Title

Evidence of a hemolymph-born factor that induces onset of maturation in *Manduca sexta* larvae

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18 **Abstract**

19 Insect metamorphosis is a complex developmental transition determined and coordinated
20 by hormonal signaling that begins at a critical weight late in the larval phase of life. Even
21 though this hormonal signaling is well understood in insects, the internal factors that are assessed
22 at the critical weight and that drive commitment to metamorphosis have remained unresolved in
23 most species. The critical weight may represent either an autonomous decision by the
24 neuroendocrine system without input from other developing larval tissues, or an assessment of
25 developmental thresholds occurring throughout the body that are then integrated by the
26 neuroendocrine tissues. The latter hypothesis predicts that there could be one or more
27 developmental threshold signals that originate from developing tissues and ultimately induce the
28 onset of metamorphosis. However, there is no evidence for such a signal in the organisms for
29 which the critical weight is well described. Here we test for the evidence of this factor in
30 *Manduca sexta* (Lepidoptera: Sphingidae) by transferring hemolymph from individuals that are
31 either post- or pre-critical weight into pre-critical weight 5th instar larvae. We found that
32 hemolymph from a post-critical weight donor induces a shortening of development time, though
33 the mass at pupation is unaffected. This suggests that metamorphic commitment occurring at the
34 critical weight is at least partially coordinated by signaling from developing tissues via a
35 hemolymph-borne signaling factor.

36 **Keywords**

37 metamorphosis, critical weight, developmental regulation, development time, body size,
38 *Manduca sexta*

1. Introduction

Insect metamorphosis is a complex life history transition that brings larval growth to a conclusion and morphologically transforms the insect to the adult phase of the life cycle. Functionally, insect larvae grow, accumulate nutritional stores, and invest in incipient development of imaginal tissues. During metamorphosis, larval nutritional stores fuel development of adult tissues and structures, thus larval growth and development are essential for both completion of metamorphosis and reproduction as an adult. As such, the decision to undergo metamorphosis should be dependent on attaining or reaching growth thresholds that ensure survival through metamorphosis and subsequent adult reproduction (Davidowitz and Helm, 2014). However, the physiological and developmental factors that are assessed to determine the timing of metamorphic onset late in the larval phase still remain unresolved for even well-studied insect models.

In the final larval instar of insect growth, the signaling and interaction between two key hormones directs the cessation of the larval period and metamorphosis to the pupa or adult—juvenile hormone and ecdysone. Ecdysone signaling causes ecdysis, or the periodic molting of the cuticle, that occurs both between larval instars and during the larva to pupa transition (Truman and Riddiford, 1974; Truman, 2005; Truman et al., 1974). The identity of the molt—either non-metamorphic as in larva to larva molts or metamorphic as in larva to pupa molts—is controlled by the presence or absence of juvenile hormone during ecdysone signaling (Truman and Riddiford, 2007). In larva to larva molts, juvenile hormone titers remain high during ecdysone signaling, inhibiting imaginal tissues from proliferating and maintaining the larval form (Truman and Riddiford, 2007). However, during the final larval instar, juvenile hormone production by the corpora allata glands is stopped, and residual hormone is cleared from the

hemolymph (Baker et al., 1987; Nijhout and Williams, 1974). The ecdysone signaling cascade is prevented until juvenile hormone titers are sufficiently low (Baker et al., 1987; Rountree and Bollenbacher, 1986), but then proceed to cause growth cessation and later ecdysis of larval cuticle. Once juvenile hormone has been cleared from the body, prothoracitropic hormone induces ecdysone signaling during a specific photoperiodic gate that recurs each day (Davidowitz et al., 2003; Truman, 1972; Truman et al., 1974). Finally, ecdysone induces development of larval epidermal tissues to pupal epidermis and proliferation of the imaginal discs (Tobler and Nijhout, 2010; Truman et al., 1974; Wolfgang and Riddiford, 1986). Taken together, metamorphic commitment occurs in the final larval instar when the corpora allata no longer produce juvenile hormone and the subsequent cascade directs the developmental processes giving rise to metamorphosis.

Hormonal metamorphic commitment in larval insects co-occurs with attainment of a critical weight near the end of larval growth (Davidowitz et al., 2003; Nijhout and Williams, 1974; Shingleton, 2011; Stieper et al., 2008). At this critical weight, the hormonal interactions described above are irreversibly initiated, and the developing larvae will cease growth and pupate under a normal time course even in absence of additional feeding (Davidowitz and Nijhout, 2004; Davidowitz et al., 2003; Nijhout and Williams, 1974). Prior to the critical weight, starvation induces a delay in the timing of growth cessation and metamorphosis, and the severity of the delay decreases as size at starvation approaches the critical weight (Davidowitz et al., 2003; Nijhout and Williams, 1974; Stieper et al., 2008). These observations suggest that the critical weight represents a developmental threshold that responds to one or more internal or external factors that co-occur with growth and development (Davidowitz and Helm, 2014). Why this cascade occurs when a particular body size is reached, however, remains an unsolved puzzle

in insect development. Currently, there are two general models for how the critical weight may be assessed by the growing larvae and then triggered by the neuroendocrine system.

