Received: 20 April 2021 | Revised: 5 May 2022 | Accepted: 24 May 2022

DOI: 10.1002/jez.b.23165

BRIEF COMMUNICATION

JEZ-B MOLECULAR AND DEVELOPMENTAL EVOLUTION WILEY

Evaluating old truths: Final adult size in holometabolous insects is set by the end of larval development

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Funding information
National Science Foundation, Grant/Award Numbers: IOS 1256689, IOS 1901680, IOS-1557341; Discovery Grant from the Natural Sciences and Engineering Research Council of Canada; Australian Research Council Centre of Excellence in Advanced Molecular Imaging, Grant/Award Number: FT170100259; Fonds de Recherche du Québec - Nature et Technologies (FRQNT)

Abstract

For centuries, it has been understood that the final size of adult holometabolous insects is determined by the end of the larval stage, and that once they transform to adults, holometabolous insects do not grow. Despite this, no previous study has directly tested these "old truths" across holometabolous insects. Here, we demonstrate that final adult size is set at the end of the last larval stage in species representing each of the four orders of holometabolous insects: the fruit fly *Drosophila melanogaster* (Diptera), the tobacco hornworm *Manduca sexta* (Lepidoptera), the dung beetle *Onthophagus taurus* (Coleoptera), and the Florida carpenter ant *Camponotus floridanus* (Hymenoptera). Furthermore, in both *D. melanogaster* and *C. floridanus*, we show that the size of adult individuals fluctuates but does not significantly change. Therefore, our study finally confirms these two basic assumptions in the biology of insects, which have for centuries served as the foundation for studies of insect growth, size, and allometry.

KEYWORDS
allometry, body mass, *Camponotus*, holometabolous insects, insect growth, *Drosophila*, morphometrics, *Manduca*, *Onthophagus*

1 | INTRODUCTION

Holometabolous insects, which are insects that undergo complete metamorphosis, have undergone remarkable diversification and now make up 80%–90% of all extant insect species (Yang, 2001). These insects pass through three postembryonic stages: the larval, pupal, and adult stage. Insect larvae are highly specialized for feeding and growth in terms of their morphology and behavior. Larval development proceeds through multiple larval stages called instars, each of which is separated by a molting event (Hall & Wake, 1999). These molting events accommodate the extraordinary mass gain acquired during the larval feeding phase by producing a progressively larger cuticle, which in turn accommodates subsequent growth within each instar (Nijhout, 1981). During the terminal larval stage, feeding stops and the larvae enter the pupal stage in response to changes in endocrine signaling (Gilbert, 2010; Nijhout, 2003; Riddiford, 1996).

Lisa Hanna and Tom Lamouret contributed equally to this study.

During the prepupal and pupal stages, larval structures degenerate and primordial tissues destined to give rise to adult structures begin to proliferate (Hinton, 1963). The final molting event occurs during metamorphosis when pupae transition to adults. At the adult stage, the cuticle hardens to become a hard exoskeleton in most body regions (i.e., head, thorax, appendages). The formation of a hard exoskeleton in the adult limits room for future growth. These observations have led to two basic assumptions about holometabolous insect growth and development: (1) adult size is determined by the size of the larva when it stops feeding and begins pupation and (2) once the adult stage is reached, adults no longer grow and their size is fixed.

These basic assumptions have been made by naturalists and philosophers for centuries. In the *History of Animals* (350 B.C.E), Aristotle writes: "For the grub of the bee, the anthrena, and the wasp, whilst it is young, takes food and voids excrement; but when it has passed from the grub shape to its defined form and become what is termed a 'nympha', it ceases to take food and to void excrement, and remains tightly wrapped up and motionless until it has reached its full size, when it breaks the formation with which the cell is closed, and issues forth." In the 1730s, the French entomologist René-Antoine Fercheault De Réaumur (1735) recorded the weight of two Lepidopteran individuals before metamorphosis and then reweighed the emerging adults after metamorphosis, finding no considerable change in weight. Finally, in his account on butterflies, the Scottish naturalist James Rennie (1829) states that "butterflies do not, like the larger animals, increase in size as they grow older" and "it is only during the caterpillar state that the insect eats voraciously and grows in proportion." To our knowledge, however, there exists no modern study that has empirically tested these historical assertions across holometabolous insects.

