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Research

Host Plant and Thermal Stress Induce Supernumerary Instars in Caterpillars

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Abstract

Environmental stressors may induce variation in the number of larval instars of holometabolous insects. Host plant quality and ambient temperature can both induce this life history shift in the silver-spotted skipper, *Epargyreus clarus* (Cramer 1775) (Lepidoptera: Hesperiidae). To better understand this phenomenon, we raised larvae on high-quality (kudzu) or low-quality (wisteria) host plants in growth chambers under three temperature regimes (20, 26, and 32°C) that were either constant or diurnally fluctuating ($T \pm 5$°C), and recorded survival and incidence of supernumerary instars. Larvae feeding on the low-quality host and/or experiencing thermal stress were more likely to show supernumerary development (SD). A subset of treatments yielded a mix of SD and TD (typical development) individuals, allowing for comparisons between phenotypes. Under the most stressful treatment (20 ± 5°C, wisteria), development time was 9 days longer in SD than in TD individuals; by contrast, at typical summer temperatures (26 ± 5°C), also on wisteria, total development time did not differ between these two phenotypes. Head capsules of both second and third instars were smaller in SD individuals. A retrospective logistic regression analysis indicated that third-instar head capsule size could be used to predict expression of the SD phenotype. By the ultimate instar, however, there were no detectable differences in head capsule size, and SD and TD individuals did not differ in pupal mass, strongly suggesting that the SD phenotype functions as a compensatory mechanism allowing *E. clarus* larvae to achieve the same size at metamorphosis (a strong fitness correlate) as TD larvae.

Keywords: insect development, instars, caterpillar

Entomologists commonly consider the number of larval or nymphal instars as a fixed characteristic of insect life cycles (Dyar 1890, Esperk et al. 2007). However, for a number of taxa representing almost all major insect orders, both hemimetabolous and holometabolous, it is not uncommon for individuals to exhibit variation in the number of immature stages required to complete development (Esperk et al. 2007). The expression of supernumerary instars during development has been shown to occur in response to a range of environmental factors, including food quality or quantity, temperature, photoperiod, rearing density, presence of parasitoids, and humidity (Morita and Tojo 1985, Esperk et al. 2007, Barraclough et al. 2014, Grunert et al. 2015). In some hemimetabolous taxa, including orthopterans (e.g., *Chortippus bunneus*; Hassall and Grayson 1987) and roaches (e.g., *Diploptera punctata*; Woodhead and Paulson 1983), individual insects can take advantage of favorable conditions and add an additional nymphal stadium prior to the molt to adulthood, enabling attainment of a larger final body size, a common fitness correlate in insects (Slansky and Scriber 1985, Honek 1993, Murphy and Lill 2010). The strongly female-biased expression of supernumerary instars (e.g., 54/56 described examples in Esperk et al. 2007, but see Penz and Corrêa Bueno 2018 for a counter-example) suggests that fecundity selection (Lande 1982) has likely played a role in the evolution of these alternative developmental phenotypes, which are characterized by prolonged larval development and sexual size dimorphism (Esperk and Tammaru 2006, Teder 2014).

Unfavorable conditions also commonly induce the production of extra instars (in both hemi- and holometabolous insects), and these phenotypes are hypothesized to represent compensatory responses in which developmental plasticity enables individuals to accommodate small size and/or slow growth (Morita and Tojo 1985, Esperk et al. 2007, Barraclough et al. 2014, Grunert et al. 2015). Among the Lepidoptera, the taxon for which we have the most information, supernumerary instars are reported to occur primarily in response to unfavorable environmental conditions and/or for taxa that require multiple growth seasons to complete development (e.g., wood-boring larvae; Solomon 1973). Under stressful circumstances, adding one or more stadia can allow a relatively small individual insect to catch up to its contemporaries and/or to reach the threshold size necessary for metamorphosis (Lee et al. 2012, Grunert et al. 2015). This phenomenon has been described for larvae from various families, including the Sphingidae (e.g., *Manduca* spp.; Kingsolver 2007), Noctuidae (e.g., *Spodoptera* spp.; Lee et al. 2012), and Cossidae

(e.g., *Prionoxytus robiniae*; Solomon 1973). In the butterfly *Papilio hectorides*, this compensatory response allows individuals exhibiting the supernumerary phenotype to attain larger sizes than their conspecifics (Penz and Corrêa Bueno 2018).

Within the Lepidoptera, the schedule of molts, which includes both the total number and timing of larval instars, is regulated by a complex interplay of environmental factors (e.g., diet quality and temperature) and internal regulators (e.g., hormone titers and neuroendocrine clock-like mechanisms); Suzuki et al. 2013, Grunert et al. 2015. For each instar, there is evidence for a 'critical weight' that, once surpassed, initiates an irreversible cascade of physiological events terminating in a molt to the next larval instar, or, for the final molt, to the pupa (Nijhout and Williams 1974, Callier and Nijhout 2011, Barraclough et al. 2014, Davidowitz et al. 2016). This critical weight is determined by a limit to the supply of oxygen that can be delivered to the tissues by a fixed volume of tracheal tissue, which is produced at the start of a given instar (Callier and Nijhout 2011). Once past the critical weight, if larvae are stressed and reduce their food intake, or are feeding on low-quality host plant tissues, their molt may result in an 'intercalary' instar that is intermediate in size between those of larvae following the typical schedule.

Previous work on the larvae of silver-spotted skippers (*Epargyreus clarus)* has demonstrated that both thermal and nutrient stress can induce supernumerary instars (Abarca et al. 2018). To examine the expression of this alternative developmental phenotype, we analyzed the responses of larvae exposed to different degrees of environmental stress (thermal, nutrient, and a combination) in the laboratory. We compared the expression of developmental phenotype [typical development (TD) vs. supernumerary development (SD)] under different degrees of environmental stress. In addition, we evaluated the costs and benefits of undergoing supernumerary development by comparing fitness components (development time and pupal mass) between TD and SD larvae that experienced the same environment (i.e., host plant and temperature regime). Finally, we measured the head capsule size of individual larvae over the course of development (under a subset of conditions) to determine when and if head capsule size portends the eventual expression of the supernumerary instar.