In the first model, the critical weight may represent a decision made by the neuroendocrine system through direct or indirect assessment of larval size. The earliest research into the hormonal regulation of metamorphosis demonstrated that distension of abdominal nerves in *Rhodnius prolixus* serves as the cue for juvenile hormone cessation by the corpora allata (Wigglesworth, 1936); however, subsequent studies suggest this to be a unique mechanism (Nijhout, 1994). In *Drosophila melanogaster*, augmented and reduced growth of the ring gland, an integrated corpora allata and prothoracic gland, can induce changes in the timing of metamorphosis consistent with changes in the critical weight, suggesting that the neuroendocrine tissues directing metamorphosis may assess size autonomously and serve as a proxy for growth overall (Mirth et al., 2005; Mirth and Riddiford, 2007). More recently, decreasing oxygen supply during larval growth has been proposed as the cue for the critical weight in larval *Manduca sexta* (Callier and Nijhout, 2011; but see Helm and Davidowitz 2013), and oxygen levels or perhaps local oxygen signaling could be assessed by the neuroendocrine system. In all of these hypotheses, body size itself is the critical factor driving metamorphosis, and the neuroendocrine system assesses size either directly or indirectly. Thus, the signal to commit to metamorphosis remains internal to the neuroendocrine system.

In an alternative, non-mutually-exclusive model, the critical weight may represent developmental thresholds occurring in tissues throughout the growing larvae, the status of which are integrated by neuroendocrine systems through hemolymph-signaled factors from the developing tissues (Mirth and Shingleton, 2012). The critical weight may not represent a body size threshold, *per se*, but rather the size at which critical developmental benchmarks are attained

in tissues important for successful metamorphosis, such as the imaginal discs and fat bodies. Damage and slowed growth of the imaginal discs is sufficient to both delay the body size at which the critical weight occurs and to prolong the period of time between the critical weight and growth cessation by ecdysone signaling (Stieper et al., 2008). Some of the imaginal discs are not innervated prior to metamorphosis (Jan et al., 1985), and thus must communicate their developmental status through secreted factors that circulate through the hemolymph. Growth of larval fat bodies may also underlie a developmental threshold co-occurring with the critical weight because they store nutritional reserves that are essential for successful metamorphosis. Fat bodies are a diffuse tissue, suspended in the hemolymph, and could communicate the status of nutritional reserves via hemolymph signaling (Arrese and Soulages, 2010; Colombani et al., 2003; Hennig et al., 2006). In these hypotheses, tissues throughout the body have individual developmental benchmarks that are necessary for metamorphosis that can only be communicated to the neuroendocrine system via hemolymph. However, there is limited evidence for a hemolymph-borne factor associated with the critical weight (Colombani et al., 2012a; Garelli et al., 2012a; Halme et al., 2010a).

The two models described above are not mutually exclusive because the neuroendocrine system is ultimately responsible for the hormonal signaling that drives commitment to and onset of metamorphosis in the larval stage. Insect metamorphosis is an incredibly complex life history transition, likely involving coordination and communication between many of the tissues in the developing insect (Mirth and Shingleton, 2012). And it is possible that both direct neuroendocrine assessment of developmental status and humoral signaling between developing tissues are involved in metamorphic commitment. However, an important advancement in understanding the mechanistic basis of metamorphosis is to characterize the roles of the

physiological systems that contribute to the metamorphic decision, and to identify the manner in which developmental status is relayed to the neuroendocrine system. In this regard, the role of humoral signaling for developmental benchmarks is a potentially important component of the critical weight and coordination of metamorphic commitment, but is implicitly assumed.

In this study we test for evidence of a hemolymph-borne critical weight factor in the tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphingidae). We assume that this factor is present after the critical weight is attained and absent prior. We then test the timing of growth cessation, or onset of ecdysone signaling, and body size consequences in *M. sexta* larvae that have received a hemolymph transfusion from donors that were either pre- or post-critical weight. If there is a critical weight signal that is communicated from developing tissues through the hemolymph, we would expect to see precocious growth cessation and a smaller final size associated with individuals which had received transfusions from post-critical weight donors. This would be consistent with early onset of the critical weight decision. Second, we predict that individuals that have received hemolymph transfusion from pre-critical weight donors should show no changes in the timing of growth cessation or body size at metamorphosis because this critical weight hemolymph-borne signaling factor should not yet be present.