Scientific knowledge often begins with proposing a hypothesis, which is a proposition or supposition about the way an observation in nature works (Platt, 1964; Popper, 1959, 1963). A hypothesis becomes a broadly accepted fact when the hypothesis has become so well supported by evidence that we feel confident in assuming it is true (Gauch, 2003; Staddon, 2017). In the case of the two assumptions or hypotheses we focus on here, both appeared to have transitioned from hypothesis to fact without formal empirical tests. However, because adult body mass in insects can fluctuate in response to changes in nutritional quality and quantity, it becomes important to test these age-old assumptions (Poças et al., 2020; Verdú et al., 2010).

Generally, insect "size" is assessed in two ways: (1) body mass and/or (2) morphometric measurement of sclerotized body parts (Daly, 1985; Davidowitz et al., 2003; Gould, 1966; Poças et al., 2020). First, when body size is defined by body mass, such as wet or dry weight, growth is defined in terms of a consistent increase in mass, which occurs during the intermolts of the larval stages. Second, when body size is defined by morphometric measurements of sclerotized body parts, growth is defined by a consistent increase in the dimensions of parts of the exoskeleton during the intermolts of the larval stages, such as the mandibles and head capsule (Nijhout, 1994; Wigglesworth, 1972). Although the sclerotized exoskeleton does not grow within an instar, the unsclerotized exoskeleton, such as the larval body wall in holometabolous insects, can grow by stretching of an initially corrugated cuticle and by intercalation (Wolfgang & Riddiford, 1981).

We therefore used these two measures of insect size to test the two basic assumptions of insect growth and development across insects representing the four Orders of holometabolous insects: the fruit fly *Drosophila melanogaster* (Diptera), the tobacco hornworm *Manduca sexta* (Lepidoptera), the dung beetle *Onthophagus taurus* (Coleoptera), and the Florida carpenter ant *Camponotus floridanus* (Hymenoptera). Specifically, we measured, using body mass and morphometric dimensions, the body size of the terminal larvae and compared it with the body size of the pupae or emerged adults for all of these species. In addition, using *D. melanogaster* and *C. floridanus*, we measured body size at different time points during the adult stage. For all of these experiments, larvae and adults were raised under constant diet and environmental variables.

2 | RESULTS

We first tested the assumption that adult size is determined at the end of larval development by measuring the size of the terminal stage larvae and the emerging pupae or adults using body mass or morphometric measurements. As expected, for all species our analysis revealed a strong positive correlation and slope between larval body length or weight and its respective pupal or adult body length or weight (Figure 1; Table 1). Furthermore, for *M. sexta*, our analysis similarly shows a positive correlation and slope between the pupal body weight and its respective adult body weight (Figure 1d′; Table 1). We then tested the assumption that holometabolous insects do not grow during the adult stage. We measured adults of *C. floridanus* and *D. melanogaster* during different time points during the adult stage under constant conditions. In *C. floridanus*, we found no significant alteration in adult head width or mesosoma length across 20 days (Figure 2a,b; $n_{head} = 25$, $\chi^2_{head} = 1.11$, $p_{head} = 0.291$, $r_{head} = 0.0033$; $n_{mesosoma} = 25$, $\chi^2_{mesosoma} = 0.018$, $p_{mesosoma} = 0.897$, $r_{mesosoma} = 0.0023$). Similarly, for *D. melanogaster*, we found that adult weight in males and females did not significantly change across 17 days (Figure 2c; $n = 89$, $\chi^2 = 1.94$, $p = 0.163$, $r = -0.0066$).

3 | DISCUSSION

Our results demonstrate that adult body size (measured by body mass or morphometric measurements) is largely determined by the size achieved during the terminal larval stage across holometabolous insects. Although we found that the correlation between terminal larval size and initial adult size is high, it is not perfect. With respect to body mass, this imperfect correlation is due to a fluctuation in mass during metamorphosis due to metabolism, tissue remodeling, meconium, and the loss of cuticles during the pupal and adult molts

