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Research Article

A Rearing Method for *Argynnis (Speyeria) diana* (Lepidoptera: Nymphalidae) That Avoids Larval Diapause

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We describe a rearing protocol that allowed us to raise the threatened butterfly, *Argynnis diana* (Nymphalidae), while bypassing the first instar overwintering diapause. We compared the survival of offspring reared under this protocol from field-collected *A. diana* females from North Carolina, Georgia, and Tennessee. Larvae were reared in the lab on three phylogenetically distinct species of Southern Appalachian violets (*Viola sororia, V. pubescens*, and *V. pedata*). We assessed larval survival in *A. diana* to the last instar, pupation, and adulthood. Males reared in captivity emerged significantly earlier than females. An ANOVA revealed no evidence of host plant preference by *A. diana* toward three native violet species. We suggest that restoration of *A. diana* habitat which promotes a wide array of larval and adult host plants, is urgently needed to conserve this imperiled species into the future.

1. Introduction

The Diana fritillary, *Argynnis (Speyeria) diana* (Cramer 1775), is a threatened butterfly species that has been extirpated from large portions of its former distribution [1–5]. The subgenus *Speyeria*, now incorporated into the genus *Argynnis* [6], has been described as challenging to rear, primarily due to the six- to nine- month overwintering period required by first instar larvae. During winter diapause, tiny first instar larvae are most vulnerable to environmental extremes such as freezing temperatures, flooding, and fire [7]. Almost 140 years ago, Edwards noted the challenges of rearing the larvae of *Argynnis cybele* (Fabricius 1775), *A. aphrodite* (Fabricius 1789), and *A. diana* [8]. Edwards obtained hundreds of *A. diana* ova from 60 females captured outside of Coalburgh, West Virginia, but none of the larvae survived to pupation [8]. The first description of bypassing larval diapause in *Argynnis* came nearly a century later from W.H. Evans' notes on rearing and breeding *A. atlantis* (Drury 1773) [9]. Evans suggested the use of mechanical stimulation to encourage feeding by "listless" *A. atlantis* larvae [9].

First instar *Argynnis* larvae are notorious for not surviving overwintering when reared in a lab, regardless of their care and treatment [9]. To address this, Matoon et al. [10] developed a rearing protocol that requires packing *Argynnis* larvae in cold storage blocks and storing them under controlled refrigerated conditions for the duration of their overwintering period [10]. Scott [11] reared *A. atlantis* from Colorado under a 24 h photoperiod and was able to stimulate first instar larvae to feed on *Viola nephrophylla* leaves after a shortened diapause period of several days to several weeks. James [12] compared acceptance of *V. adunca, V. glabella, V. labradorica,* and *V. tricolor* by first instars of five Greater Fritillaries from the Pacific Northwest, *Argynnis. cybele leto, A. coronis simaetha, A. zerene picta, A. egleis mcdunnoughi,* and *A. hydaspe rhodope*. Ours is the first study of host plant acceptance and survival of an eastern *Argynnis* species.

Argynnis (Speyeria) diana is an eastern North American fritillary species that is rare to uncommon across much of its distribution in the southeastern United States (Figure 1). Violets are the sole food plant used by *Argynnis* caterpillars; however, specific associations or preferences for *Viola* spp. have not been reported. In late fall, adult female *A. diana* will each lay thousands of eggs singly on the forest floor near or on *Viola* spp. First instar larvae eat their way out of the egg casing, and immediately burrow into the litter layer of the forest floor. There, they remain inactive through the winter months. The following spring, first instar larvae emerge from the litter layer to feed on violets, *Viola* spp., both nocturnally and diurnally [13, 14].

(a) (b)

Figure 1: *Argynnis diana* male (top, orange and black) and female (bottom, blue and black) adults are sexually dimorphic and striking with their bright coloration. *Photos by Connie Wells*.