2. Materials and methods

2.1. Rearing procedures and conditions

For all physiological manipulation experiments we reared *M. sexta* caterpillars in environmental chambers with a 16:8 day-night cycle at 25C. Caterpillars were fed a defined standard wheat-germ based artificial diet (100% diet in Davidowitz et al. 2003). Cohorts of caterpillars with common hatch dates were reared in lidded metal trays lined with paper towel.

Hardware mesh platforms suspended the larvae and food above the bottom of the tray and permitted frass to fall below. Rearing trays were checked daily to ensure *ad libitum* amounts of artificial diet and cleaned to prevent the build-up of frass and mold. Caterpillars were transferred to individual rearing cups (Solo TP12-0090, Lakeforest, IL) at the end of the 4th instar, demarcated by the cessation of feeding and head capsule slippage. The morning following transfer to individual rearing cups (6 hrs following lights on), each caterpillar was weighed and provided with a large block of artificial diet to synchronize feeding time (as in Baker et al., 1987). Individuals that had not yet molted into the 5th instar were removed from further analysis. Using this protocol, all larvae were developmentally synchronized at the onset of the last larval instar.

2.2. Measurement of the critical weight

Because the critical weight can potentially evolve in laboratory culture (D'Amico et al., 2001), we measured critical weight in our colony of *M. sexta* according to the standard protocols (see Nijhout and Williams, 1974a,b; D'Amico et al., 2001; Davidowitz et al. 2003). Colony-reared larvae were sampled throughout the growth phase of the final larval instar, weighed, and then randomly assigned to either a fed or starved diet treatment. For the fed treatment, individuals were reared individually in plastic cups and provided with artificial food *ad libitum* until the cessation of growth. For the starved treatment, individuals were reared individually in plastic cups and provided a cube of 3% agar to prevent dehydration; however, no food was provided. The critical weight is operationally defined as the minimal mass at which no further nutrition is required for a normal time course to pupation (Nijhout and Williams 1974, Davidowitz et al. 2003). To determine the body size where this occurs during growth, we tracked the time until the cessation of growth for individuals assigned to both fed and starved

treatments. In *M. sexta*, growth cessation is marked by characteristic gut purge and wandering behavior, the dorsum turns pink and the dorsal vessel becomes visually apparent (Davidowitz et al., 2003). We then assigned each larva to a 0.5g categorical size bin based on its mass at the time of treatment and compared the development time of fed and starved individuals for each mass bin. A weight bin of 0.5 g was chosen because the frass size of a large individual is about 0.3 g, which would shift larvae to a smaller bin had we used a size smaller than 0.5 g (Davidowitz et al. 2003). The critical weight was estimated as the mass bin at which a difference in development time between fed and starved treatments is no longer statistically detectable (t-test; see Davidowitz et al. 2003 for details). Where possible, 30 individuals were sampled per treatment per mass bin, and sample sizes are otherwise provided in Supplemental Table 1.

2.3. Estimation of Hemolymph Volume

In order to standardize the amount of hemolymph injected into a larva of a given size, we determined hemolymph mass and volumes of larvae across the range of sizes in the 5th instar. First we measured the relationship between total body mass and the mass of the hemolymph. Each larva was placed into a 4C refrigerator for 30 minutes and then placed into a carbon dioxide flow for approximately 5 min. Hemolymph was bled from small incisions in both the proleg and the prothorax. If individuals “woke” during this period, they were placed back into the carbon dioxide flow until anesthetization resumed. Hemolymph was gently squeezed from the body and collected in a pre-weighed Eppendorf tube and weighed once no more could be extracted. We then weighed a single volume aliquot of hemolymph from each individual. Smaller individuals had less hemolymph than larger individuals so sampled aliquots were roughly based on the amount of hemolymph extracted from each individual. We measured 100µl (n=6), 200µl (n=4), 300 µl (n=6), 400µl (n=6), 500µl (n=7), 600µl (n=4), 700µl (n=2), 800µl (n=4), 900µl (n=3),

1000 μ l (n=3), 1100 μ l (n=5). Then we calculated the linear relationship of measured masses to known aliquot volumes using R for Linux v 2.15.1 (R Core Team, 2012), generating the equation, $H_V = 0.980 \cdot H_M + 19.836$ (Supplemental Figure 1A: $R^2=0.947$, $P<0.0001$), where H_V is a known volume of hemolymph and H_M is the measured known hemolymph mass. We estimated the total volume of hemolymph from total mass of hemolymph that was extracted from each experimental individual using the equation derived above. We then predicted total hemolymph volume of the larvae from the 5th instar as $H_B = 193.06 \cdot B_M - 257.77$ (Supplemental Figure 1B: $R^2=0.813$, $P<0.0001$), where B_M is body mass and H_B is estimated body hemolymph mass.