Methods

Study System

Epargyreus clarus (Lepidoptera: Hesperiidae) is a multivoltine butterfly native to North America. Its range includes the continental United States and southern Canada, where it typically exhibits two to three generations per year. Caterpillars feed on the foliage of several species of legumes in the family Fabaceae (Wagner 2005), on which they build characteristic leaf shelters. Each caterpillar inhabits a shelter for several days; once it outgrows the shelter, the larva leaves and constructs a new one, building a total of four or five during the course of larval development (Weiss et al. 2003). The larvae of *E. clarus* typically pass through five larval instars. A distinct morphological shift occurs at the start of the fourth instar, when large, brightly colored orange false 'eye spots' appear on the anterior portion of the head capsule (Fig. 1). Larger orange false eye spots are present in fifth instar larvae. *Epargyreus clarus* are locally multivoltine, with one to two generations that undergo direct development (i.e., development proceeds uninterrupted through the pupal stage, with adult butterflies emerging after about 10 days), and, later in the season, a generation that undergoes diapause during the pupal stage, with adult butterflies emerging the following spring, after several months. Changing photoperiod is the environmental cue that most strongly determines whether or not larvae induce diapause (Abarca 2019).

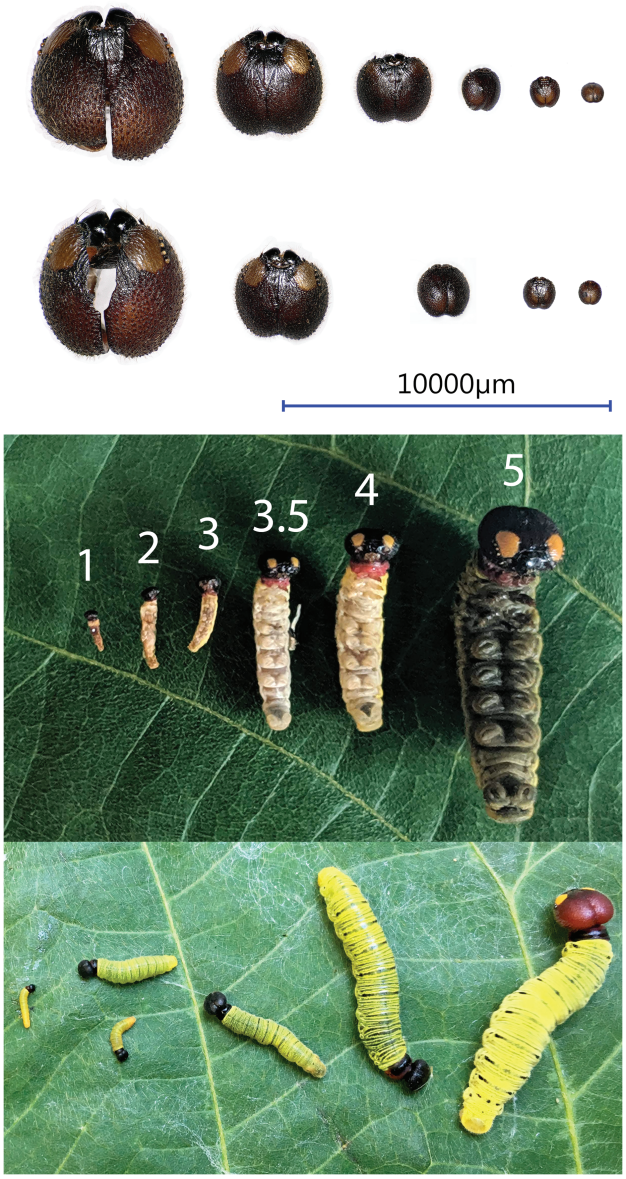

Fig. 1. Top panel: Head capsules of *Epargyreus clarus* larvae exhibiting supernumerary development (SD, top) and typical development (TD, bottom) phenotypes. Each set of head capsules was collected from the same individual. Bottom panel: Larvae of each of the six instars that characterize supernumerary development.

Because the expression of supernumerary instars is induced by stress, which often results in low survival, studying this phenomenon can be challenging. Here, we took advantage of several existing data sets (collected in 2016 and 2017 to examine the interactive effects of temperature regimes, host plant, and photoperiod on insect fitness and diapause induction; Abarca et al. 2018, Abarca 2019), in order to explore the phenomenon of supernumerary development. Both years we reared larvae for the duration of their development in growth chambers (models 136 VL and 130 VL, Percival Scientific, Perry, IA) under a range of temperature and photoperiod regimes. Larvae were fed on high-nitrogen (kudzu) or low-nitrogen (wisteria) host plants (Rosenwald et al. 2017), both of which are commonly used by wild populations of *E. clarus*, and which are known to influence larval days to pupation (Abarca et al. 2018) and developmental phenotype (Abarca 2019).

Larvae were reared individually in 16 oz. plastic deli cups containing moist filter paper, and were fed *ad libitum* with cut foliage of their assigned host plant (a salad of leaves collected from more than five individual plants growing in and around the Georgetown University campus, Washington, DC). In 2016, we reared individuals under three constant temperature regimes [20, 26, and 32°C, $N = 40$ per treatment, photoperiod of 16:8 (L:D) h]; in 2017, we implemented three fluctuating temperature regimes with diel sinusoidal oscillation (20 ± 5, 26 ± 5, 32 ± 5°C, $N = 80$ per treatment). In 2017, half of the larvae within each treatment experienced a constant photoperiod [14:10 (L:D) h] and the other half a decreasing photoperiod (decreasing day length from 14 to 12 h 50 min, over 7 wk). Different combinations of host, temperature and photoperiod implemented during 2017 resulted in a mix of larvae undergoing diapause versus nondiapause phenotypes. Because diapause induction affects development time and pupal mass (Teder et al. 2010), we separated diapausing and nondiapausing individuals for analyses involving these response variables.

We recorded survival and incidence of developmental phenotypes in all treatments. Only certain combinations of temperature, photoperiod and host plant prompted expression of the supernumerary instar phenotype; our focus was to contrast the developmental phenotypes (SD vs TD); thus, we restricted our analyses to the subset of treatments that produced phenotypic mixtures (i.e., that had more than five individuals of each phenotype; these treatments are indicated in Fig. 2). First, we compared development time and pupal mass between individuals demonstrating SD vs.TD phenotypes growing under two temperature regimes (20 ± 5 and 26 ± 5°C) and feeding on the low-quality host (wisteria). To avoid confounding effects of diapause induction, we examined developmental time and pupal masses only of individuals that initiated diapause (from both photoperiod treatments). Next, we compared head capsule size between SD and TD phenotypes, which involved collecting the head capsules of caterpillars feeding at 20 ± 5°C on wisteria ($N = 44$ caterpillars, 212 head capsules) and on kudzu ($N = 71$ caterpillars, 303 head capsules); we measured the area (mm^2) using ImageJ software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD, https://imagej.nih.gov/ij/, 1997–2018), and from that, estimated diameter (assuming circular head capsules). In addition, we tested whether head capsule size during earlier instars predicted the subsequent expression of the SD phenotype. For this analysis we used only larvae feeding on wisteria, as only a small proportion of larvae feeding on kudzu expressed the supernumerary phenotype.