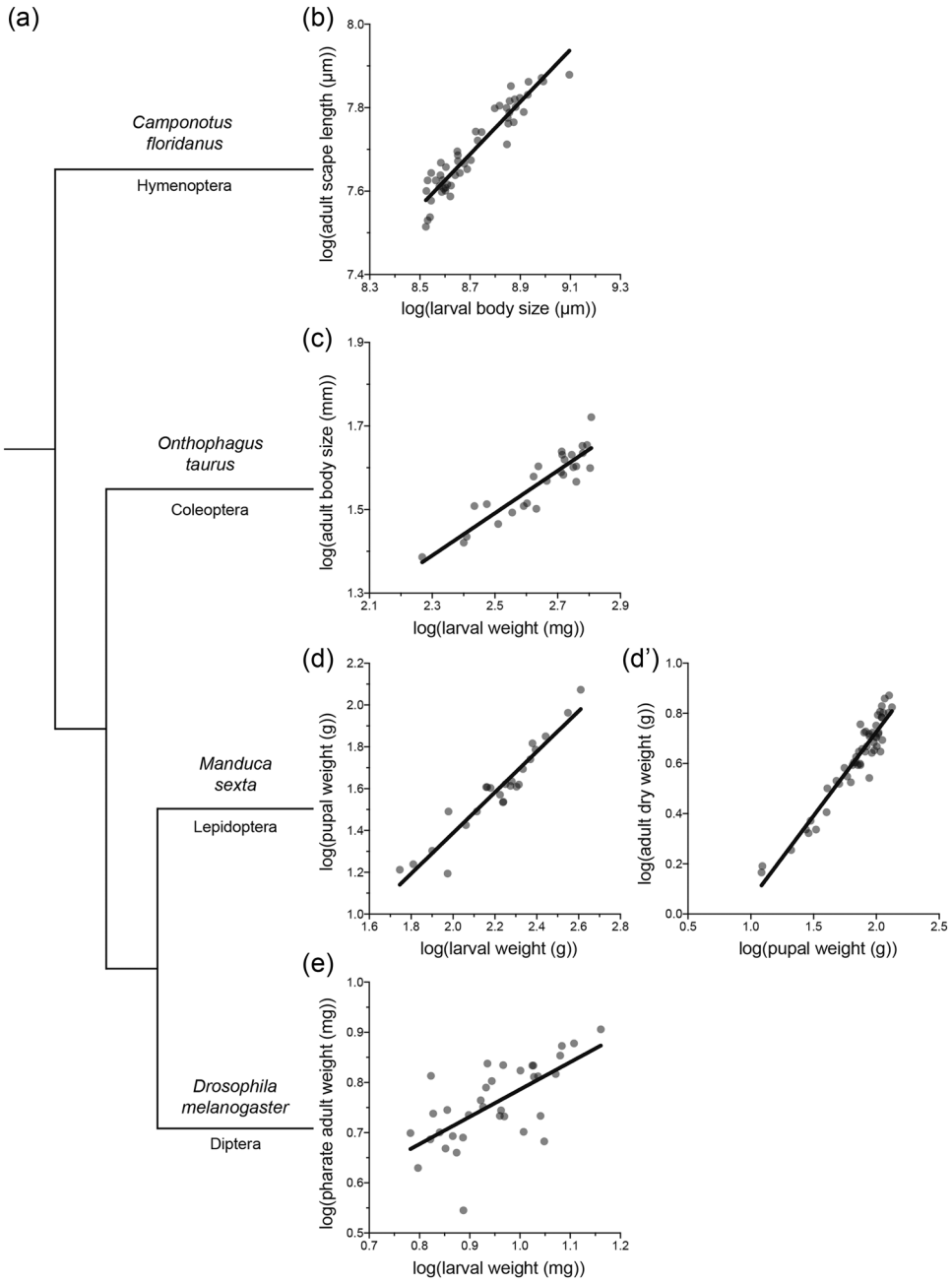

FIGURE 1 Correlation between body size in the terminal larva and the pupa or adult stage. (a) Phylogenetic relationship between *Camponotus floridanus*, *Onthophagus taurus*, *Manduca sexta*, *and Drosophila melanogaster*. (b) Log–log plot of larval body size and adult scape length in *C. floridanus*. (c) Log–log plot of larval weight and adult body size in *O. taurus*. Log–log plot of larval weight and pupal weight (d), and pupal weight and adult dry weight (d′) in *M. sexta*. (e) Log–log plot of larval weight and pharate adult weight in *D. melanogaster*.

