dark more quickly. When diet turns dark, it is a sign that some of the ingredients are degrading, which can affect the nutritional quality of the diet. Thus, there are several other dietary iron sources that are bioavailable to *L. dispar* and could be used as viable replacements for ferric phosphate. Alternatives are needed since there are two forms of ferric phosphate and only one is bioavailable. Also, the amorphous ferric phosphate is no longer available for purchase and venders of ferric phosphate or salt mixtures that contain it do not differentiate between the two forms. The bioavailability of the different compounds did not seem to be affected by the valence of the iron, even though the ferrous ion is generally more available than the ferric ion (Lee and Clydesdale 1979). There were three diets that the test results for bioavailable iron were lower than expected: $Fe_2(SO_4)_3$, $Fe_2(C_4H_4O_6)_3$, and $C_{10}H_{12}N_2O_8FeNa$. This may be because the solubility and stability of the iron is affected by other dietary ingredients that complex with it. Several

dietary ingredients are also known to affect absorption of iron. In human foods, ascorbic acid and proteins enhance absorption, whereas phytate, calcium, and some polyphenols inhibit absorption (Hallberg and Hulthen 2000).

Two of the iron compounds ($Fe(NH_4)(SO_4)_2$ and $Fe_2(SO_4)_3$ that caused minor issues for *L. dispar* both contained sulfate. This may indicate that sulfates are not ideal for lepidopterans, although ferrous sulfate has been shown to be better than ferric phosphate for humans and other vertebrates (Lee and Clydesdale 1979). The other iron compound, $C_{10}H_{12}N_2$ O_8FeNa, which caused the diet to darken quickly, contains a potent metal chelator, ethylene diamine, whose function is to prevent discoloration, so the cause of the discoloration is unknown. The chelating effects of $C_{10}H_{12}N_2$ O_8FeNa were evident because the amount of available iron in the diet (63 mg/l) was below the expected amount (100 mg/l); this is another potential reason to avoid using it as a replacement for amorphous ferric phosphate. Ferric citrate ($FeC_6H_5O_7$), which is readily available and exists in only one form and is one of the cheaper options (Table 3), has recently been successfully used by *L. dispar*-rearing facilities as a replacement over multiple generations, once the more bioavailable amorphous ferric phosphate was no longer sold. Ferric phosphate represents another example of a product used in insect rearing that became unavailable and requires research to find a viable alternative. Alternatives must be assessed over more than one generation to ensure no unforeseen deleterious effects occur.

Acknowledgments

I thank H. Nadel, and J. Richards, and the two anonymous reviewers for their critical review of this paper. I thank A. Bridgeforth, P. Moore, C. Odell, D. Roberts-Paschall, and S. Taylor who provided technical assistance. I thank W. Wallner and B. Weston for assistance in obtaining egg masses. This study was funded by the United States Department of Agriculture Forest Service Northern Research Station.

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