Data Analysis

We performed all analyses in R 3.5.1 (R Core Team 2018). We used contingency tables to compare the frequencies of mortality and expression of the SD phenotype between rearing hosts within each oscillating temperature regime. We used two-way ANOVA to compare larval development time (egg hatch to pre-pupa) and pupal mass between SD and TD larvae fed on wisteria at 20 and 26°C, with developmental phenotype (SD vs TD), temperature treatment, and their interaction as explanatory class variables. We used analysis of variance (ANOVA) to compare head capsule size between SD and TD larvae. To compare the head capsule size of the supernumerary instar (designated here as instar 3.5; see 'Discussion') between host plant treatments, we used a one-way ANOVA. Individual two-way ANOVAs were run for instars 1, 2, 3, and 4, and included developmental phenotype (SD vs TD) and rearing host (wisteria vs kudzu) as explanatory variables. Due to reduced availability of fifth-instar head capsules from individuals feeding on kudzu, we compared fifth-instar head capsule size between SD and TD development with a one-way ANOVA, including only larvae feeding on wisteria. Data satisfied the assumptions of ANOVA in all cases. Our previously published work on these same data sets indicated that performance

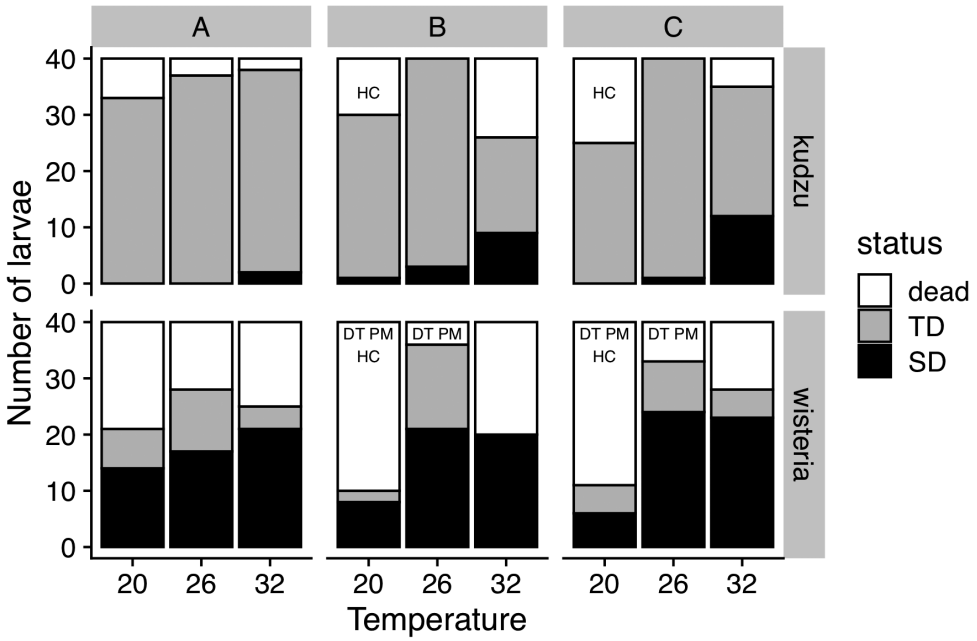

Fig. 2. Larval survival (instar 1 to pre-pupa) and phenotype incidence (TD: typical development, SD: Supernumerary development) of larvae growing under A) constant temperature and constant photoperiod, B) oscillating temperature and constant photoperiod, and C) oscillating temperature and decreasing photoperiod. In all three cases, the initial sample was 40 larvae per temperature treatment. Letters indicate the treatments included in subsequent analyses. DT: development time; PM: pupal mass; HC: head capsule size. We pooled larvae from groups B and C (different day lengths) that were growing under the same temperature and host conditions and that induced diapause.

variables were not sexually dimorphic (Rosenwald et al. 2017, Abarca et al. 2018), so we did not include sex as a predictor variable in these analyses.

Finally, to determine the stage in the growth trajectory at which we could first predict the expression of the SD phenotype, we performed logistic regression (GLM, binomial distribution, logit link), using head capsule size as the continuous predictor variable and developmental phenotype (SD or TD) as the binomial response variable (after Etilé and Despland 2008). For these analyses, we included data from the single experimental treatment (larvae reared on wisteria at 20 ± 5°C) that produced both SD and TD phenotypes in comparable numbers. We performed analyses for instars 2 and 3 to determine retroactively if and when the novel phenotype could be detected. These analyses enabled us to ask whether larvae that are falling behind their peers in terms of growth are predisposed to express the SD phenotype. Because neonate larvae (first instars) eclose from their eggs with sclerotized head capsules in place, experimental treatments should not affect first instar head capsule size.

Results

Across all treatment combinations, we observed strong effects of host plant on both mortality and the proportion of larvae expressing the SD phenotype (Fig. 2). Under optimal rearing temperatures (26 ± 5°C; middle bars in Fig. 2B and C), larval mortality was low on both host plants (0% on kudzu, ~20% on wisteria), as anticipated. Under cold stress (20 ± 5°C), both mortality (χ^2 = 28.97; P < 0.0001) and the proportion of SD larvae (χ= 40.34; P < 0.0001) were significantly higher for larvae fed on wisteria (first bars in Fig. 2B and C). At that temperature regime, mortality of caterpillars feeding on kudzu was 31% (N = 80), and of the survivors, only a single individual exhibited the SD phenotype. At the same temperature, mortality of caterpillars feeding on wisteria was considerably higher (~74%; N = 80), and a much higher proportion of the survivors (67%) exhibited the SD phenotype. Under heat stress (32 ± 5°C), half of the larvae feeding on wisteria died (N = 80), and of the survivors, 91% expressed the SD phenotype. For larvae feeding on kudzu at these high temperatures, mortality was about 25%, and only about a third of the survivors exhibited the supernumerary phenotype (third bars in Fig. 2B and C).

Development time of larvae feeding on wisteria (number of days from egg hatch to pre-pupa) was significantly affected by developmental phenotype (SD vs TD), temperature treatment, and their interaction ($F_{3,50}$ = 217.1, P < 0.0001, N = 54; Table 1, Fig. 3). Individuals feeding at 20 ± 5°C on wisteria required ~30 d longer to reach the pre-pupal stage (an increase of 86%) than individuals feeding at 26 ± 5°C. For larvae reared at 20 ± 5°C, total larval development time was significantly increased in SD caterpillars relative to TD caterpillars, and *all* of the difference in development time between SD and TD caterpillars (~9 d or 16% increase in SD caterpillars) was explained by the presence of the supernumerary instar (Table 2). By contrast, at optimal temperatures (26 ± 5°C), SD caterpillars had only a slightly longer total development time (~2 d or ~6% increase) relative to TD caterpillars and this difference did not correspond with the length of the intercalary instar, which lasted on average ~5 d (Supp Tables 1 and 2 [online only]).