(Nijhout, 1994; Wigglesworth, 1972). With respect to sclerotized body parts, this imperfect correlation may be due to the deposition and thickening of the endocuticle. Our results also demonstrate that adult size does not significantly change with age. The fluctuation we observe in adult mass is likely due to feeding, desiccation, and the change in ovary mass following oviposition, while the fluctuation in morphometric measurements is likely due to measurement error caused by the difficulty of capturing accurate measurements on live ants. Regardless of the source of these fluctuations, they are not statistically significant, and therefore, are consistent with the assumption that insects do not grow in the adult stage.

Our findings that adult body size is largely determined by the size achieved during the terminal larval stage are consistent with a previous study focusing on dung beetles (Rohner & Moczek, 2021). Furthermore, our findings are also consistent with Poças et al. (2020), who showed that both adult body weight and appendage size do not significantly increase when both larvae and adult *D. melanogaster* are maintained on the same diet. In addition, when Poças et al. (2020)

TABLE 1 Regression analysis summary for the relationship between larval to pupal or adult body size and pupal to adult body size

Species	Stage	Sample number (*n*)	Coefficient of determination (r^2)	β	*T* value	*p* value
Camponotus floridanus	Larval-to-adult	50	0.946	0.626	20.2	***
Onthophagus taurus	Larval-to-adult	27	0.911	0.507	11.0	***
Drosophila melanogaster	Larval-to-adult	35	0.669	0.544	5.18	***
Manduca sexta	Larval-to-pupal	25	0.952	0.972	15.0	***
Manduca sexta	Pupal-to-adult	53	0.973	1.45	30.1	***

***$p < 0.001$.

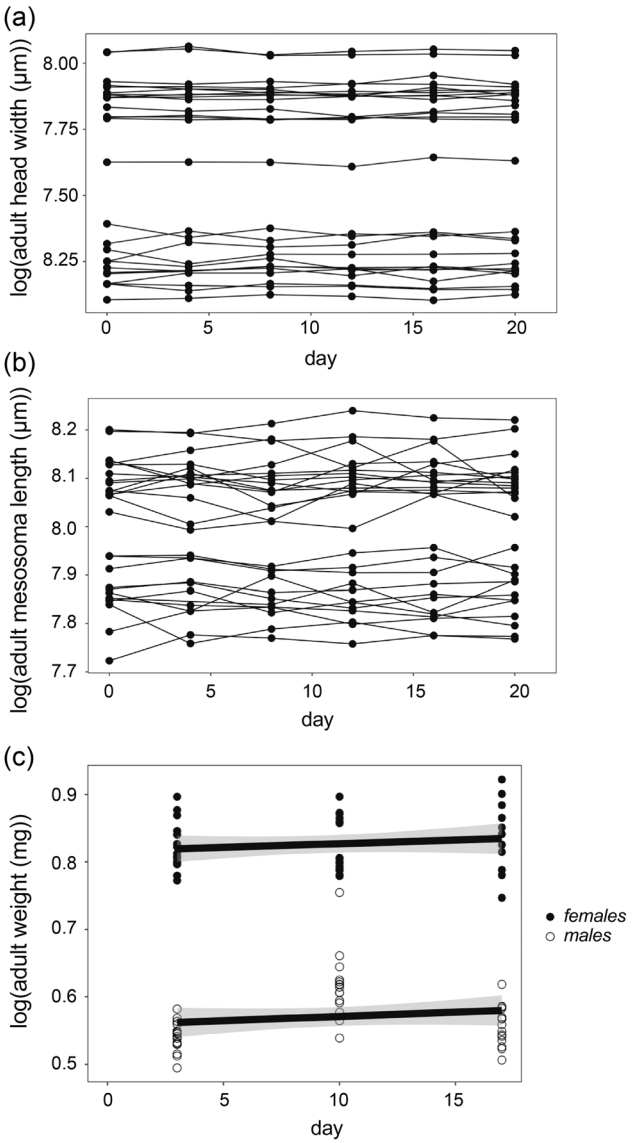

FIGURE 2 Adult size does not change over time. Adult head width (a) and mesosoma length (b) measured over time (days) in *Camponotus floridanus*. Individuals represent both minor and major workers. (c) Adult weight measured over time (days) in *Drosophila melanogaster*.

experimentally increased or decreased nutritional quality between the larval and adult stages they found that: (1) appendage size fluctuates but does not significantly increase; and (2) adult weight fluctuates up or down, which means that the observed increase is likely due to gains in ovary mass and digestive organs and not due to growth. Collectively, our data support both of the long-held truths: adult size is determined at the terminal larval stage, and once the adult stage is reached, holometabolous insects no longer grow. By finally confirming these assumptions across all four holometabolous insect orders, they will continue to confidently serve as the foundation for studies of insect growth, size, and allometry.

