

# Larval growth and allometry in the cabbage butterfly *Pieris brassicae* (Lepidoptera: Pieridae)

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## Abstract

By adopting a longitudinal study design and through geometric morphometrics methods, we investigated individual and ontogenetic variation in size, shape and timing during larval development of the cabbage butterfly *Pieris brassicae* under laboratory conditions. We found that ontogenetic size progression departs modestly, but significantly, from growth at a constant rate and that size at hatching contributes considerably to determine the size of the individual at all subsequent stages. As for the shape, ontogenetic allometry is much more conspicuous than static allometry, the latter in many cases being close to isometry. Analysis of developmental timing revealed a stage of apparently more effective developmental control at stage 3, supported by both the relatively small variance in cumulative developmental time up to stage 3 and by the pattern of correlation between duration of single stages. While presenting detailed quantitative aspects of growth in *P. brassicae*, in particular with respect to individual variation, this study and the associated dataset can provide a basis for further explorations of the post-embryonic development in this insect and contribute to the ongoing investigations on growth regulation and control in insects.

## KEYWORDS

developmental regulation, Dyar's rule, geometric morphometrics, ontogenetic allometry, static allometry

## 1 | INTRODUCTION

Recent advances in the study of the molecular-genetic and physiological mechanisms that regulate body growth in insects, including the relative growth of different body parts (reviewed in Shingleton & Frankino, 2018; see also Beukeboom, 2018 and references therein), are fuelling a renewed interest in the developmental mechanisms controlling body size and shape, especially in the light of their potential to influence phenotypic evolution (Casasa, Schwab, & Moczek, 2017; Nijhout & McKenna, 2017). Unfortunately, these leading studies, which are by necessity conducted on a restricted set of model organisms, like the fruit fly *Drosophila melanogaster* (e.g. Shingleton, 2010) or the tobacco hawk moth *Manduca sexta* (e.g. Grunert, Clarke, Ahuja, Eswaran, & Nijhout, 2015), stand out in a landscape where observational,

quantitative ontogenetic data, even at the level of growth patterns, are not so common outside these taxa. This limits the possibility for comparative analyses and the incorporation of available information into an evolutionary context.

With the aim of contributing to an assessment of the operational repertoire in growth patterns and regulation in insects, we investigated individual and ontogenetic variation in size, shape and timing during larval development of the lepidopteran *Pieris brassicae* (Linnaeus, 1758) under laboratory conditions. The study, conducted through geometric morphometrics methods (Zelditch, Swiderski, & Sheets, 2012), has a longitudinal design, that is individuals were reared separately to produce a dataset consisting of the measurements of the same individual in subsequent stages (*longitudinal data*; Cock, 1966). Geometric morphometrics analytical techniques have proven to be very effective in the

study of developmental variation (Mayer, Metscher, Müller, & Mitteroecker, 2014), and quantitative ontogenetic data accounting for individual variation are considered to give access to a deeper understanding of developmental processes (Cooper & Albertson, 2008; Fusco, 2015).

*Pieris brassicae*, the cabbage butterfly (also known as the large white), is a major pest on cultivated crucifers (mustard, cabbage, cauliflower and allies), which is common throughout Europe, Asia and North Africa. As an insect of economic importance, many aspects of its biology have been explored quite in detail (reviewed in Feltwell, 1981). These include observations on behaviour, morphology and phenology of larval and/or pupal stages, both in captivity and in natural conditions (e.g. David & Gardiner, 1962; Kristensen, 1994; Lafont, Mauchamp, Boulay, & Tarroux, 1975; Srihari, 1972), analyses of growth under synthetic and natural diet (e.g. Chew, 1980; Chlodny, 1967; David & Gardiner, 1965), and studies that investigated the effect of several extrinsic factors on larval development (e.g. Ali & Rizvi, 2007; Breugnon, 1972; Klein, 1932; Long, 1953; Maercks, 1934). However, little attention has been paid so far to detailed quantitative aspects of its growth, in particular with respect to individual variation. Nonetheless, *P. brassicae* is particularly suitable for studying some general aspects of post-embryonic development in holometabolous insects, as it is easily cultured under laboratory conditions, has a fixed number of moults under a wide range of culturing parameters, and, of particular interest for morphological studies, the sclerotized head capsule can be recovered intact after each of the first four moults, so that, by means of a simple individual-culture system, it is possible to obtain data on individual developmental trajectories.

By exploiting the effectiveness and power of longitudinal experimental designs for quantitative developmental studies, we conducted a first batch of growth analyses on several aspects of size, scaling and timing of *P. brassicae* post-embryonic development. While presenting detailed metric aspects of growth in this insect, in particular with respect to individual variation, this study and the associated dataset can provide a basis for further explorations of the post-embryonic development in the same species and contribute to the ongoing investigations on growth regulation and control in insects.

## 2 | MATERIALS AND METHODS

This study is based on a longitudinal dataset of morphometric and developmental time measurements on 65 specimens of the lepidopteran *P. brassicae*. Although rearing started from a sample of more than 100 specimens, only data from specimens that completed larval development within five stages, which finalized pupal development until adult eclosion and with perfectly preserved exuviae, were used.

Morphometric data cover the head capsule and include the first four larval stages (L1–L4), whereas time data include all the five larval stages (L1–L5).

### 2.1 | Rearing and exuviae collection

Larvae were obtained from a stock population at the insect farm “Smart Bugs” (Ponzano Veneto, Italy). The stock, which derives from Northern East Italy natural populations and includes more than 5,000 reproductive individuals, has been maintained for more than 50 generations. At each generation, at least one individual from a natural population is introduced in the stock, following the widely accepted “one migrant per generation rule,” which provides the appropriate level of gene flow to minimize losses in genetic diversity and to ensure low levels of drift and inbreeding (Wang, 2004).

Eggs were collected from 19 different families (egg masses) to increase individual variation in the sample. Although initially an approximately equal number of eggs per brood was selected, the final dataset, which includes complete individual series of exuviae only, sees a variable number of individuals per family, mostly between 2 and 6.