For these same treatment combinations, *E. clarus* pupal mass was strongly affected by temperature ($F_{3,48}$ = 11.71, P < 0.0001, N = 52, Table 3, Fig. 4) with individuals reared at 26 ± 5°C producing pupae that were on average 18% heavier than pupae reared at 20 ± 5°C. However, unlike development time, pupal masses did not differ between developmental phenotypes (Table 3, Fig. 4).

As expected, the two-way ANOVA model for head capsule size was not significant for 1st instar caterpillars ($F_{3,99}$= 0.9211, P = 0.4336; Fig. 5), since head capsule size is set at egg hatch, prior to the implementation of experimental treatments. However, we did detect significant main effects (but not interactive effects) of both host plant and developmental phenotype on the diameter of *E. clarus* head capsules in instars 2 and 3 (Developmental Phenotype: second: $F_{1,88}$= 12.8636, P = 0.0005; third: $F_{1,82}$ = 17.9, P <0.0001; host: second instar: $F_{1,88}$= 8.8960, P = 0.004; third instar: $F_{1,82}$ = 54.00, P < 0.0001). For individuals expressing the SD phenotype (i.e., those adding an extra larval molt), head capsule diameter did not differ between rearing hosts ($F_{1,23}$= 3.761, P = 0.06, N = 25). Upon reaching the fourth or penultimate instar, developmental phenotype significantly predicted head capsule diameter ($F_{1,82}$ = 23.66, P < 0.0001, N = 86), with SD larvae having 12–20% *larger* head capsules (depending on host plant) than TD larvae, but neither rearing host ($F_{1,82}$ = 0.11, P = 0.74) nor its interaction with phenotype ($F_{1,82}$ = 1.55, P = 0.22) significantly predicted head capsule diameter. Finally, in the ultimate instar (fifth), we did not detect a significant effect of phenotype on head capsule diameter of individuals feeding on wisteria ($F_{1,24}$ = 2.75, P = 0.1, N = 26).

By retrospectively examining the growth trajectories of second- and third-instar larvae reared at 20 ± 5°C feeding on wisteria (a subset of the data analyzed in the two-way ANOVA described above), we found that head capsule size of third instar-larvae predicted future developmental phenotype (Wald χ_1^2 = 8.9571, P < 0.01, N = 37, Fig. 6), but this signal was not yet evident in second instars (Wald χ_1^2 = 2.2, P = 0.14).

Discussion

Environmental stressors that negatively affect larval survival to metamorphosis (e.g., feeding on wisteria and/or being reared at temperatures above or below the thermal optimum for *E. clarus*; Fig. 2) also tend to induce the expression of the SD phenotype. At all temperature regimes, larvae reared on wisteria were much more likely to add an extra instar than were those reared on kudzu; wisteria leaves have a lower leaf nitrogen and water content than do kudzu leaves (Rosenwald et al. 2017), and are also likely to have a different secondary compound profile (Wink and Mohamed 2003). Even under optimal temperatures, feeding on low-quality wisteria was sufficient to induce at least half of the surviving larvae to express the SD

Table 1. Analysis of variance (type II test) for the effect of phenotype (TD vs SD) and temperature (20 ± 5 vs 26 ± 5 °C) on development time (hatch to pre-pupae) of *Epargyreus clarus*

Source	df	SS	*F*	*P*
Phenotype	1	252.5	19.468	<0.0001
Temperature	1	8194.5	631.7	<0.0001
Phenotype * temperature	1	154.5	11.91	0.001
Residuals	50	648.6		

phenotype; adding thermal stress (either high or low) increased SD expression even more (up to 80%; Fig. 2B, wisteria, 20°C).

The vast majority of larval mortality occurred in the first and second larval instars, and thus we cannot know which developmental phenotype those larvae would have followed, and therefore, whether or not plasticity in developmental phenotype allows for survival under stressful conditions. This hypothesis could be tested in future studies by comparing selected lines (monomorphic for developmental phenotype), as was done with *Manduca sexta* (Kingsolver 2007, Diamond et al. 2010).

Under oscillating regimes (treatments B and C), larvae had to endure temperatures that may have surpassed the thermal maxima or minima for some individuals: as low as 15°C for a portion of their day (in the 20°C treatments) and as high as 37°C (in the 32°C treatments). Caterpillar responses to temperature stress were clearly not symmetrical, since heat stress induced the SD phenotype considerably more often than cold stress, particularly when larvae were reared on kudzu. Within each treatment (A–C), more larvae survived when experiencing mean temperatures of 26°C, indicating this was the least stressful regime.

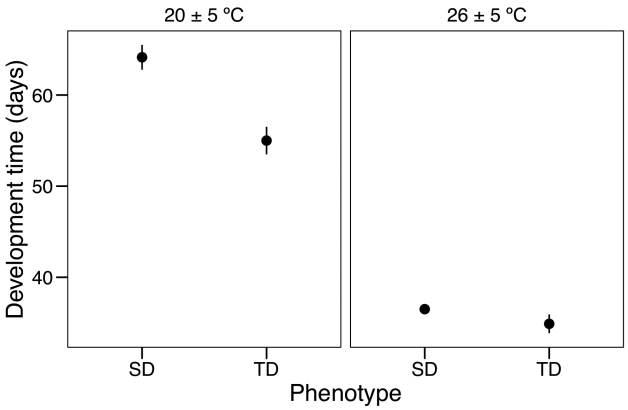

Fig. 3. Development time (hatch to pre-pupae) by temperature (20 ± 5 and 26 ± 5°C) and phenotype (SD: supernumerary development, TD, typical development) of larvae feeding on wisteria.

To interpret these results, it is important to consider the physiological processes that occur during larval development. In each instar the larvae grow until they reach a critical weight, about halfway through the molt (Suzuki et al. 2013), at which point the corpora allata (CA) stop releasing juvenile hormone (JH). Once the remaining JH is metabolized in the hemolymph, a pulse of another hormone, ecdysone, regulates the molt (Nijhout and Williams 1974, Davidowitz et al. 2016). After the CA switch off, the cascade of hormonal and physiological events that lead to molting cannot be reversed; if, during this 'post-commitment phase', the larvae are stressed by temperature or nutritional intake, they will still molt but will do so at a smaller size. It is plausible that this process results in the production of the extra (intercalary) instar in *E. clarus*.