2.4. Hemolymph Transfers

On day 2 of the 5th instar, 196 pre-critical weight individuals were weighed and total hemolymph volume was estimated using the body mass to hemolymph volume calculation as described above (Supplemental Figure 1B). Prior to manipulations, each individual larva was anesthetized by placing it into a chamber with a low-rate carbon dioxide flow for ~5 min. We then transfused recipient larvae with a 25% volume addition of their estimated hemolymph for each of the following treatments: 1. hemolymph from donor larvae that were larger than the critical weight (Post-CW, >7 grams, n=66), 2. hemolymph from donor larvae that had not yet attained the critical weight (Pre-CW, <7 grams, n=64), or 3. *Manduca* physiological saline as an experimental sham (Control, n=66). In a pilot study for working out protocols presented here, we had transfused 25%, 50%, and 75% volume additions of hemolymph and found consistent effects on larval development across treatments (*data not shown*). We chose 25% for this study because it was the least invasive method for the larvae and the most conservative treatment.

A small incision was made on the 4th proleg, and volumes of hemolymph were injected using a 200µl pipette with a sterile tip. The wound was immediately sealed with a liquid tissue adhesive (3M Vetbond, St. Paul, MN). Larvae were monitored directly following treatment until normal movement and feeding resumed, at which time they were returned to their environmental chamber. We then recorded the day that cessation of growth occurred, marked by characteristic changes associated with PTTH and ecdysone signaling (Davidowitz et al. 2003). Each larva was then weighed seven days after onset of wandering which is the first day pupae can be handled without damage. For all individuals we recorded the initial mass of the 5th instar, mass at time of treatment, 5th instar development time and body size.

2.5. Data Analysis

In *M. sexta*, cessation of growth is a consequence of the clearance of juvenile hormone from the hemolymph and the photoperiodically-gated release of prothoracic hormone (PTTH), which then induces subsequent ecdysone signaling (Davidowitz and Nijhout, 2004; Davidowitz et al., 2004, 2003; Truman and Riddiford, 1974; Truman, 1972). Assessing changes in development time is complicated by the fact that cessation of growth can only occur during a discrete period of time that recurs each day due to the photoperiodic gating of PTTH release (Davidowitz et al., 2003; Nijhout et al., 2006). At the population scale, development time approximates a normally distributed trait; however, photoperiodic gating results in a set of discrete times at which larval feeding could stop due to the photoperiodic gating of PTTH-20E signaling (see Nijhout et al. 2006). As a consequence, shifts in development time for any one particular individual larva must occur in discrete 24 hour jumps. Shifts in the average time at which PTTH release occurs in experimental treatments may not correctly capture shifts in developmental timing unless this gating effect is accounted for.

To test for shifts in development timing while also accounting for the recurring discrete outcome of PTTH photoperiodic gating, we analyzed development time as a product of hemolymph treatment using general linearized mixed models (GLMM) with probit link function. Statistical models were fit using the “lme4” package (Bates et al., 2013) in R for Linux version 2.15.1 (R Core Team, 2013). We assessed the incidence of growth cessation as a binary outcome (0 = no growth cessation, 1 = growth cessation) for each day and each individual in our experiment. Because this could be scored indefinitely, we restricted our scoring to the days in which growth cessation was observed across experimental treatments, days 4-9 of the 5th instar. Because we were interested in how the incidence of growth cessation varied as a consequence of hemolymph transfusion treatment, we included treatment—either post-critical weight donor, pre-critical weight donor, or control—as a fixed effect. The incidence of growth cessation increases with days spent feeding so day in 5th instar was also included as a second fixed effect. Because we assessed the occurrence of growth cessation for each individual multiple times, we included model terms for both within and between individual variance as random effects to account for repeated measures. To calculate, average values of development time from each treatment, we fit a probit binomial regression for each of our hemolymph transfusion treatments separately and calculated average time of growth cessation and standard error of this average using the P_{50} value of fitted models using the “dosep” function in the package “MASS” (Venables and Ripley, 2002) for R. We assessed the overall effect of treatment by comparing a full GLMM with a reduced GLMM model that did not include a model term for treatment using AIC scores and a likelihood ratio test (Zuur et al., 2009); BIC scores and likelihood values were reported for comparative purposes. We subsequently inferred the effects of specific treatments using model parameter

estimates and z-tests, given that treatment was found to have an overall significant effect in the models.