4 | MATERIALS AND METHODS

4.1 | *C. floridanus*

Colonies of the ant *C. floridanus* were collected in Gainesville, Florida, USA. The colonies were housed at 25°C, 60% humidity, and 12 h day:night cycle. Individual nests were provided with several water-filled glass tubes plugged with cotton. Ants were fed sugar-water, Bhatkar–Whitcomb yellow diet (Bhatkar & Whitcomb, 1970), and frozen mealworms three times a week.

To obtain individuals in the highest part of the worker size distribution, nearly all adult major workers were removed from five colonies, as this has been previously demonstrated to stimulate increased production of major workers in the next generation (Gregg, 1942). Following this procedure, terminal stage larvae were identified and collected for measurement. The black pigmentation of the gut, as well as density of the fat cells surrounding the gut were used to identify terminal stage larvae (Alvarado et al., 2015; Wheeler & Wheeler, 1976). Eighty-two terminal staged larvae from five colonies were collected and total body length along their ventral, lateral, and dorsal axes were measured. Following measurements, each larva was individually isolated and was placed in a water filled tube plugged with cotton along with three minor workers. The isolated larvae were fed with Bhatkar–Whitcomb diet every 3 days (Bhatkar & Whitcomb, 1970). Out of the 82 larvae, 71 spun a cocoon

and 50 molted into adults. For adult measurements, since adult ants curl inwards when they die, the entire body length could not be reliably measured. Therefore, the length of the scape (first segment of antenna) was measured as it is isometric and can be used as a proxy for body size (Alvarado et al., 2015; Diniz-Filho et al., 1994). All images were taken using a Zeiss Discovery V12 microscope, and measurements were performed using AxioVision SE64.1994.

To measure individuals from the onset of the adult stage, pupae that were near adult eclosion were identified and collected. In *C. floridanus*, cuticle coloration becomes darker as the pupae nears eclosion, which allowed selection of the oldest individuals. A total of 25 pupae were collected and individually placed in water filled tubes plugged with cotton along with three adult minor workers. The minor workers were dabbed with paint to distinguish them from the newly molted adults. Following eclosion, live adults were placed in a cotton filled petri dish for immobilization, imaging, and measuring. Measuring scape length on live ants is challenging because they cannot be made completely stationary without dissecting the antenna. Therefore, measurements of 25 live adults were taken along the width of the head (at the widest part) as well as along the dorsal axes (mesosoma length). Measurements were performed every 4 days for a period of 20 days. Images were taken using a Zeiss Discovery V12 microscope, and measurements were performed using AxioVision SE64.

4.2 | *O. taurus*

Adult *O. taurus* were collected from cow dung pads at Marble Hill Farm in Bloomington, IN (39°3′8″ N, 86°36′12″ W), and maintained in laboratory colonies in a moist sand–soil mixture at 24°C and fed cow dung ad libitum as described previously (Ledon-Rettig et al., 2017). To generate larvae, adult beetles were bred in plastic containers (25 cm tall × 20 cm diameter) filled 75% with a moist sand–soil mixture. Three male and six female beetles were added to each container and provisioned with ~0.5 L of cow dung. Following 6 days of breeding, adult beetles were recaptured and brood balls, each containing a single larva, were collected and placed into separate plastic containers. Offspring were maintained within their natal brood balls for approximately 3 days, at which time they were transferred to individual artificial brood balls (ABBs) within 12-well tissue culture plates containing cow dung ad libitum, and maintained within stand-alone incubators at constant 24°C as detailed in Shafiei et al. (2001). To quantify larval growth, larvae were briefly removed from their ABB using featherweight forceps, and weighed individually every 48 h until pupation using a Mettler Toledo (AL54 Ohio, USA, $d = 0.1$ mg) scale. Pronotum width was used as a proxy for adult body size and measured as in previous studies (e.g., Kijimoto & Moczek, 2016). Pronotal measurements were to the nearest 0.01 mm and collected by APM.