Effects of Host Plant

Because our two rearing hosts differ in the overall concentration of leaf nitrogen, the strong host plant effects on SD expression are most parsimoniously explained by nutrient limitation of larval growth; once larvae surpass the critical weight, their ability to add biomass may be compromised on a lower-quality host like wisteria, forcing them to molt at a smaller size. The significantly longer instar durations of *E. clarus* reared on wisteria, relative to kudzu (Table 2), support this interpretation. The composition and concentration of secondary chemicals in leaves of the two host plants, which were not measured in this study, may also contribute to these differential responses. While our quantitative inferences are limited to the two contrasting host plants described here, we have also reared *E. clarus* larvae on a wider assortment of leguminous host plants used by local skipper populations (American hog peanut, *Amphicarpa bracteata*; cultivated soybean, *Glycine max*; showy tick-trefoil, *Desmodium canadense*; and black locust, *Robinia pseudoacacia*) that differ in N content. Larvae reared on these host plants in the laboratory at least occasionally added an extra instar, and incidence roughly tracked host plant quality, as measured by % foliar N: plants with lower N added extra instars more commonly than did higher-N plants, consistent with what we report here for wisteria relative to kudzu.

Table 2. Duration of larval stadia by developmental phenotype of caterpillars growing at oscillating temperature (20 ± 5°C) and feeding on either kudzu or wisteria, days ± SE (*N*)

Phenotype	Instar 1	Instar 2	Instar 3	Instar 3.5	Instar 4	Instar 5	Total larva
kudzu							
TD	6.2 ± 0.17 (54)	7.06 ± 0.22 (54)	6.22 ± 0.25 (54)	-	8.37 ± 0.29 (54)	14.24 ± 0.55 (53)	42.05 ± 0.9 (54)
SD	6 (1)	17 (1)	4 (1)	3 (1)	9 (1)	19 (1)	58(1)
wisteria							
TD	6.71 ± 0.42 (7)	8.6 ± 0.37 (7)	8.14 ± 0.67 (7)	-	12.29 ± 1.06 (7)	19.28 ± 0.56 (7)	55 ± 1.51 (7)
SD	7.08 ± 0.56 (13)	9 ± 0.35 (12)	8.15 ± 0.52 (13)	9.26 ± 0.52 (14)	12.21 ± 0.61 (14)	18.43 ± 0.84 (14)	64.14 ± 1.36 (14)

Table 3. Analysis of variance (type II test) for the effect of phenotype (TD vs SD) and temperature (20 ± 5 vs 26 ± 5°C) on *Epargyreus clarus* pupal mass

Source	df	SS	*F*	*P*
Phenotype	1	6030	2.07	0.16
Temperature	1	88838	30.44	<0.0001
Phenotype * temperature	1	2051	0.70	0.4060
Residuals	48	140095		

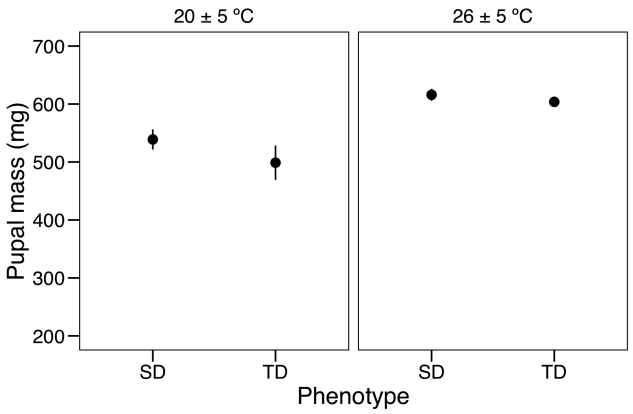

Fig. 4. Pupal mass (mean ± SE) by temperature (20 ± 5 and 26 ± 5°C) and phenotype (SD: supernumerary development, TD, typical development) of larvae feeding on wisteria.

Effects of Temperature

Low temperatures are expected to reduce metabolic activity in poikilotherms, which in our system was clearly evident in the extended development times of larvae at 20°C. Larvae feeding at this temperature regime are forced to stretch the time spent in each instar during the pre-commitment phase (i.e., prior to reaching the critical weight for that instar), but once they reach critical weight, the time to molting is physiologically constrained (Esperk and Tammaru 2010, Davidowitz et al. 2016). While this post-commitment phase is predicted to be longer under low temperatures (due to the dynamics of enzyme kinetics), it may be insufficiently long to allow third instar *E. clarus* larvae to reach the size of their contemporaries feeding at optimal temperatures, forcing them to molt at a smaller size and thereby producing the SD phenotype. At local field sites, we have observed that *E. clarus* larvae experimentally reared on a range of host plants varying in host quality all exhibit some SD development in the late season (i.e., October), when temperatures often approach or drop below 20°C.