To test for differences in body mass, while also accounting for potential sources of variation, we analyzed pupal mass as a product of hemolymph treatment using generalized linear mixed model with a Gaussian link function. Individual models were fit using the “lme4” package in R for Linux v. 2.15.1. As above, treatment, was included as a fixed effect. We then included both initial mass in the 5th instar and total days spent in growth as random effects. We then estimated each individual’s growth rate by calculating the difference of natural log-transformed values for mass at the time of injection and initial mass of the 5th instar over time. Because growth rate could be altered by treatments, we assessed differences in growth rate following treatment using an analysis of variance. Regardless, growth rate was included as a random effect in our model analyzing body mass. The effect of hemolymph transfusion in our model was assessed by comparing a full GLMM that included treatment as a variable with a reduced model that did not include treatment using AIC scores and log-likelihood ratio tests (Zuur et al., 2009); BIC scores and likelihood scores were reported for comparative purposes.

Finally, we examined the relationship between development time and body size because of the potential for variation as a consequence of days spent growing. We analyzed the relationship between these traits using standard analysis of variance and ordinary least squares regression in R for Linux v. 2.15.1.

3. Results

3.1. The Critical Weight

When we compared the development times of *M. sexta* larvae that had been either starved or fed, we found that the body mass at which there was no longer a difference in the time until growth cessation was 7 grams (Figure 1, Supplemental Table 1). When starved at body sizes greater than 7 grams, the mean time until growth cessation did not differ from larvae that had been fed. Below 7 grams, cohorts of larvae that had been starved prolonged the time until cessation of growth compared to fed treatment.

Figure 1

3.2. *Effects of hemolymph transfusions on the timing of growth cessation*

In the hemolymph transfusion experiment the incidences of growth cessation began on day 4 of the 5th instar and increased through day 9 (Figure 2A, Table 1B: $\beta=4.82$, $z=8.51$, $P<0.001$). Based on the P_{50} values obtained by generalized linear models, individuals that had received post-CW transfusion took 5.63 ± 0.08 days, pre-critical weight transfusion took 5.90 ± 0.09 days, and controls took 5.89 ± 0.08 days until growth cessation (Figure 2B). To assess whether these differences in development time constitute statistically significant differences, we compared the full GLMM with a reduced GLMM (no term for treatment), we observed that treatment had a significant influence on the incidence of growth cessation in our experiment (Table 1A: *log-likelihood ratio test*, $X^2=10.29$, $P=0.0058$). Individuals that had received a hemolymph transfusion from a donor that had already attained the critical weight were more likely to stop growth at an earlier development time than controls (Table 1B: $\beta=2.01$, $z=2.78$, $P=0.0055$). Individuals that received hemolymph transfusion from a donor that had not yet reached the critical weight showed no difference in the timing of growth cessation as compared with controls (Table 1B: $\beta=0.75$, $z=1.05$, $P=0.29$).

309 **Figure 2**

310

311 **Table 1**

A. Comparison of full and reduced GLMMs							
Model	df	Model Fit Scores			LRT		
		AIC	BIC	logLik	X^2	P	
Full	26	534.57	666.26	-241.29	10.29	0.0058	***
Reduced (No Treatment)	24	540.86	662.42	-246.29			
Notes: Full and reduced model were compared using a likelihood ratio test (LRT). A reduction in the AIC score for our full model indicated that adding a term for treatment was the more appropriate model for explaining development time. BIC scores and likelihood values were reported for comparative purposes.							
B. Parameter estimates of full GLMM							
Fixed Effects	Estimate	Std. Err.	z	P			
Intercept	-33.93	3.93	-8.63	<0.0001	****		
Day	4.82	0.57	8.51	<0.0001	****		
Treatment (Post-CW)	2.01	0.72	2.78	0.0054	***		
Treatment (Pre-CW)	0.75	0.72	1.05	0.29			

Notes: Model parameters were estimated using maximum likelihood, and coefficient values were evaluated with z-tests. Significant z-tests ($P<0.05$) suggested that specific model coefficients had an effect on development time.

3.3. Effects of hemolymph transfusions on the pupal mass

The average pupal mass of individuals that had received hemolymph transfusions from post-CW donors, pre-CW donors, or controls were 4.71 ± 0.75 g, 4.69 ± 0.58 g, and 4.63 ± 0.67 g respectively (Figure 3A). When we compared a full GLMM with a reduced GLMM that excluded treatment, there was no statistically detectable effect of hemolymph transfusion on pupal mass (Table 2A: *log-likelihood ratio test*, $X^2=1.83$, $P=0.40$). Control pupal masses did not differ from post-critical weight hemolymph transfused larvae (Table 2B: $\beta=0.16$, $t=1.36$, $P=0.47$) nor from pre-critical weight hemolymph transfused larvae (Table 2B: $\beta=0.093$, $t=0.79$, $P=0.57$).