Two days after hatching, the first-stage larvae were transferred to the Department of Biology of the University of Padova for culturing. They were kept individually in petri dishes (Ø 35 mm) and fed ad libitum with a solid-paste mix of vegetables containing dried powdered green *Brassica* leaves (the same semi-synthetic diet used to maintain the stock; see David & Gardiner, 1965), which was replaced and hydrated once a week. Larvae were reared into an incubator (Sanyo Versatile Environmental Test Chamber MLR-352H) at steady temperature (25°C), humidity (50% RH) and photoperiod (16L:8D) under the control of an automated system (DAMSystem3 FileScan Software). These environmental parameters reproduce optimal condition for larval development in *P. brassicae*. Maercks (1934) indicated an optimal culturing temperature in the range 20–26°C. Since development at higher temperatures within this range is faster and has no detrimental effects (David & Gardiner, 1962), temperature was set to 25°C. Humidity was set only slightly lower than the value indicated by David and Gardiner (1962) for group rearing (60% RH), to avoid the formation of condensation inside the individual petri dishes. Photoperiod was set to approximately match that of the season at the time (June–July 2017) and latitude (ca. 45°N) of the experiment, to prevent possible interference with larval biological clock.

Larvae were checked for moult twice a day and upon a moult the exuvia of the cephalic capsule was collected and stored in 70% ethanol. Individuals were sexed at the stage of pupa by observing the presence/absence of female genital openings (Feltwell, 1981).

## 2.2 | Time measurements

Larvae were checked approximately 12 hr apart, thus stage duration data have a resolution of half a day. Time zero was set at the morning of hatching, and 0.5 days were added at each subsequent check. The recovery of the exuvia was recorded as the time of the moult. The end of the fifth stage was marked when the cephalic capsule was found outside the upper part of the newly formed pupa. Two time series were derived for each individual: single stage duration ( $T1$ – $T5$ ) and cumulative developmental time ( $TC1$ – $TC5$ ), that is the total larval time until a given moult.

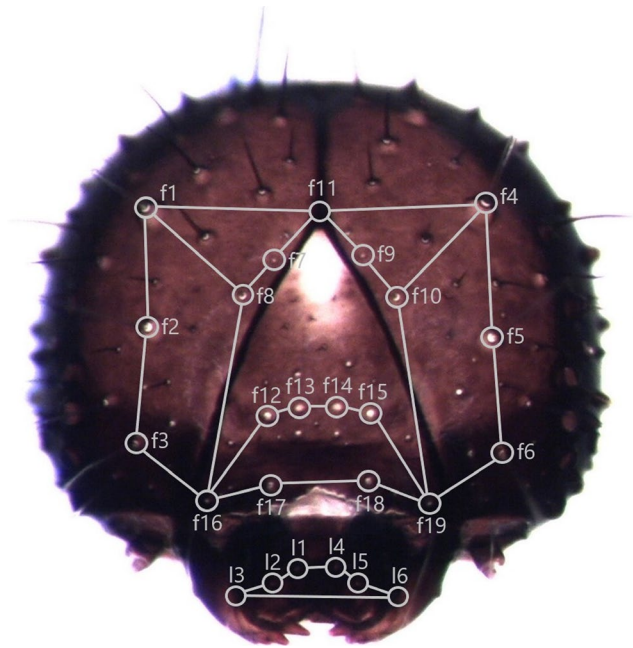
## 2.3 | Morphometric measurements

Measurements were made on the exuviae of the cephalic capsule of stages 1–4, because the ecdysis to the pupa regularly brakes the cephalic capsule apart. The fifth-stage cephalic capsule could have been obtained by sacrificing the animal during that stage, but with no possibility to sex individuals and to control for (a) possible extra moults before pupation (we recorded two cases of one additional moult) and (b) successful metamorphosis (we had 22 cases of death at the pupa stage). By refraining to get measurements of stage 5, we could verify the homology of the first four stages (Minelli, Brena, Deflorian, Maruzzo, & Fusco, 2006; Minelli & Fusco, 2013a) and ascertain that morphometric data represent a segment of the ontogeny of viable individuals.

Two different parts of the larval head were measured and analysed: the plaque formed by the two *genae* plus the *frons* and the *labrum*. For simplicity, the two parts are indicated here as “frons” and “labrum”, respectively. Both parts were measured and analysed through geometric morphometrics, a collection of analytical tools that provide a statistical description of biological forms in terms of their size and geometric shape based on a configuration of landmarks (Klingenberg, 2010).

### 2.3.1 | Image acquisition

Digital images of the frons and labrum were taken separately for each individual and stage by the same person (AS). The cephalic capsule was reversibly glued to the floor of a petri dish filled with ethylene glycol to avoid refraction and desiccation, and the latter was aptly oriented to have the part of interest coplanar with focal plane of the microscope. Exuviae were photographed using a digital camera (LEICA DFC 400) mounted on a stereomicroscope (LEICA MZ12.5) at variable magnification depending on stage (20X–50X). Images were acquired through the Leica Application Suite software (V.2.8.1) at the size of  $2,048 \times 1,536$  pixels.



**FIGURE 1** Position of landmarks (circles) on the frons (upper wireframe) and on the labrum (lower wireframe) in *Pieris brassicae*. Landmark definitions are in Table S1 [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

As a standard practice for controlling for measurement error (Viscosi & Cardini, 2011), two images of each part were taken separately for each individual and stage, following a new placement of the specimen under the stereomicroscope.

### 2.3.2 | Landmark selection and digitalization

Landmarks on the two parts were chosen to be approximately coplanar, in order to mitigate against the error stemming from the projection of a three-dimensional structures onto the plane of the image (Cardini, 2014). One medial landmark was placed at the vertex of the frons, while all the other two-sided landmarks were positioned in correspondence of the basis of nine pairs of idionymic setae for the frons and three pairs idionymic setae for the labrum (Figure 1). These are setae that are homologous across stages and individuals within a species (Savriama, Gerber, Baiocco, Debat, & Fusco, 2017) and thus fulfil the criteria of homology and correspondence required for the geometric morphometric methods.

To assess measurement error due to digitalization, landmarks were digitized twice on each of the two images by the same operator (AS), in two independent working sessions on different days, using TPSDig 2 (ver. 2.30; Rohlf, 2015) (for a total of four sets of data for each part, individual and stage). The program tpsUTIL (ver. 1.74; Rohlf, 2015) was used to build the final (.NTS) data files by combining all data into a single dataset for each part.

## 2.4 | Time analyses

Ontogenetic variation in stage duration was analysed for two time variables: *single stage duration* ( $T$ ) and *cumulative developmental time* ( $TC$ ). Time progressions were summarized by averaging all individual histories, and variation was studied with one-way ANOVA complemented by Tukey's HSD post hoc test. Possible within-individual associations in the duration of stages were investigated by correlation analysis (Pearson's product-moment correlations).