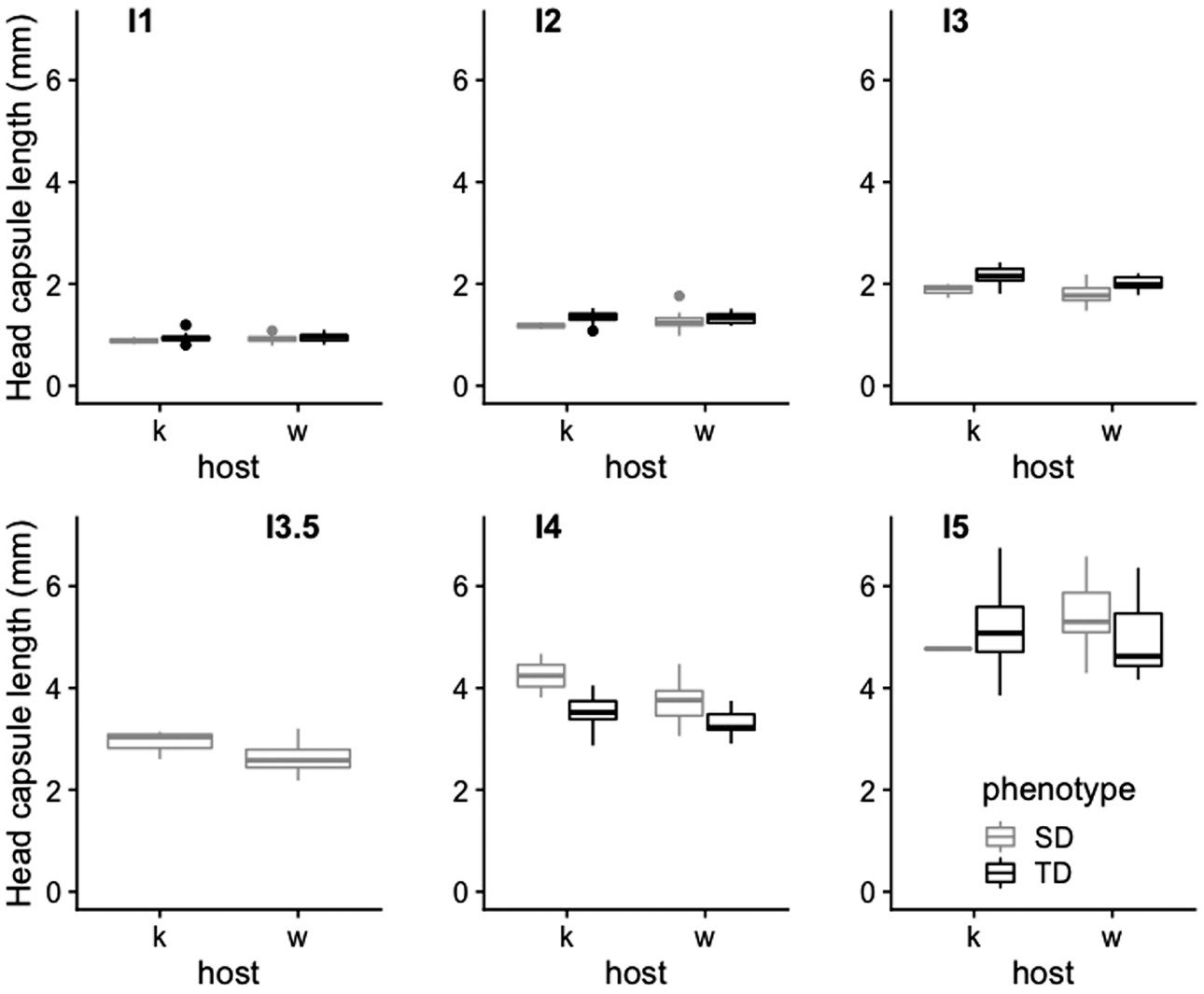

Fig. 5. Head capsule size (diameter) of each larval instar by host (k, kudzu; w, wisteria) and phenotype (SD: supernumerary development, TD, typical development) of individuals growing at 20 ± 5°C.

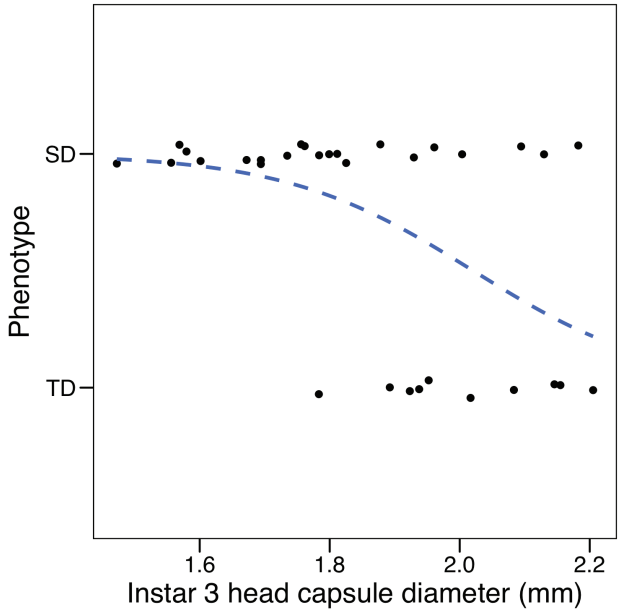

Fig. 6. Logistic regression showing the relationship between phenotype (SD: supernumerary development, TD, typical development) and head capsule diameter of third instar larvae feeding on wisteria.

In general, as rearing temperatures approach an insect's thermal maximum, growth rates remain relatively high, so the expression of the SD phenotype under high temperature stress is somewhat more challenging to interpret. At very high temperatures (38°C), survival in *E. clarus* has been shown to be severely reduced at all life stages (Abarca et al. 2018); however, our 32°C high temperature is below the thermal maximum for local populations of *E. clarus* (Abarca et al. 2018), which routinely encounter temperatures this high or higher. The duration of the post-commitment 'window' within which larval growth can be completed is presumably much shorter at 32°C than at 26°C; thus *E. clarus* larvae may be either time-constrained or energy-constrained (due to increased respiratory demands; e.g., Reynolds et al. 1985, Kingsolver and Woods 1997), resulting in molting at a smaller-than-typical size.

To explore the consequences of expressing SD versus TD phenotypes on traditional performance measures (e.g., developmental trajectory, pupal mass), we narrowed our scope of inference to a smaller set of host plant × temperature combinations that yielded a reasonable mix of these dimorphic individuals (see 'Methods').

Development Time

On wisteria, total development time (egg hatch to pre-pupa) of *E. clarus* larvae expressing the SD phenotype was significantly longer than that of larvae expressing the TD phenotype only at 20 ± 5°C, which was the most stressful treatment; by contrast, we did not detect an effect of phenotype on total development at 26 ± 5°C (i.e., there was a temperature × phenotype interaction). At 20 ± 5°C, the additional nine days required to complete larval development in SD individuals compared to TD individuals was entirely accounted for by the additional supernumerary instar (which lasted a mean of 9 d; Table 2). Low temperatures reduce the rate not only of metabolism, but also movement, both of which can extend the molting process. In other Lepidoptera Esperk and Tammaru (2004) found that the period of inactivity preceding a molting event is costly in terms of growth, as it results in a significant arrest of biomass accumulation (because larvae do not feed). While both SD and TD larvae would have experienced prolonged periods of inactivity surrounding each molt under cold stress, SD larvae had to endure six of these periods whereas TD larvae had to endure only five. Etilé and Despland (2008) also found that total development time increased with the number of larval instars in *Malacosoma disstria*.

Unexpectedly, under more benign temperature conditions (26 ± 5°C), the mean development time of SD caterpillars was just 2 d longer than that of TD caterpillars; the 6% difference was statistically indistinguishable with our limited sample size. These similar development times came about because in SD larvae, third and fourth instar durations were two days shorter than those stages in the TD phenotype, suggesting that the ability of *E. clarus* larvae to temporally compensate for adding an intercalary instar is at least partly temperature-dependent. Quantifying the duration of circum-molt periods of inactivity for each instar at different temperatures would help clarify the dynamics of larval development time.