2. Methods

We reared offspring of *A. diana* females collected from three Southeastern field sites on three native *Viola* species: *Viola sororia* (Willd), *Viola pubescens* (Aiton), and *Viola pedata* (Linnaeus). There are approximately 80 species of *Viola* native to North America, all of which are small herbaceous plants producing five-petaled flowers with distinct petal spurs [15]. The three *Viola* species chosen for our experiment are found throughout the geographic range of *A. diana* and are easily distinguishable in appearance. These three species represent independent phylogenetic branches within the genus, *Viola* [16]. Violets were purchased and donated from a native nursery near our study area and transplanted into one-gallon buckets containing organic potting soil mixed with worm castings and perlite. Plants were exposed to 12 h photoperiod under 60 Watt grow bulbs at 72°C and watered daily.

2.1. Butterfly Rearing. We captured eight *A. diana* females from a field site in Henderson County, North Carolina in late September 2007. Live females were placed in glassine envelopes and held in a cooler for transport back to Clemson University, Clemson, SC. Within six hours of capture, we placed each female butterfly inside an individual paper bag containing clippings of fresh *Viola* foliage from *V. sororia*, *V. pubescens*, or *V. pedata* to trigger oviposition. We fed female butterflies twice daily by saturating cotton balls with a commercially available sports drink, Gatorade, containing a combination of water, sugar, salt, carbohydrates, and electrolytes. Ova were deposited daily on the inside surface of the paper bags, so we transferred butterflies to fresh paper bags every 24 h in order to collect and count ova each day. Small pieces of paper bag containing ~10 to 50 ova were transferred to sterile petri dishes lined with 50 mm Whatman filter paper. We misted ova daily with distilled water to prevent desiccation and changed filter paper every 24–48 hrs to prevent accumulation of mold, which can be detrimental to eggs and newly emerged larvae. We tallied the total number of ova produced daily by each of the eight North Carolina *A. diana* females. In addition to these ova, colleagues kindly donated one hundred fifty *A. diana* ova from three females captured in Carter County, Tennessee and one hundred fifty ova from three females captured in Rabun County, Georgia in late September 2007.

Upon hatching, we evenly divided 1st instar larvae from each of our three field sites into petri dishes containing one of the three native violet species (Figure 2(a)). In order to bypass the six- to nine- month overwintering period, we exposed all newly hatched larvae to continuous lighting, and mechanically stimulated individual larvae 3 to 4 times daily with a camel-hair brush to encourage feeding. Our lighting system consisted of a 1.2 m fluorescent grow light fixture holding two 40 Watt, 122 cm fluorescent tubes, set 0.4 m above the larvae and producing a constant temperature of 27°C. We misted growing larvae twice daily with distilled water and provided fresh filter paper and fresh *Viola* clippings each day to reduce infection from pathogens. Once larvae reached the fifth instar, they were transferred to individual 0.25 L clear pupation chambers (Figure 2(a)). Once chrysalids formed, they were misted daily with distilled water to prevent desiccation. Chrysalids were often observed twitching and pulsing in response to the stimulation of misting. Adult butterflies emerged in these containers. Butterflies emerged in winter, and therefore could not be released to their maternal field sites. Instead, butterflies were killed by freezing after their wings had fully expanded and dried to preserve them for future study.