All the statistical procedures were carried out in R (R Core Team, 2019).

## 2.5 | Morphometric analyses

Size and shape development was investigated with geometric morphometric (GM) methods. Both the frons and labrum have *object symmetry*, that is a form of bilateral symmetry in which a structure is symmetric in itself, and landmark configurations were analysed accordingly (Klingenberg, Barluenga, & Meyer, 2002; Klingenberg, 2015).

Independently for the two parts, configuration of raw coordinates of the landmarks was translated, scaled and rotated through a generalized Procrustes least square superimposition, to obtain a standard measure of linear size and scale-independent shape coordinates (Rohlf & Slice, 1990). Size of each part was estimated as the centroid size (CS), the square root of the sum of squared distances between each landmark and the centroid of the configuration (Bookstein, 1991). Procrustes methods allow to partition symmetric and asymmetric components of shape variation. All the statistical procedures for the extraction of size and shape data and the analyses of shape were carried out in the package MorphoJ (ver. 1.06d; Klingenberg, 2011), while all the other analyses (in particular size analyses) were carried out in R (R Core Team, 2019).

### 2.5.1 | Sex and family effects

The effects of sex and family on larval size, growth rate and stage duration were checked using the analysis of variance (one-way ANOVA).

### 2.5.2 | Growth progression

Logsize variables of this study are the natural logarithm of the CS ( $\ln CS$ ) of the frons and labrum averaged by individual and stage. Individual per-moult growth rates were computed as the antilogarithm of the difference in  $\ln CS$  between

successive stages. For each specimen, three values of growth rates ( $GR1$ ,  $GR2$  and  $GR3$ ) for each of the two parts were obtained, corresponding to the postmoult/premoult size ratio at the first, second and third moult, respectively.

The *Average per-moult Growth Rate* ( $AGR$ ) was estimated as the antilogarithm of the arithmetic mean of the three log-transformed  $GRs$  ( $\ln GR$ ). This is equivalent to the geometric mean of the three  $GRs$  (Fusco, Garland, Hunt, & Hughes, 2012).

Individual deviation of the ontogeny from the growth progression at a constant rate (Dyar's rule, Dyar, 1890) was tested with a one-way ANOVA, using  $\ln GR$  as single factor, followed by Tukey's HSD post hoc test, and quantified with the *Index of conformity to Dyar's rule* ( $IDC$ , Fusco, Garland, Hunt, & Hughes, 2012). This metric takes values between 0 (maximal divergence from Dyar's rule, the whole growth realized in a single stage) and 1 (perfectly constant growth rate) inclusively.

### 2.5.3 | Shape analyses

*Static allometry* refers to scaling relationship in the individuals of a species at the same developmental stage, whereas *ontogenetic allometry* refers to scaling relationship in the individuals of a species at different developmental stages. Multivariate regression analyses (Klingenberg, 2016) were used to study both relationships, considering the symmetric component of shape versus log-transformed centroid size ( $\ln CS$ ) within each part. Shape and  $\ln CS$  data were averaged by individuals and stages. Statistical significance of the relationships was assessed by a permutation test with 10,000 rounds. Direction of the vectors of allometric shape variation was compared by computing the angles between them in the shape space (Klingenberg et al., 2002). Statistical significance of the angular differences between vectors was assessed by a permutation test with 10,000 rounds.

Allometry between the frons and the labrum was analysed through reduced major axis (RMA) regression of  $\ln CS$  of the two parts (Smith, 2009).

## 3 | RESULTS

### 3.1 | Check of sex and family effects

#### 3.1.1 | Effect of sex

One-way ANOVAs on  $\ln CS$  and  $\ln GR$  for both labrum and frons and the time variables  $T$  and  $TC$  showed that the factor "sex" has no effect on these variables during larval growth for both parts and in all stages (Tables S2 and S3).

### 3.1.2 | Effect of family

One-way ANOVAs on  $\ln CS$  and  $\ln GR$  showed that the factor “family” has effect on size variables (marginally non-significant only in stage 4 labrum) but not on growth rates. The one-way ANOVA performed on the time variable  $T$  showed that the family has an effect on stage duration of the first and fourth stage, whereas for the time variable  $TC$  the family has an effect on developmental time only until the third stage (Tables S2 and S3).

The inclusion of individuals of different sex and from different families in our sample was aimed at increasing individual variation, but the experiment was not designed for specifically investigating the effects of these factors (see Discussion). Thus, the subsequent analyses were carried out pooling all the individuals of the sample.

## 3.2 | Growth progression and conformity to Dyar's rule

*Pieris brassicae* growth progression (Figure 2), summarized as the sample mean of the individual Average per-moult Grow Rate ( $AGR$ ), was 1.616 for the frons (individual range 1.575–1.651) and 1.497 for the labrum (individual range 1.461–1.533). One-way ANOVAs computed to test the equality of the postmoult/premoult ratios showed significant differences between the three values of  $\ln GR$  in both parts ( $p < .0001$ ). Post hoc tests showed that for the frons both the pairs  $\ln GR1$ — $\ln GR2$  and  $\ln GR1$ — $\ln GR3$  differed significantly ( $p < .0001$ ), whereas for the labrum all  $\ln GRs$  differ significantly from each other ( $p < .0001$ ). Despite significant differences in  $\ln GR$ , the index of conformity to Dyar's rule revealed that in both parts the deviations of size progression from the growth at a constant rate are modest, with sample mean  $IDC = .967$  for the frons (individual range .936–.998) and  $IDC = .956$  for the labrum (individual range .852–.994).

Variance in  $\ln CS$  increases steadily until the third stage (Figure 2; Fisher's  $F$  tests,  $p < .045$ ), to decrease (although not significantly) at stage 4, both for the frons and the labrum. Values of  $\ln CS$  are all positively and significantly correlated between stages, both for the frons ( $r = .52$ – $.86$ ,  $p < .0001$ ) and the labrum ( $r = .58$ – $.84$ ,  $p < .0001$ ). Stated differently, those specimens that have larger (or smaller) sizes at the beginning of post-embryonic development tend to retain their relative size throughout larval growth. The substantial correlation coefficients between individual size at stage 1 and 4 (frons:  $r = .52$ , labrum:  $r = .58$ ) show that larval size at the first stage contributes considerably to determine the final larval size: about 27% (frons) and 33% (labrum) of size variation at stage 4 can be explained by the first-stage size variation.