Pupal Mass

Unlike development time, pupal mass did not differ between SD and TD phenotypes, suggesting that the expression of the SD phenotype is likely a compensatory mechanism that allows *E. clarus* to reach the same size as TD larvae at metamorphosis. This finding is similar to that reported in larvae of *Man. sexta* (Kingsolver 2007) and *Mal. disstria* (Etilé and Despland 2008). Although some species of grasshoppers, beetles, and roaches take advantage of favorable growing conditions to add an extra instar at the end of immature development, resulting in a larger adult, we do not see this pattern in *E. clarus* or in most other Lepidoptera (Esperk et al. 2007; but see Penz and Corrêa-Bueno 2018), even under ideal conditions (i.e., on kudzu at 26 ± 5°C).The fact that individuals expressing alternative developmental phenotypes (SD vs TD) did not differ in their mass at pupation suggests that plasticity in developmental phenotype has evolved primarily to allow individuals to compensate for suboptimal growing conditions. While most studies have demonstrated that size at metamorphosis is under positive directional selection (Roff 1992, Taylor et al. 1998, Eck et al. 2015), some studies have also found evidence for stabilizing selection (e.g,. Lill 2001), suggesting that tradeoffs in fitness-related traits (Stearns 1992, Roff 2002) or physiological limitations may constrain pupal mass (Davidowitz and NIjhout 2005).

Larval Size

Growth patterns of individual larvae across instars also support the proposition that the SD phenotype functions as a compensatory mechanism in *E. clarus*. As measured by head capsule diameter, larvae that would eventually go on to include an extra instar had significantly smaller head capsules in both the second and third instars relative to larvae that would express the TD phenotype, on both host plants. Thus, the difference in growth trajectory can be initiated by the end of the first instar, after only a few days of feeding. The size of the extra instar did not differ between larvae reared on the two host plants. However, by the start of the fourth instar (or its size equivalent in SD larvae, which numerically is their fifth instar), SD larvae had more than compensated for their earlier small size, as they exhibited significantly larger head capsule diameters than did TD larvae, on both hosts (12% larger on wisteria and 20% larger on kudzu). The difference disappeared by the ultimate instar, at which point head capsule diameters of SD and TD larvae were again equivalent in size. For the subset of caterpillars that were fed on wisteria and thus had similar proportions of SD and TD individuals, we also took a retrospective approach (using logistic regression) to

determine whether head capsule size early in development (i.e., in instars 2 and 3) could be used to predict the resulting developmental phenotype. Head capsule size predicted developmental phenotype only for third instar individuals, in which those with head capsules below about 1.8-mm diameter all followed an SD developmental trajectory. Notably, individuals with head capsules larger than that threshold were able to undergo either an SD or a TD developmental trajectory (Fig. 6). It is likely that larvae with head capsules larger than 1.8-mm diameter that followed an SD trajectory were lighter in weight than those with the same range of head capsule sizes that followed the TD pathway; i.e., they failed to realize their full growth potential during the instar. These results are similar to those reported for forest tent caterpillars (*Mal. disstria)*, in which head capsule size and mass at the start of the instar predicted developmental phenotype (Etilé and Despland 2008).

An important finding in this study is that both SD and TD phenotypes were simultaneously observed in many of the host plant × temperature treatment combinations. Both genetic and environmental variation, as well as gene × environment interactions, are likely to have contributed to this pattern. Because we reared larvae on a diet of field-collected foliage, uncontrolled differences in nutritional quality and/or defensive chemistry may represent an important source of phenotypic variation. Similarly, subtle differences in the temperature within the chambers is another possible source of variation. Genetically based variation in: 1) the propensity to follow a SD versus TD phenotype; and 2) the ability to tolerate environmental stress (nutritional and/or temperature; a type of G × E interaction) are likely sources of uncontrolled variation in this study. For each experiment, larvae originated from haphazardly collected eggs produced by a large breeding colony of *E. clarus*, and thus likely included a variety of genotypes. The existence of additive genetic variance for developmental strategy is supported by work on laboratory colonies of *Man. sexta* that have been selected to be canalized for the TD phenotype, relative to wild-type *Man. sexta*, which have the ability to add supernumerary instars when stressed (Kingsolver 2007, Diamond et al. 2010). In our system, it is also possible that the SD phenotype is genetically linked to a gene or genes involved in stress tolerance, such that individuals that survive stressful conditions have a greater likelihood of expressing the SD phenotype. For larvae reared at very high temperatures (32°C), all of those individuals that survived to adulthood expressed the SD phenotype, which is at least consistent with this idea.

Expression of the SD phenotype may increase under global change as both higher temperatures and exposure to suboptimal hosts (including non-native invasive plant species in the case of *E. clarus*) trigger its expression. Investigating the genetic basis for the expression of this strategy, as well as the ecological consequences stemming from its expression (relative to TD phenotypes) are necessary to improve predictions of how insects will respond to the novel scenarios resulting from climate change.

Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

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References Cited

Abarca, M. 2019. Herbivore seasonality responds to conflicting cues: untangling the effects of host quality, temperature, and photoperiod. In press. PLoS One. 14.9: e0222227.

Abarca, M., E. A. Larsen, J. T. Lill, M. Weiss, E. Lind, and L. Ries. 2018. Inclusion of host quality data improves predictions of herbivore phenology. Entomol. Exp. Appl. 166: 648–660.

Barraclough, E. I., E. P. J. Burgess, A. M. Kean, and L. A. Malone. 2014. Growth and development in a lepidopteran with variable instar number, *Pseudocoremia suavis* (Geometridae), under standard rearing conditions and when parasitised by *Meteorus pulchricornis* (Hymenoptera: Braconidae). Eur. J. Entomol. 111: 501–511.

Callier, V., and H. F. Nijhout. 2011. Control of body size by oxygen supply reveals size-dependent and size-independent mechanisms of molting and metamorphosis. Proc. Natl. Acad. Sci. U. S. A. 108: 14664–14669.

Davidowitz, G., D. A. Roff, and H. F. Nijhout. 2005. A physiological perspective on the response of body size and development time to simultaneous directional selection. Integr. Comp. Biol. 45: 525–531.

Davidowitz, G., D. Roff, and H. F. Nijhout. 2016. Synergism and antagonism of proximate mechanisms enable and constrain the response to simultaneous selection on body size and development time: an empirical test using experimental evolution. Am. Nat. 188: 499–520.

Diamond, S. E., S. D. Hawkins, H. F. Nijhout, and J. G. Kingsolver. 2010. Evolutionary divergence of field and laboratory populations of *Manduca sexta* in response to host-plant quality. Ecol. Entomol. 35: 166–174.