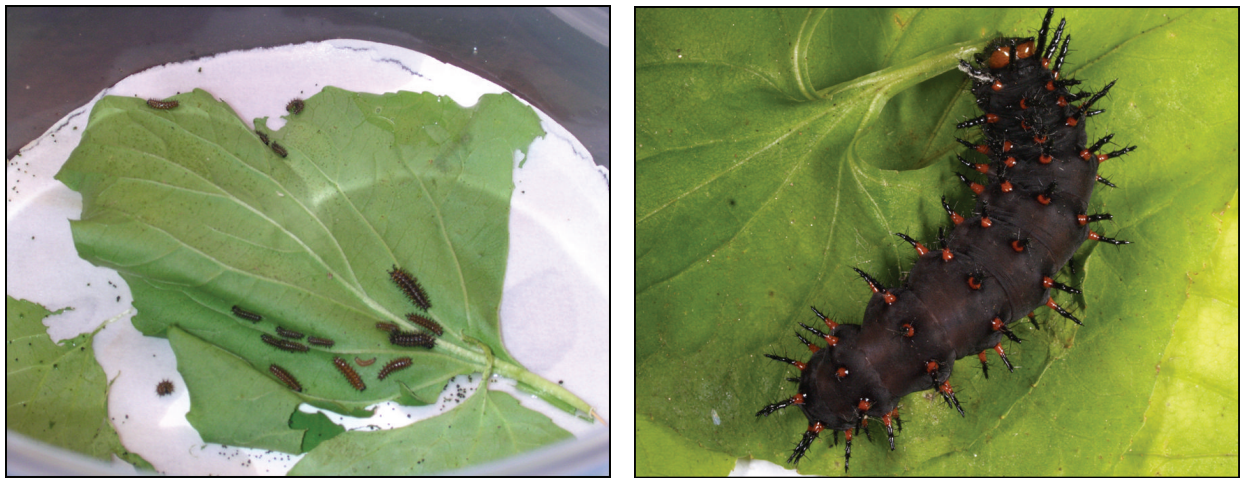
3. Results

We were able to bypass first instar diapause with our rearing protocol, reducing the 10–12-month life cycle of *A. diana*

Table 1: Egg production and survival of *Argynnis diana* females held in captivity for rearing and their progeny.

Butterfly ID	Total number of eggs	Number of hatched eggs (hatch rate)	Number of larvae surviving to second instar	Number of larvae surviving to last instar	Number of pupating larvae	Number of adults (% ecolosion)
NC-1	1605	746 (47%)	203 (27%)	54	24	6 (25%)
NC-2	0	0 (0%)	—	—	—	—
NC-3	1499	428 (29%)	115 (27%)	—	—	—
NC-4	1574	881 (56%)	155 (18%)	36	13	7 (54%)
NC-5	1041	771 (74%)	211 (27%)	72	19	3 (16%)
NC-6	1163	834 (72%)	427 (51%)	40	10	—
NC-7	1265	816 (65%)	280 (34%)	55	8	2 (25%)
NC-8	1386	963 (70%)	307 (32%)	—	—	—
NC Totals	9533	5440 (57%)	1698 (27%)	257	74	18 (24%)
GA-1	50	25 (50%)	15 (60%)	9	7	5 (71%)
GA-2	50	20 (40%)	6 (30%)	5	—	—
GA-3	50	29 (58%)	20 (69%)	16	9	3 (33%)
*GA Totals	150	74 (49%)	41 (53%)	30	16	8 (50%)
TN-1	50	28 (56%)	12 (43%)	4	—	—
TN-2	50	35 (70%)	29 (83%)	17	8	4 (50%)
TN-3	50	30 (60%)	25 (83%)	13	5	3 (60%)
*TN Totals	150	93 (62%)	66 (69%)	34	13	7 (54%)

* Ova collected from three females in Rabun County, GA ($N = 150$), as well as three females in Logan County, TN ($N = 150$), were donated from colleagues, E. Smith and I. Finkelstein.



(a) (b)

Figure 2: (a) First and second instars (*Argynnis diana*), approximately 10 days old; (b) sixth instar *A. diana* larvae, approximately 90 days old.

down to 3-4 months. The eight females collected from North Carolina lived between 12 and 32 days (mean = 26 days, SE = 2) and produced a total of 9,533 ova (mean = 1,362 ova/female, SE = 81) (Table 1). One female from North Carolina died without ovipositing; this female was included in all analyses. Peak egg production by captive females occurred between the 19-20th of September, with a maximum of 323 ova collected on Sept 20th (Figure 3).