## 3.3 | Allometry in shape space

### 3.3.1 | Static allometry

The multivariate regressions of shape on centroid size in each stage showed a significant change in the symmetric component of shape with size during the first two stages in the frons and in the third stage in the labrum. However, the percentage of shape variance explained by size is modest in all cases ( $<7\%$ ) (Table 1).

### 3.3.2 | Ontogenetic allometry

Multivariate regressions showed a significant association between the symmetric component of shape and centroid size in both parts of the larva head ( $p < .0001$ ; Figure 3). Size accounted for 38.28% of the total amount of shape variation in the frons and 18.36% in the labrum. For the frons, ontogenetic shape variation mainly consists in a lateral elongation of the structures in the upper part of the genae, complemented by a parallel medial elongation of the structures in the lower half of the frons in the strict sense. In the labrum, a subtle elongation of the middle part of the structure is observed during ontogeny.

### 3.3.3 | Vectors directions

The comparisons between the regression vector of the ontogenetic allometry and the four regression vectors of static allometry gave significant angles only for the frons, ranging from  $47.4^\circ$  to  $63.5^\circ$  ( $p < .032$ ; Table 2). Differences between the static allometric vectors were all significant for the frons, with angles varying from  $29.4^\circ$  to  $60.9^\circ$  ( $p < .020$ ), whereas in the labrum, the significant angles between static vectors were on average smaller ( $9.8^\circ$ – $29.6^\circ$ ) (Table 2).

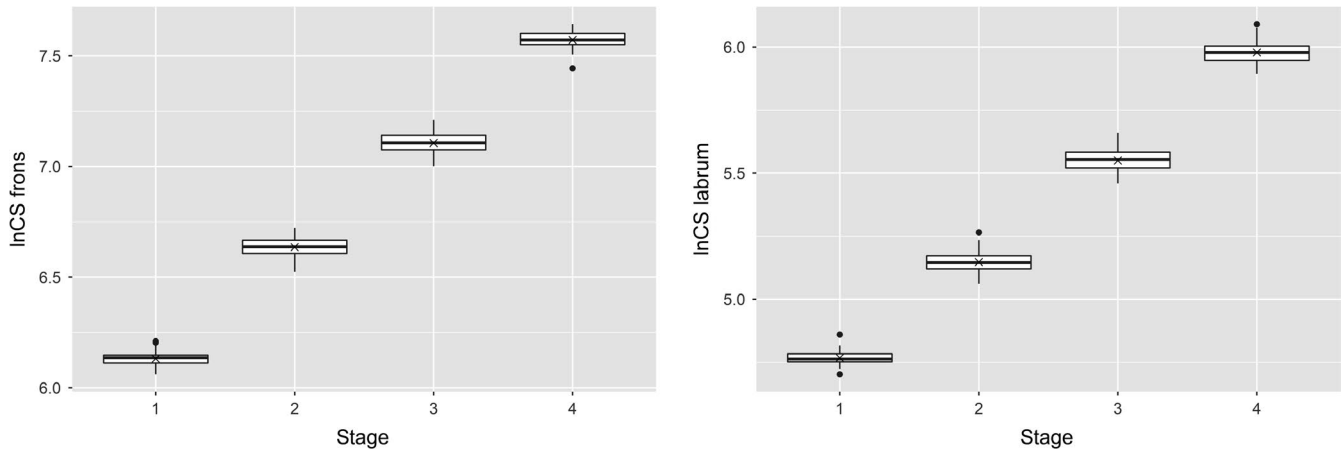
## 3.4 | Allometry of labrum versus frons

### 3.4.1 | Static allometry

Allometry of the labrum on frons varies from negative in stage 1 (slope  $\pm$  s.e.,  $b = .781 \pm .089$ ) to positive in stage 4 ( $b = 1.127 \pm .093$ ), with a monotonic trend for slopes to become steeper with stage progression (Figure S1). However, only stage 1 relationship is significantly different from isometry (two-tailed Student's  $t$  tests,  $p = .016$ ).

### 3.4.2 | Ontogenetic allometry

This is significantly negative ( $b = .841 \pm .004$ ,  $p < .0001$ ; Figure S1), deriving from the higher value of  $AGR$  in the



**FIGURE 2** Growth progression during the first four larval stages for the natural logarithm of the centroid size ( $\ln CS$ ) of the frons (left panel) and labrum (right panel) in *Pieris brassicae*. Boxes represent the interquartile interval, with median (transverse line) and mean (cross); vertical lines are ranges of variation, to the exclusion of outliers (dots)

**TABLE 1** Static allometry: results of the multivariate regressions of shape on CS. var%, percentage of explained shape variance;  $p$ ,  $p$ -values from permutation tests (10,000 rounds)

Stage	FRONS		LABRUM	
	var%	$p$	var%	$p$
1	4.17	<b>.012</b>	3.03	.130
2	6.41	<b>&lt;.001</b>	1.99	.275
3	2.30	.151	4.66	<b>.026</b>
4	1.54	.434	2.99	.108

Note: Statistically significant  $p$ -values ( $p < .05$ ) are in bold.

frons with respect to the labrum. The ontogenetic slope has a value in between the static slopes of the larval stages 1 and 2, but it differs significantly only from the slopes of stages 3 and 4 (two-tailed Student's  $t$  tests).

## 3.5 | Timing

### 3.5.1 | Duration of stages

Average stage duration in the five stages ( $T1$ – $T5$ ) was 3.7, 3.2, 3.5, 5.1 and 10.4 days, respectively, and the average cumulative developmental time ( $TC1$ – $TC5$ ) was 3.7, 6.9, 10.4, 15.4 and 25.9 days (Figure 4). Stages 4 and 5 are significantly longer than the other stages (one-way ANOVA and Tukey post hoc test,  $p < .0001$ ). Variance in stage duration does not increase significantly during the first three stages, to start increasing only thereafter (Fisher's  $F$  tests,  $p < .02$ ). For the cumulative developmental time, variance tends to increase from one stage to the next with the exception of  $TC2$  versus  $TC3$ ,

which do not differ significantly from each other (Fisher's  $F$  test), with the result that the coefficient of variation of  $TC3$  (6.6%) is almost one half of that of  $TC2$  (12.5%).