Dyar, H. G. 1890. The number of molts of lepidopterous larvae. Psyche. 5: 420–422.

Eck, D. J., R. G. Shaw, C. J. Geyer, and J. G. Kingsolver. 2015. An integrated analysis of phenotypic selection on insect body size and development time. Evolution. 69: 2525–2532.

Esperk, T., and T. Tammaru. 2004. Does the "investment principle" model explain moulting strategies in lepidopteran larvae? Physiol. Entomol. 29: 56–66.

Esperk, T., and T. Tammaru. 2006. Determination of female-biased sexual size dimorphism in moths with a variable instar number: the role of additional instars. Eur. J. Entomol. 103: 575–586.

Esperk, T., and T. Tammaru. 2010. Size compensation in moth larvae: attention to larval instars. Physiol. Entomol. 35: 222–230.

Esperk, T., T. Tammaru, and S. Nylin. 2007. Intraspecific variability in number of larval instars in insects. J. Econ. Entomol. 100: 627–645.

Esperk, T., T. Tammaru, S. Nylin, and T. Teder. 2007. Achieving high sexual size dimorphism in insects: females add instars. Ecol. Entomol. 32: 243–256.

Etilé, E., and E. Despland. 2008. Developmental variation in the forest tent caterpillar: life history consequences of a threshold size for pupation. Oikos. 177: 135–143.

Grunert, L. W., J. W. Clarke, C. Ahuja, H. Eswaran, and H. F. Nijhout. 2015. A quantitative analysis of growth and size regulation in *Manduca sexta*: the physiological basis of variation in size and age at metamorphosis. PLoS One. 10: e0127988. https://doi.org/10.1371/journal.pone.0127988.

Hassall, M., and F. W. L. Grayson. 1987. The occurrence of an additional instar in the development of *Chorthippus brunneus* (Orthoptera: Gomphocerinae). J. Nat. Hist. 21: 329–337.

Honek, A. 1993. Intraspecific variation in body size and fecundity in insects : a general relationship. Oikos. 66: 483–492.

Kingsolver, J. G. 2007. Variation in growth and instar number in field and laboratory *Manduca sexta*. Proc. Biol. Sci. 274: 977–981.

Kingsolver, J. G., and H. A. Woods. 1997. Thermal sensitivity of growth and feeding in Manduca sexta caterpillars. Physiol. Zool. 70: 631–638.

Lande, R. 1982. A quantitative genetic theory of life history evolution. Ecology. 63: 607–615.

Lee, K. P., S. T. Kwon, and C. Roh. 2012. Caterpillars use developmental plasticity and diet choice to overcome the early life experience of nutritional imbalance. Anim. Behav. 84: 785–793.

Lill, J. T. 2001. Selection on herbivore life-history traits by the first and third trophic levels: the devil and the deep blue sea revisited. Evolution. 55: 2236–2247.

Morita, M., and S. Tojo. 1985. Relationship between starvation and supernumerary ecdysis and recognition of the penultimate-larval instar in the common cutworm, *Spodoptera litura*. J. Insect Physiol. 31: 307–313.

Murphy, S. M., and J. T. Lill. 2010. Winter predation of diapausing cocoons of slug caterpillars (Lepidoptera: Limacodidae). Environ. Entomol. 39: 1893–1902.

Nijhout, H. F., and C. M. Williams. 1974. Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta* (L.): cessation of juvenile hormone secretion as a trigger for pupation. J. Exp. Biol. 61: 493–501.

Penz, C. M., and R. L. Corrêa Bueno. 2018. Supernumerary larval molts are male-biased and lead to larger pupae size in *Papilio hectorides* (Papilionidae). J. Lep. Soc. 72: 185–191.

R Core Team. 2018. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna.

Reynolds, S. E., S. F. Nottingham, and A. E. Stephens. 1985. Food and water economy and its relation to growth in fifth-instar larvae of the tobacco hornworm, *Manduca sexta*. J. Insect Physiol. 31: 119–127.

Roff, D. A. 2002. Life history evolution. Sinauer Associates Inc, Sunderland, MA.

Roff. 1992. The evolution of life histories. Chapman and Hall, New York.

Rosenwald, L. C., J. T. Lill, E. M. Lind, and M. R. Weiss. 2017. Dynamics of host plant selection and host-switching by silver-spotted skipper caterpillars. Arthropod. Plant. Interact. 11: 833–842.

Slansky, F., and J. Scriber. 1985. Food consumption and utilization, pp. 87–163. *In* Comprehensive Insect Physiology, Biochemistry and Pharmacology. Vol. 4. Pergamon Press, Oxford, United Kingdom.

Solomon, J. D. 1973. Instars in the Carpenterworm, *Prionoxystus robiniae*. Ann. Entomol. Soc. Am. 66: 1258–1260.

Stearns, S.C., 1992. The evolution of life histories. Oxford University Press, Oxford.

Suzuki, Y., T. Koyama, K. Hiruma, L. M. Riddiford, and J. W. Truman. 2013. A molt timer is involved in the metamorphic molt in *Manduca sexta* larvae. Proc. Natl. Acad. Sci. U. S. A. 110: 12518–12525.

Taylor, B. W., C. R. Anderson, and B. L. Peckarsky. 1998. Effects of size at metamorphosis on stonefly fecundity, longevity, and reproductive success. Oecologia. 114: 494–502.

Teder, T. 2014. Sexual size dimorphism requires a corresponding sex difference in development time: a meta-analysis in insects. Funct. Ecol. 28: 479–486.

Teder, T., T. Esperk, T. Remmel, A. Sang, and T. Tammaru. 2010. Counterintuitive size patterns in bivoltine moths: late-season larvae grow larger despite lower food quality. Oecologia. 162: 117–125.

Wagner, D. L. 2005. Caterpillars of Eastern North America: a guide to identification and natural history. Princeton University Press, Princeton, NJ.

Weiss, M. R., E. M. Lind, M. T. Jones, J. D. Long, and J. L. Maupin. 2003. Uniformity of leaf shelter construction by larvae of *Epargyreus clarus* (Hesperiidae), the silver-spotted skipper. J. Insect Behav. 16: 465–480.

Wink, M., and G. Mohamed. 2003. Evolution of chemical defense traits in the Leguminosae: mapping of distribution patterns of secondary metabolites on a molecular phylogeny inferred from nucleotide sequences of the rbcL gene. Biochem. Syst. Ecol. 31: 897–917.

Woodhead, A. P., and C. R. Paulson. 1983. Larval development of *Diploptera punctata* reared alone and in groups. J. Insect Physiol. 29: 665–668.