3.1. Larval Survival. Viable eggs darkened and turned opaque prior to hatching within two weeks of being laid, while inviable ones remained light in color and often collapsed. First instars were observed eating their way out of their egg casings. The mean larval hatch rate was highest in ova from Tennessee-collected females (62%, SE = 0.03), followed by North Carolina-collected females (57%, SE = 0.09), and Georgia-collected females (49%, SE = 0.03) (Table 1).

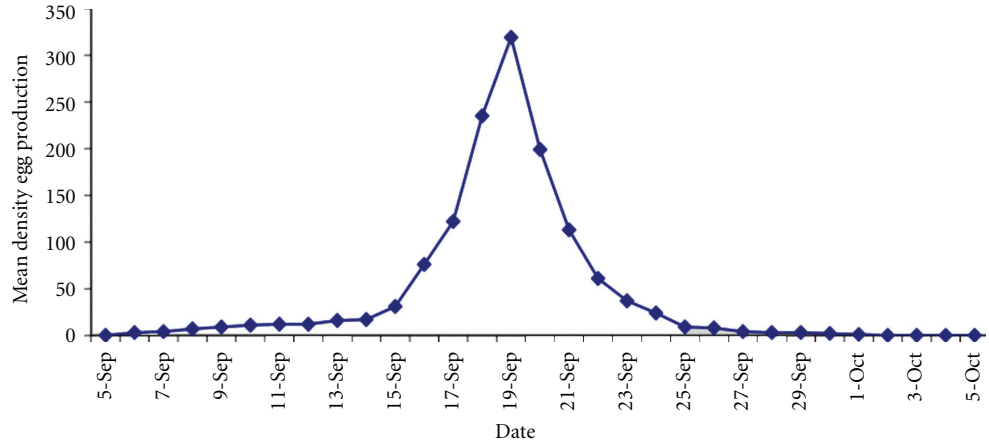


Figure 3: Mean daily egg production for eight Diana fritillary females collected from western North Carolina, fall 2007.

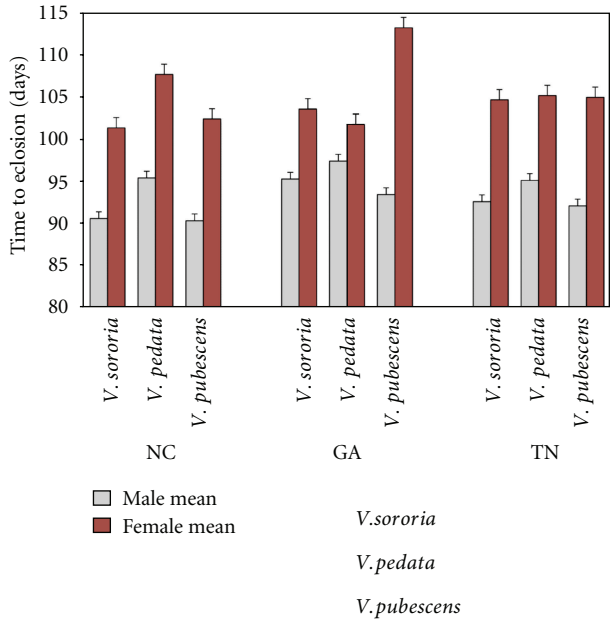


Figure 4: Mean time to eclosion (+/− SE) for *A. diana* offspring from North Carolina, Georgia, and Tennessee reared on three Southern Appalachian violet (*Viola*) species, *V. sororia, V. pedata,* and *V. pubescens*. The only significant differences in time to eclosion was between the sexes ($df = 1, F = 54.11, P = 0.0$).

3.2. Adult Survival. A total of 16 females and 13 males were reared to adulthood. The male to female ratio from the North Carolina site was 3 : 6; in Georgia was 4 : 1, and in Tennessee was 1 : 3. ANOVA tests were conducted to determine the effects of location, host plant species, and sex on the time to adult eclosion. The only significant difference in time to eclosion was between the sexes ($df = 1, F = 54.11, P = 0.0$) with males always emerging significantly earlier than females, regardless of location and host plant species (Figure 4).