### 3.5.2 | Relationship between stage durations

A complex pattern of correlations emerged both in the duration of the single stages ( $T$ ) and in the cumulative developmental time ( $TC$ ) (Figure 5).

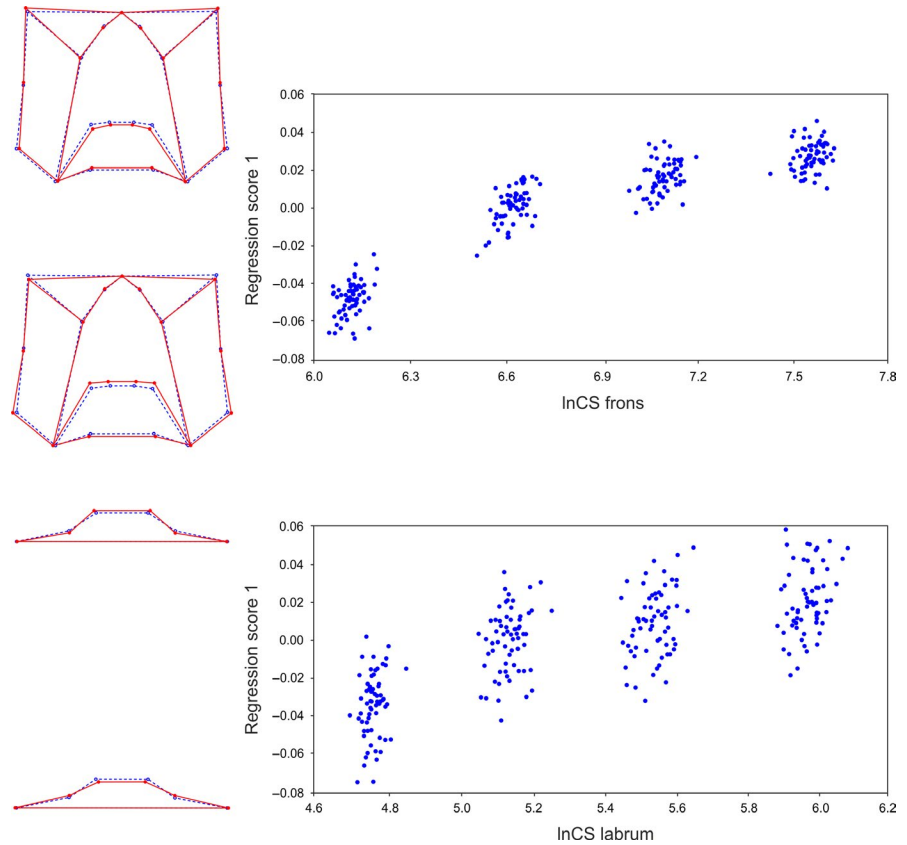
For the variable  $T$ , we found significant correlations only between certain stages, and the correlation coefficients are either positive ( $T1$ – $T2$ ,  $r = .33$ ,  $p = .007$ ;  $T3$ – $T4$ ,  $r = .43$ ,  $p = .0004$ ) or negative ( $T1$ – $T3$ ,  $r = -.56$ ,  $p < .0001$ ;  $T1$ – $T4$ ,  $r = -.29$ ,  $p = .021$ ;  $T2$ – $T3$ ,  $r = -.46$ ,  $p < .0001$ ). All the other relationships between stage durations are weak and non-significant.

For the variable  $TC$ , the significant correlation coefficients are all positive. These extend for two successive stages for stage 1 and 2 ( $TC1$ – $TC2$ ,  $r = .77$ ,  $p < .0001$ ;  $TC1$ – $TC3$ ,  $r = .61$ ,  $p < .0001$ ;  $TC2$ – $TC3$ ,  $r = .87$ ,  $p < .0001$ ;  $TC2$ – $TC4$ ,  $r = .29$ ,  $p = .018$ ) and for one stage only for stages 3 and 4 ( $TC3$ – $TC4$ ,  $r = .55$ ,  $p < .0001$ ;  $TC4$ – $TC5$ ,  $r = .45$ ,  $p = .0001$ ).

## 4 | DISCUSSION

Longitudinal ontogenetic studies, where the developmental trajectory of single individuals is tracked, allow to directly observe variation in individual growth progression and developmental timing, but unfortunately, they are not very common among entomology studies (e.g. Klingenberg, 1996). Although in most longitudinal experimental settings, like the one implemented here, the environmental component

**FIGURE 3** Multivariate regressions of shape (Procrustes coordinates) on centroid size for the frons (upper panel) and the labrum (lower panel) during the first four larval stages in *Pieris brassicae*. Solid-line wireframes represent the opposite extremes of shape variation with respect to the average shape (dashed-line wireframes) [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



**TABLE 2** Angular comparisons between the regression vectors of static and ontogenetic allometry

Vectors comparison	FRONS		LABRUM	
	Angle	<i>p</i>	Angle	<i>p</i>
Vontogenetic–V1static	56.02	<b>.008</b>	63.13	.222
Vontogenetic–V2static	47.37	<b>.001</b>	40.95	.070
Vontogenetic–V3static	49.74	<b>.002</b>	49.48	.117
Vontogenetic–V4static	63.50	<b>.032</b>	31.60	.335
V1static–V2static	42.48	<b>&lt;.001</b>	77.60	.364
V1static–V3static	46.32	<b>&lt;.001</b>	102.23	.634
V1static–V4static	60.91	<b>.020</b>	72.91	.316
V2static–V3static	29.39	<b>&lt;.001</b>	27.40	<b>.022</b>
V2static–V4static	46.66	<b>&lt;.001</b>	9.85	<b>.001</b>
V3static–V4static	35.28	<b>&lt;.001</b>	29.56	<b>.028</b>

Note: *p*, *p*-values of the test against the null hypothesis that the vectors have random directions in the shape tangent space.