4.3 | *M. sexta*

Larvae of *M. sexta* were reared on an artificial diet (Davidowitz et al., 2003). To produce a series of final larval sizes, larvae were removed from the diet at weights corresponding to the critical weight and above. Starvation above the critical weight does not affect the normal temporal pattern of hormone secretion that initiates metamorphosis (Davidowitz et al., 2003; Nijhout & Williams, 1974), and the timing of metamorphosis was identical in starved and feeding individuals. The critical weight of the strain we used was 6.5 g and the typical maximum mass of larvae fed ad libitum was 13 g. Starved larvae gradually lose weight, and all animals, starved and fed, were weighed daily. The weight in the late afternoon before the initiation of the wandering stage was recorded. This was about 6–8 h before wandering. Normally-feeding larvae had already stopped feeding at that time, in preparation for wandering and pupation. The pupal weight was taken on Day 3 after pupation. Adult dry weights were determined as follows. Adults were killed 1 day after eclosion, before any feeding occurred, by freezing and then dried in an oven at 70°C for 7 days to a constant weight. All weights were measured to the nearest 0.01 g, using a Mettler AE50 analytical balance.

4.4 | *D. melanogaster*

A wild type Oregon R strain of *D. melanogaster* was used, which was maintained on sucrose yeast diet (100 g of yeast extract, 50 g of sucrose, 10 g of water in 1 L of water). Both Nipagen (3%) and proprionic acid (0.3%) were added to the diet to prevent bacterial and fungal growth. Fly cultures are kept at 25°C, 60% relative humidity, and under 12:12 light:dark cycles. To obtain larvae for these studies, approximately 100 flies were transferred to egg laying chamber (100 ml plastic cups) fitted with a 60 mm culture dish filled with apple juice medium (30% sucrose, 40% apple juice, and 30% agar weight/volume) smeared with yeast paste. Flies were left to lay eggs for 4 h. After removing the eggs with a paint brush, and washing them in phosphate buffered saline to remove food and yeast paste. Forty eggs were transferred into fly vials containing 7 ml of sucrose yeast diet. Larvae were allowed to develop until they reached the wandering stage, where they emerge from the food in search for a site to pupariate. Wandering larvae were individually collected, washed, and weighed on a Melter XPU2 Ultramicrobalance. Each larva was transferred to its own fly vial to undergo metamorphosis. These same individuals were weighed again when they reached the pharate adult stage.

To explore how adult size changed with age, data from Poças et al. (2020) were reanalyzed. These individuals were reared under similar conditions as outlined above. However, in this case the flies were left to eclose and individuals were sexed and transferred into new vials containing sucrose yeast diet. Eighty-nine adults were subsampled and weighed on Days 3, 5, and 17 after eclosion.

4.5 | Statistical analysis

To examine the relationship between final larval size and adult size, data were first log-transformed before fitting with linear models. These models used the adult size measurements as dependent variables and terminal larval stage size measurements as independent variables. Similarly, the relationship between adult size with adult age was determined by fitting linear models, using adult size measurements as the dependent variable and using age as the fixed effect and individual as a random effect to account for repeated measures (*C. floridanus*) or age and sex (*D. melanogaster*) as the fixed effect variables. For all data sets, correlation estimates were obtained using Pearson's product–moment correlation tests. All data analysis was conducted using RStudio (version 3.4; R Core Team, 2017) using the tidyverse package (Wickham, 2017) for data wrangling and visualization.

ACKNOWLEDGMENTS

We thank two anonymous reviewers for comments, and members of the Abouheif lab for comments and insights into experimental design for ants. This study was supported by a Discovery Grant from the Natural Sciences and Engineering Research Council of Canada to E. Abouheif, an Australian Research Council FT170100259 to C. K. Mirth, a National Science Foundation grants IOS 1256689 and 1901680 to A. P. Moczek, an IOS-1557341 to F. H. Nijhout, and a Doctoral Fellowship from Fonds de Recherche du Québec—Nature et Technologies (FRQNT) to L. Hanna.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/jez.b.23165

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How to cite this article: Hanna, L., Lamouret, T., Poças, G. M., Mirth, C. K., Moczek, A. P., Nijhout, F., & Abouheif, E. (2022). Evaluating old truths: Final adult size in holometabolous insects is set by the end of larval development. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, 1–7. https://doi.org/10.1002/jez.b.23165