4. Discussion

Results of our rearing trial demonstrate that first instar larval diapause can be broken in *Argynnis* (*Speyeria*) *diana* larvae, shortening the life cycle of this species by several months in a controlled laboratory. These results are likely applicable to many other Greater Fritillary species and should prove useful to future studies and rearing efforts within this group of butterflies. The hatch rate of *A. diana* eggs from our three Southeastern field sites was approximately 50%, which is not atypical of other nymphalid laboratory rearing protocols [12, 17].

Females retained their ova after being captured, as egg production was very low during the first week of captivity. A sharp peak of oviposition activity occurred for all females between Sept 19 and Sept 21. During these 48 h, the majority of all fertilized ova were deposited, and levels steadily declined after this time. Most females expired within 4 weeks after capture, and within three days of their last eggs being produced. Fritillaries are widely known to hold eggs in the fall so as to increase survival of first instars [3, 4]. Our results support the hypothesis that *A. diana* females carry a majority of their eggs through the hot dry summer, laying eggs later in the season when temperatures are cooler and rains more frequent. Our findings help explain why *A. diana* males die off on average much earlier than females which can persist into early October [18]. We acknowledge, as well, that the extremely sharp peak in oviposition observed in our study could alternatively be an artifact of gravid females being held captive in small paper bags after their capture.

We were quickly overwhelmed caring for the large number of ova produced by the North Carolina females, and strongly suggest that future applications of this protocol adequately prepare for the possibility of each female producing over a thousand fertilized ova. It has been suggested to the authors that following some of the rearing procedures of James [12], who used overwintering blocks, would likely have resulted in higher overall adult survivorship, perhaps up to a higher order of magnitude [10]. Diapause can be broken after 2-3 months of larvae being refrigerated. If adults could be mated in captivity, carried through additional lab generations, and then brought into synchrony, some generations later, they could then be used to augment wild populations, which is often the aim for the conservation

of certain species. In our case, however, we aimed to study the basic biology of an imperiled butterfly species to better understand and document its larval behavior, not to repopulate natural areas using our procedure.

Our study revealed no preference toward *V. sororia V. pubescens,* and *Viola pedata*. Future studies should aim at identifying host plant preference during each individual instar in order to understand preference and survival throughout the course of larval development. Controlled feeding experiments assessing *Viola* preference by *A. diana* with a larger number of regional violet species, and across different larval instars would provide a clearer picture of the *A. diana* life cycle. Specific measurements of larval performance, such as tracking mass of each larva over time, and/or frass collection from each larvae, would also add some clarity to our results.

With record numbers of butterfly species facing extinction, captive rearing programs have become a popular management tool to replenish and repopulate certain threatened populations. The captive breeding of at-risk butterfly populations has played an important role in the recovery and ongoing conservation of many threatened and endangered species [17–20]. We emphasize that our protocol breaks the butterfly's natural larval diapause and is therefore in no way intended as a long-term conservation plan for *A. diana*. *Argynnis* butterflies are univoltine insects that overwinter as unfed first instars, mate in June or July, and then enter a reproductive diapause until August-September. Forced development, by breaking larval diapause prematurely, actually results in removing reproductive individuals from the population. We suggest that aggressive habitat preservation, with ample larval and adult host plants, is a more appropriate management strategy for protecting *A. diana* for the long term. While larval host plant availability can result in quick and drastic population declines in endangered butterfly species [20, 21], nectar plant diversity can also be a limiting factor for adult butterfly survival [22]. Although we did not detect a significant difference in *A. diana* larval feeding on violets in this study, previous researchers have suggested that *A. diana* adults preferentially feed from high quality nectar sources such as milkweed, compass plant, and coneflower [23, 24]. Maintaining a diverse array of native nectar and *Viola* plants should be a conservation priority for this species.

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