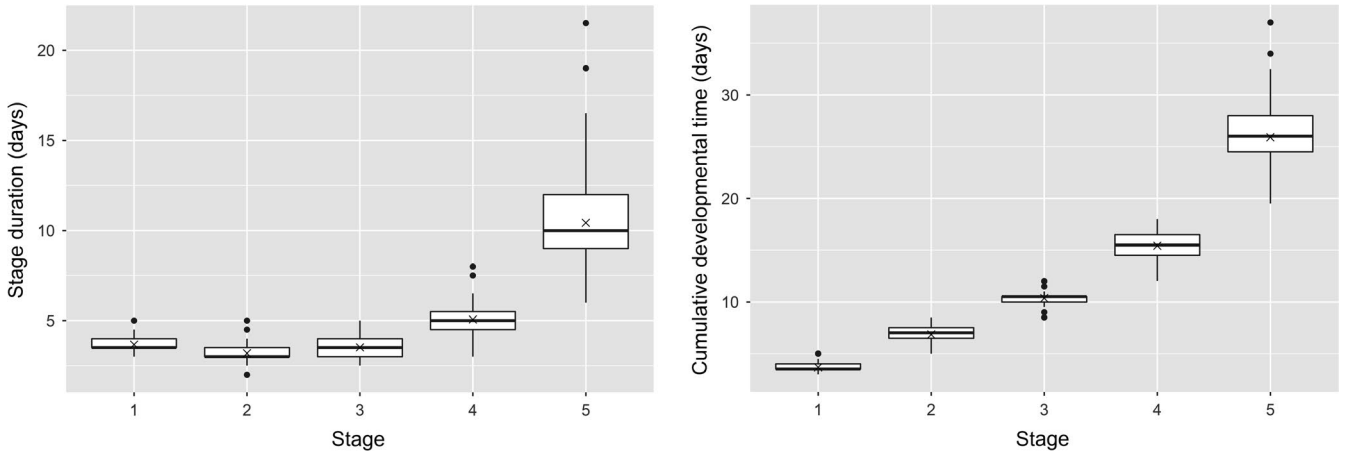
of individual developmental variation is almost annulled, the data one can obtain are on the other hand suitable for investigating detailed quantitative aspects of growth and growth regulation. We produced a novel dataset of longitudinal growth data for the lepidopteran *P. brassicae* and conducted a first batch of growth analyses on several aspects of size, shape

and timing, which exposes some underlying mechanisms of post-embryonic developmental control in this insect.

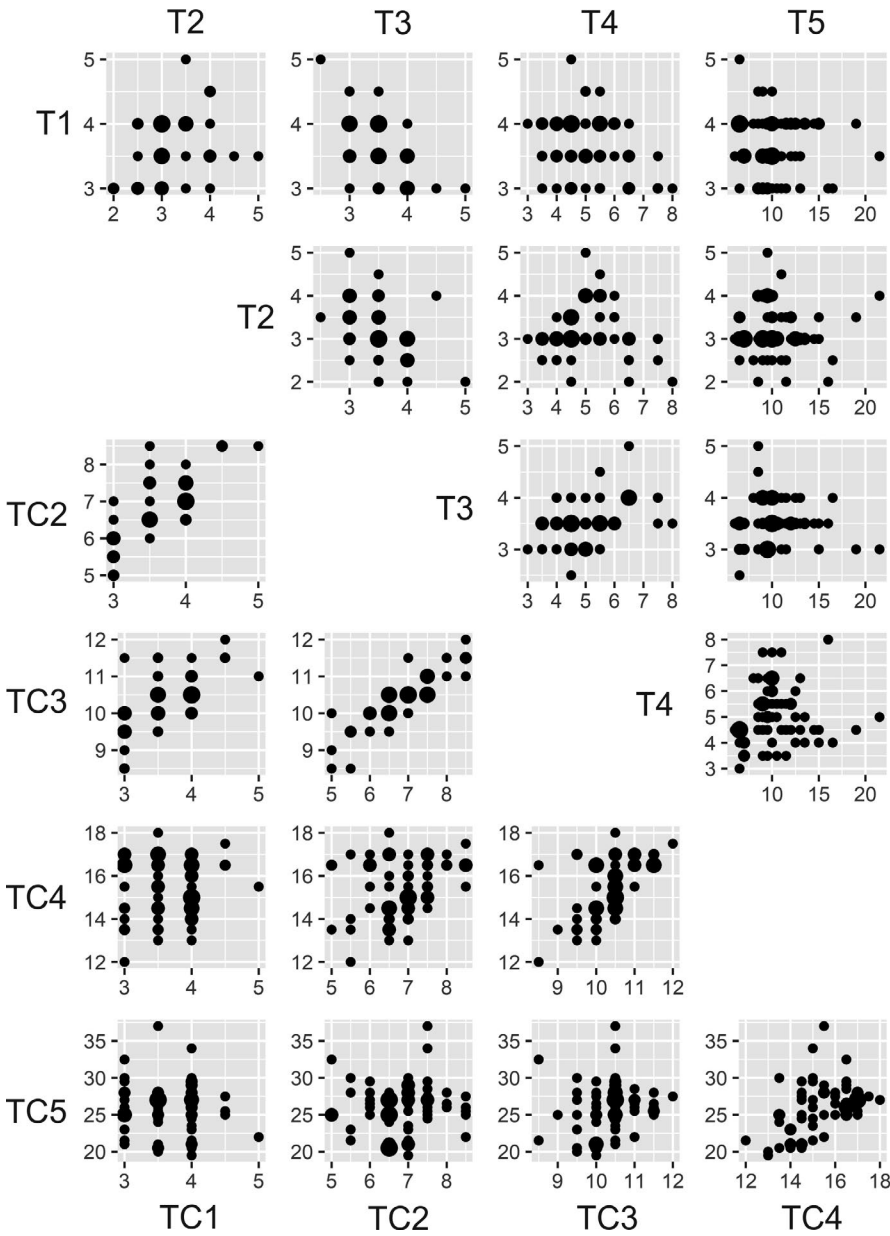
#### 4.1 | Sources of individual variation

The features of the stock from which the study sample derives and the collection of individuals from numerous families make confident that the level of genetic variation in the sample is representative of that present in natural populations of the species.

With respect to the investigated developmental characters, no significant differences in growth progressions between sexes, either for size or time, were found. On the contrary, the belonging to a given family showed a significant effect on size (but not on growth rates) and, although less consistently, on developmental times. This suggests an hereditary component for the size at hatching and for developmental times at specific stages. This is in agreement with the fact that there are positive maternal effects on offspring egg size and larval size at hatching (Azevedo, Partridge, & French, 1997). These effects may include a mix of genetic and environmental components (Bonduriansky & Day, 2018; Fox, 1994; Mousseau & Dingle, 1991; Mousseau & Fox, 1998), but only specifically designed experiments, which involve genetic analyses (e.g. Meister, Hämäläinen, Valdma, Martverk, & Tammaru, 2018;



**FIGURE 4** Duration of larval stages (left panel) and cumulative developmental time (right panel) in *Pieris brassicae*. Boxes represent the interquartile interval, with median (transverse line) and mean (cross); vertical lines are ranges of variation, to the exclusion of outliers (dots)



**FIGURE 5** Relationships between the durations of stages ( $T$ ) and cumulative developmental time ( $TC$ ) in *Pieris brassicae*. The diameter of the points is proportional to the number of individuals with the same combination of stages' duration (resolution 1/2 day)



Mensch et al., 2008), could shed light on these hereditary aspects of growth.