Figure 3

Table 2

A. Comparison of full and reduced GLMMs						
Model	df	Model Fit Scores			LRT	
		AIC	BIC	logLik	X²	P
Full	7	354.85	377.05	-170.43	1.83	0.40
Reduced (No Treatment)	5	352.69	368.54	-171.34		
Notes: Full and reduced model were compared with a likelihood ratio test (LRT). An increase in the AIC score for our full model indicated that adding a term for treatment made the full model less appropriate than the reduced for explaining pupal mass. BIC scores and likelihood values are reported for comparative purposes.						
B. Parameter estimates of full GLMM						
Fixed Effects	Estimate	Std. Err.	t	P		
Intercept	4.59	0.14	31.98	<0.0001	****	
Treatment (Post-CW)	0.16	0.12	1.36	0.40		
Treatment (Pre-CW)	0.093	0.12	0.79	0.57		
Notes: Model parameters were fit using maximum likelihood and their significance was assessed using t-tests. Significant t-tests (P<0.05) would indicate that individual model coefficients explained variance in body mass; however, coefficient estimates were not significant.						

3.4 Correlation between Body Size and Development Time

There was a significant effect of development time on pupal mass across all of our treatment groups (Figure 3B: ANOVA, $F=6.55$, $P=0.011$). Pupal mass was positively correlated with the duration of the growth period; however, the correlation between pupal mass and growth duration was low (Figure 3B: *ordinary least squares regression*, $\beta=0.126$, $R^2=0.036$). Growth duration only explained approximately 4% of the overall variance in pupal mass across hemolymph treatments and controls.

4. Discussion

This study demonstrates that there is a putative hemolymph factor, present after the critical weight decision has been made and prior to ecdysone signaling, which can induce precocious growth cessation in larvae that have not yet reached their own critical weights. We

demonstrated this potential because hemolymph injected from post-critical weight donors shortened the time until growth cessation significantly in comparison to larvae that had received hemolymph from pre-critical weight donors and saline sham controls. Receiving hemolymph from post-critical weight donors resulted in a ~10% (about 12 hours) reduction in the average time until growth cessation, but this likely represents an even larger effect for individuals because growth duration can only be shortened by discrete 24hr jumps. Thus, this downward shift in the average time is better interpreted as many individuals stopping growth at earlier photoperiodic gates, and is evidenced by the observation that the frequency of individuals that were transfused with hemolymph from post-critical weight donors stopped growth in greater numbers at the earliest growth cessation times observed (Figure 2A). In fact, post-critical weight hemolymph transfusion induced growth cessation at development times when neither pre-critical weight hemolymph transfusion nor saline sham controls were observed to stop growth (Figure 2A). These results support our hypothesis that a critical weight hemolymph factor may be responsible for signaling the critical weight, because this experimental treatment alone induced a shorter duration of time until growth cessation.

At first glance, this downward shift in the duration of growth is rather subtle and is apparent only when the dynamics of photoperiodic gating were accounted for in analysis. However, the observation that transfused hemolymph induced a downward shift in development time at all is a substantial result. It is quite difficult to physiologically alter an insect along its developmental trajectory in ways that induce reductions in total development time. More importantly, the critical weight typically occurs on day 3 of 5th instar, and we transfused larvae on day 2 of the 5th instar. This would mean that the maximum downward shift we could observe would be ~24 hours, and we observed a downward shift in development time that made up half

of this potential (~12 hrs). While studies have demonstrated delayed metamorphosis with regards to experimentally-induced effects (Stieper et al., 2008) or altered trajectories as a whole (Mirth et al., 2005), few studies directly manipulate insect physiologies to affect reduced development time. In fact, this result harkens back to the initial, classic studies of the critical weight in *R. prolixus*, which showed that metamorphosis could be physically induced by injection of saline (Wigglesworth, 1936). To our knowledge, these classic studies are the only precedent for our observed reductions in the duration of growth associated with post-critical weight hemolymph transfusion.

Our results clearly showed that pupal size following growth cessation and metamorphosis were not influenced by our hemolymph transfusions, even with a significant downward shift in the timing of growth cessation in larvae that had received post-critical weight hemolymph. This contradicts our predictions because we expected that transfusion of post critical weight hemolymph should cause final size to be smaller because the duration of growth would be shortened; however, this was not observed in our study. No significant shifts in final pupal mass were observed among our experimental treatments. However, our experiment showed a significant positive, although very weak, correlation between growth duration and pupal size. Only 4% of the variation in body size could be accounted for by differences in development time (Figure 3B). This suggests that the predictions for shifts in timing and changes in mass after hemolymph transfusions should be decoupled and assessed independently. In which case, we show that hemolymph transfusion from a post-critical weight donor significantly impacted the duration of the larval period; however, it accounts for very little variation in pupal size.