## 4.2 | Growth progression and conformity to Dyar's rule

The Average per-moult Grow Rate for the frons (1.616) is very close to the value observed by David and Gardiner (1962) ( $AGR = 1.638$ , our calculation on their Table I) on the ontogenetic progression of the width of the cephalic capsule at 25°C. This value is close to the median value of 1.52 reported for different measures of the head in the larvae of holometabolous insects (Cole, 1980) and the median value for the Lepidopterans (1.56, our calculation on Cole's Table 1).

Dyar's rule, the constant rate of size increase between moults, is considered a “null model” of arthropod growth, although several cases of deviation from perfect constant growth have been recorded, often in the form of declining growth rates with later stages (e.g. Morales-Ramos, Kay, Rojas, Shapiro-Ilan, & Tedders, 2015). We recorded a significant, although modest, deviation from constant growth rate in both the frons and labrum of *P. brassicae*. The two deviation patterns are different, and only the frons show declining growth rates with stage (Figure S2). Significant deviations from constant growth rates with variable patterns were also recorded in other insects (Brown & Davies, 1972; Grunert, Clarke, Ahuja, Eswaran, & Nijhout, 2015; Klingenberg & Zimmermann, 1992; Savopoulou-Soultani & Tzanakakis, 1990) and other arthropod taxa (Minelli & Fusco, 2013b).

Variance in  $\ln CS$  increases steadily during the first three stages, but not at the fourth. In any case, stage-specific ranges of size variation are small in comparison with growth rates, so that size distributions of different stages do not overlap (Figure S1). Values of  $\ln CS$  are all positively and significantly correlated between stages, both for the frons and labrum. Stated differently, those specimens that have larger (or smaller) sizes at the beginning of post-embryonic development tend to retain their relative size throughout larval growth, to the point that size at the first stage is a good predictor of size in all later stages. Klingenberg (1996) found a similar growth pattern in the waterstrider *Limnoporus canaliculatus* (Heteroptera: Gerridae).

## 4.3 | Allometry

Understanding the relationship between static and ontogenetic allometry is crucial to understanding shape evolution (Klingenberg, 2016; Pélabon et al., 2013; Shingleton & Frankino, 2018). However, very few observational data are available for insects (Klingenberg & Zimmermann, 1992; Nijhout & McKenna, 2017).

In *P. brassicae*, even when significant, static allometry is modest with respect to ontogenetic allometry, both in the frons and the labrum. The angles between allometric vectors in the shape space reveal that, especially in the frons, the regression vectors of static allometry differ significantly in direction from the ontogenetic vector. However, since the analysis on static allometry showed that, in both parts, just some of the static allometric vectors are significantly different from isometry and that the variation explained in any case is modest, the differences found in these comparisons have overall a little impact on shape.

Regression lines of static allometry of labrum on frons show an ontogenetic trend to becoming progressively more steep, but in most cases not significantly departing from isometry, as for the shape allometry within each single part. In contrast, the significantly negative ontogenetic allometry depends on the different morphology of the two structures; while the frons has an important elongation during growth, the labrum, less developed in height than in width, is little affected by antero-posterior elongation, which results in an overall lesser growth.

## 4.4 | Timing

Average duration of larval development (25.9 days) is sizably longer than the value observed in David and Gardiner (1962) and Breugnon (1972) rearing experiments at 25°C (12 and 13 days, respectively), even considering that these figure were obtained on a diet on leaf and that there is an expected delay for a synthetic diet in the order of 1–2 days on total larval time (David & Gardiner, 1965). This discrepancy is largely explained by the fact that individually reared larvae of *P. brassicae* are known to take considerably more time to complete their development, up to a week (Long, 1953). This is a well-known effect in gregarious caterpillars, like *P. brassicae*, which develop faster and survive better in larger groups (e.g. Clark & Stanley, 1997; Kuussaari & Singer, 2017). However, the delay in developmental times with respect to other studies is quite evenly distributed across all stages, and longer developmental times did not impact per-moult growth rates (see above).

Interestingly, the analysis of  $TC$  variation revealed a stage of apparently more effective developmental control at stage 3. This is also supported by the pattern of correlations between single stage durations. The positive correlation between  $T1$  and  $T2$  supports the idea of the presence of “slow-” and “fast-progressing” individuals during the first two stages. In parallel, the negative correlations between  $T1-T3$  and  $T2-T3$  are in agreement with a compensating duration of stage 3 and explain the reduction of variation in  $TC3$ . However, this compensation is only partial, as  $TC1$  and  $TC2$  are still a positively correlated with  $TC3$ . For all we know, no other clearly documented cases of a stage-localized control of developmental timing have been recorded so far, or these might not have been recognized as such. A

possible analogous case transpires from the sizable reduction in the variance of the cumulative time to the second larval stage (out of three) in the high-altitude species *Calliphora nigribasis* (Diptera: Calliphoridae), reared at low temperatures (Vélez & Wolff, 2008). In contrast, in a well-documented longitudinal study on the growth of the waterstrider *L. canaliculatus*, extensive positive correlations among the durations of all stage were found (Klingenberg, 1996).