The content of hemolymph in our post-critical weight and pre-critical weight donors varies with respect to factors that may have influenced the outcomes of our experiment,

irrespective of changes in the critical weight. First, post-critical weight donors would have significantly less or even no juvenile hormone, whereas pre-critical weight donors would have high concentrations of juvenile hormone (Baker et al., 1987; Bhaskaran et al., 1986). However, the presence or absence of juvenile hormone was unlikely to have confounded our results because its presence should have prolonged growth duration, and we observed delay in growth duration between saline shams and pre-critical weight treated larvae. If juvenile hormone had played a role, then we would have expected larvae that had received pre-critical weight hemolymph to be delayed, not shortened as was observed.

A second potential confounding factor for the observed responses to hemolymph transfusions was that post-critical weight donors may have already started PTTH or ecdysone signaling, whereas pre-critical weight donors would not yet have had these hormones in circulation. However, the presence of juvenile hormone in pre-critical weight larvae inhibits the growth cessation driven by PTTH and ecdysone (Baker et al., 1987; Nijhout et al., 2014). As a consequence we suspect that PTTH and ecdysone did not affect larvae that had received post-critical weight hemolymph because our recipient larvae were still pre-critical weight and should have had active juvenile hormone biosynthesis at the time of transfusion. More importantly, the effects of PTTH and ecdysone on development are immediate with ecdysone signaling causing growth cessation, gut purge, and wandering when there are low titers of juvenile hormone. However, we did not observe immediate responses observed to hemolymph transfusions; rather, growth usually stopped days after the hemolymph transfusion itself.

A third potential confounding factor that may have affected the response to hemolymph transfusions is a difference in the relative content of juvenile hormone esterase in hemolymph from pre- and post-critical weight donors. Juvenile hormone esterase is responsible for breaking

down juvenile hormone that is secreted into the hemolymph, and its production is upregulated after the critical weight is reached (Baker et al., 1987). This could have potentially resulted in a reduced development time in larvae that were transfused with hemolymph from post-critical weight donors as our study observed. However, juvenile hormone esterase is active throughout the larval instar and reduced juvenile hormone titers after the critical weight occur because the corpora allata is no longer producing new juvenile hormone (Baker et al., 1987). Thus, juvenile hormone esterase would be unlikely to reduce overall development time, unless the corpora allata had stopped production of juvenile hormone.

Hemolymph could have varied with respect to the nutritive content or driven changes in nutrient signaling in response to hemolymph transfusions. In terms of nutrients themselves, post-critical weight donors may have contained accumulated carbohydrate, lipid and/or protein that was not present in pre-critical weight larvae. Though we do not report the results in this manuscript, direct injection of trehalose, amino acids, or a combination resulted in significant delays in the cessation of growth compared with saline controls (Helm and Davidowitz, *unpub.*). Thus, injecting excess nutrients had different effects compared to larvae treated with hemolymph from either a pre- or post-critical weight donor.

Beyond nutrient resources themselves, hemolymph transfusion may have induced changes in growth that were mediated through various cellular growth regulators such as insulin, insulin-like peptides, or TOR signaling (Colombani et al., 2003; Edgar, 2006; Hennig et al., 2006; Oldham and Hafen, 2003; Oldham et al., 2000). Enhanced or diminished growth following hemolymph transfusion due to changes in these cellular growth regulators could potentially cause the critical weight to be reached at different time points because of overall changes in growth rate. Unfortunately, we did not monitor growth rates following treatments, and thus we

cannot completely refute that any one of these growth mediators, and/or bulk changes in growth rate itself, drove changes in development time. However, nutritional signaling and coordination of growth is of key importance for developing larvae of all sizes (Colombani et al., 2003; Edgar, 2006; Hennig et al., 2006; Oldham and Hafen, 2003; Oldham et al., 2000), thus these factors were likely present in the hemolymph of both hemolymph transfusion treatments. Additionally, growth rates are sigmoidal in form with respect to time and larval body size (Nijhout et al., 2006), which means that growth rates are highest in the early 5th instar and decline as the larva approaches growth cessation. Assuming that growth factors are likely to reflect overall patterns of growth with respect to development, this would suggest that growth regulators, such as insulin, were more abundant in pre-critical weight larvae than in post-critical weight larva. Thus, we may have expected a shift in growth cessation to be stronger for pre-critical weight larva had these growth factors been involved. However, larvae transfused from pre-critical donors did not stop growth any earlier or later than saline sham controls, which contained no growth signaling factors. Nevertheless, the study of nutritional signaling has unveiled tremendous complexity in growth coordinating mechanisms of developing larvae (Colombani et al., 2003; Edgar, 2006; Hennig et al., 2006; Oldham and Hafen, 2003; Oldham et al., 2000), and individual growth factors or their combined effect on development time may play a role in relative timing of growth cessation as well as the critical weight.