On the whole, these findings point to the little considered phenomenon of the unequal meaning of moults along an arthropod's post-embryonic development (Minelli, 2003:64) and can contribute to the recent progresses in our understanding of the mechanisms controlling size, scaling and timing in insects development and their evolution.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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## Larval growth and allometry in the cabbage butterfly *Pieris brassicae* (Lepidoptera: Pieridae)

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### Supporting Information

**Table S1.** Definition of landmarks positioned on the two parts of the cephalic capsule in *P. brassicae*.

part	landmark	definition
<b>FRONS</b>	f1	right gena upper seta
	f2	right gena central seta
	f3	right gena lower seta
	f4	left gena upper seta
	f5	left gena central seta
	f6	left gena lower seta
	f7	right gena upper paramedial seta
	f8	right gena lower paramedial seta
	f9	left gena upper paramedial seta
	f10	left gena lower paramedial seta
	f11	top vertex of the frons
	f12	right frons central seta
	f13	right frons central paramedial seta
	f14	left frons central paramedial seta
	f15	left frons central seta
	f16	right frons lower seta
	f17	right frons lower paramedial seta
	f18	left frons lower paramedial seta
	f19	left frons lower seta
<b>LABRUM</b>	l1	right labrum upper paramedial seta
	l2	right labrum central seta
	l3	right labrum lower seta
	l4	left labrum upper paramedial seta
	l5	left labrum central seta
	l6	left labrum lower seta

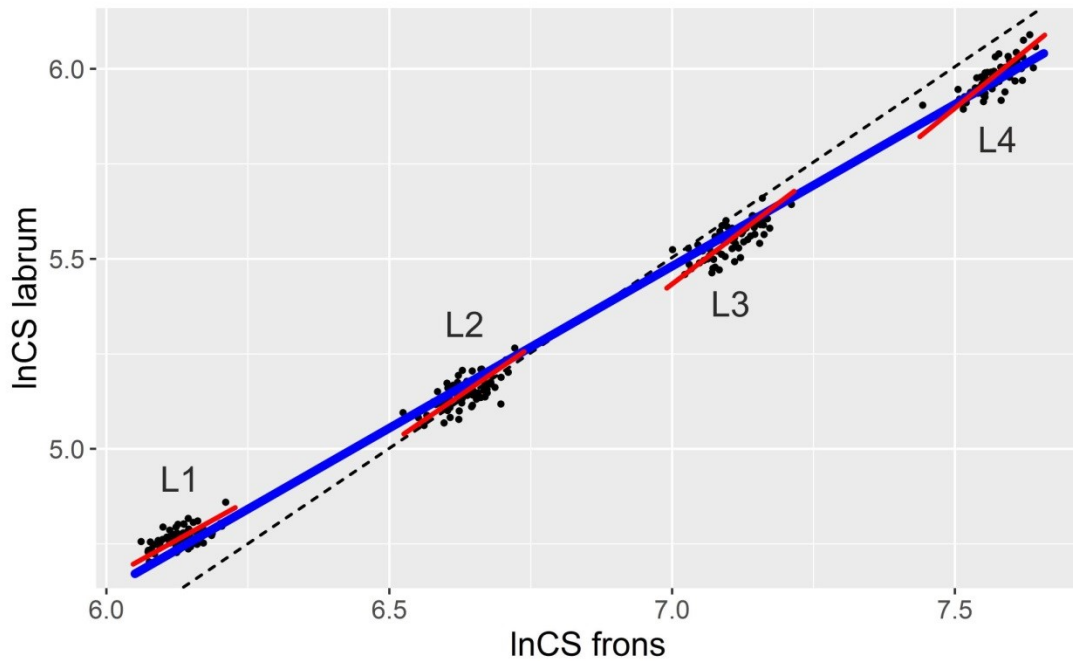
**Table S2.** Results of one-way ANOVAs with the classification factors “sex” and “family” for two morphometric developmental variables (logarithm of the centroid size, *lnCS*; logarithm of the per-stage growth rate, *lnGR*) at different larval stages (1-4) in *P. brassicae*. df, degrees of freedom; F, Fisher’s test value; p, p-value.

factor ‘sex’						
	FRONS			LABRUM		
variable	df	F	p	df	F	p
lnCS1	1	0.84	0.364	1	0.85	0.361
lnCS2	1	2.29	0.135	1	1.46	0.232
lnCS3	1	3.20	0.078	1	2.18	0.145
lnCS4	1	3.86	0.054	1	2.17	0.146
lnGR1	1	1.19	0.279	1	0.66	0.419
lnGR2	1	0.47	0.494	1	0.45	0.504
lnGR3	1	0.01	0.906	1	0.04	0.842
factor ‘family’						
	FRONS			LABRUM		
variable	df	F	p	df	F	p
lnCS1	18	3.22	<b>0.001</b>	18	2.70	<b>0.003</b>
lnCS2	18	2.95	<b>0.002</b>	18	2.97	<b>0.001</b>
lnCS3	18	2.65	<b>0.004</b>	18	2.22	<b>0.015</b>
lnCS4	18	2.46	<b>0.007</b>	18	1.61	0.098
lnGR1	18	1.70	0.074	18	2.39	0.089
lnGR2	18	0.78	0.716	18	0.84	0.649
lnGR3	18	1.66	0.084	18	1.45	0.153

**Table S3.** Results of one-way ANOVAs with the classification factors “sex” and “family” for two developmental time variables (stage duration, *T*; cumulative developmental time, *TC*) at different larval stages (1-5) in *P. brassicae*. df, degrees of freedom; F, Fisher’s test value; p, p-value.

<b>factor ‘sex’</b>			
<b>developmental time</b>	<b>df</b>	<b>F</b>	<b>p</b>
T1	1	0.57	0.451
T2	1	0.02	0.881
T3	1	0.37	0.548
T4	1	2.31	0.133
T5	1	1.83	0.181
TC1	1	0.58	0.451
TC2	1	0.26	0.611
TC3	1	0.07	0.798
TC4	1	1.27	0.263
TC5	1	2.40	0.127
<b>factor ‘family’</b>			
<b>developmental time</b>	<b>df</b>	<b>F</b>	<b>p</b>
T1	18	4.65	<b>&lt;0.001</b>
T2	18	1.72	0.071
T3	18	1.65	0.085
T4	18	3.07	<b>0.001</b>
T5	18	0.82	0.663
TC1	18	4.66	<b>&lt;0.001</b>
TC2	18	2.88	<b>0.002</b>
TC3	18	1.83	<b>0.050</b>
TC4	18	1.81	0.053
TC5	18	0.81	0.679

**Figure S1.** Static (thin solid lines) and ontogenetic (thick solid line) allometry of labrum on frons during the first four larval stages (L1-L4) in *P. brassicae*. RMA regressions on the logarithm of the centroid size ( $\ln CS$ ). Isometric slope (dashed line) is shown as a reference.



**Figure S2.** Log-transformed per-stage growth rates ( $\ln GR$ ) of the frons (left panel) and labrum (right panel) during the first four larval stages in *P. brassicae*. Boxes represent the interquartile interval, with median (transverse line) and mean (cross); vertical lines are ranges of variation, to the exclusion of outliers (dots).