The regulation of growth as well as the mechanistic basis for the critical weight are complex. As a consequence there are many confounding factors determining development time that cannot be completely refuted to play a role in the shift in growth cessation that we observed. Though we did not directly measure the critical weight of larvae that had been transfused, we argue that our results are indicative that the critical weight was induced earlier in development

time when post-critical weight hemolymph was transfused into pre-critical weight larvae. In terms of physiological modeling (Davidowitz et al., 2003; Mirth and Shingleton, 2012; Nijhout et al., 2014; Shingleton, 2011), the timing of growth cessation is determined by three factors at play here—the duration of time until the critical weight is attained, the interval of time until juvenile hormone clearance, and photoperiodic gating of PTTH/ecdysone signaling. The shift of growth cessation in response to post-critical weight transfusion is inconsistent with changes in growth rate (see above), juvenile hormone esterase activity (see above), and we statistically accounted for photoperiodic gating. Thus, we believe that the likely explanation is that the critical weight occurred earlier in development, inducing early growth cessation. However, teasing apart the complexity underlying the critical weight is perhaps one of the greatest challenges that remains for understanding the developmental causes of metamorphosis and mechanisms generating variation in body size and development time more generally.

One key question that remains following our study is whether this critical weight factor represents an already identified growth and development regulator or if it is a novel factor for insect developmental physiology to identify. In *D. melanogaster*, damage to imaginal discs can induce delays in the onset of metamorphosis, which are mediated by secreted growth factors *retinoid* and *dILP8* (Colombani et al., 2012b; Garelli et al., 2012b; Halme et al., 2010b), and they have been implicated in the control of the critical weight. But these factors have been identified during injury, rather than the critical weight itself, and are suspected to regulate compensatory growth for the imaginal discs (Colombani et al., 2012b; Garelli et al., 2012b; Halme et al., 2010b). In this study, we found that hemolymph transfusion induced precocious development, rather than inhibited development. Perhaps these growth factors and/or unidentified growth factors work in tandem to both induce or delay the critical weight to ensure that all necessary

developmental benchmarks throughout the growing larvae are attained (Mirth and Shingleton, 2012; Nijhout et al., 2014).

While our study is a first step in examining hemolymph-borne critical weight factors, future work should focus on disentangling the overall complexity of growth and developmental regulation as well as characterize the identity of and interactions among growth mediators. In terms of understanding the regulation of metamorphic commitment overall, our hypothesis provides support for lines of evidence that tissues throughout the body as well as the neuroendocrine system are jointly involved in metamorphic commitment (Mirth and Shingleton, 2012). This signaling is hypothesized to occur at least partially through the hemolymph and supports hypotheses that suggest that tissues such as the imaginal discs and fat bodies could play a role in determining the critical weight (Colombani et al., 2003; Stieper et al., 2008). This hypothesis is not mutually-exclusive with neuroendocrine assessment of the critical weight as mentioned above, but rather becomes an alternative mechanism that could occur in insects where an absence of neuroendocrine control of the critical weight is suspected. More importantly, while studies have shown a delay in the critical weight when peripheral tissues are damaged, we present evidence that a circulating factor can shorten growth duration. Circulating growth factors not only inhibit the critical weight when tissues aren't prepared, but may also induce the critical weight when their own developmental thresholds are reached.

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Figure and Table Legends

Figure 1.

The critical weight estimated as the body mass at which there is no difference in the mean time until growth cessation between larvae that were fed (filled circle) or starved (open circles) at various body masses. Bars represent standard error of the mean (see Supplemental Table 1 for significance values). As indicated in this graph, the critical weight is 7 grams (arrow).

Figure 2.

Cumulative frequency of growth cessation of 5th instar *Manduca sexta* larvae plotted as a logistic regression for individuals that had received hemolymph transfusions from pre-cw (diamonds, dashed line), post-cw (open squares, dotted line), or saline control injection (circles, solid line). The light gray horizontal dashed line represents the P50 that was used to estimate average times until growth cessation (see Section 2.5 Data Analysis). Curves were fit for each treatment using predicted values estimated from our separate binomial regression fits for each treatment (see Section 2.5 Data Analysis). In cases where blue diamonds are not immediately apparent, they appear behind the black circles.

Figure 3.

A. Comparison of pupal masses across three treatments and B. the relationship between pupal mass and growth duration across all treatments.

Table 1.

A comparison of our full and reduced GLMMs to test for the overall effect of hemolymph transfusion on the timing of growth cessation in 5th instar *M. sexta* (A) with a report of the fitted parameters of the full GLMM (B).

Table 2.

Analysis summary of GLMMs that describe pupal mass in response to hemolymph transfusion from post-CW donor, pre-CW donor, and saline sham control. A full model, including all model terms described in Section 2.5, was compared to a reduced model that excluded the fixed effect hemolymph transfusion treatment (A). Estimated model parameters for fixed effects are then provided to describe specific effects of hemolymph transfusion treatment compared to saline sham control (B).

638

639 Graphical Abstract