Original research article

Head Capsule Width, Body Weight and Length Measurements for Instar Determination in Mason Bee (*Osmia cornifrons*) Larvae

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Abstract *Osmia cornifrons* is a cavity-nesting solitary species used as an apple pollinator in Korea. We collected *Osmia* spp larvae to examine potential correlations of larval stage. The head widths of from 1st to the 5th instar ranged from 0.4 ± 0.1 to 1.3 ± 0.3 mm, and growth rate of each instar was significantly highest between 1st and the 2nd instar. The fitness regression model for the head capsule width was analyzed. The head capsule width plotted against the number of instars resulted in a regression line of $y = 0.146x + 0.551$, $R^2 = 0.838$. The body weights of the larvae increased with larval developmental stage, and the coefficient of variation of larval weights was high. However, the calculated regression line was $y = 5.122x^2 - 12.154x + 11.444$, $R^2 = 0.900$. The growth ratio of the larval length was clearly maximal between the 3rd and 4th instars, while that of 5th instar larvae was decreased. The calculated regression line is $y = 2.595x + 0.472$, $R^2 = 0.920$. The result suggests that measurements of head capsule width, weight and length may be useful as a method to determine larval instar.

Keywords Mason bee, *O. cornifrons*, Larval development, Head width, Weight, Length, Instar

INTRODUCTION

Osmia cornifrons (Radoszkowski) (Hymenoptera: Megachilidae), commonly known as the Japanese orchard bee or hornfaced bee, is an important alternative pollinator of various crops, such as apples, apricots, cherries, nectarines, peaches, pears, plums, etc. (Yamada *et al*., 1971; Park *et al*., 2018). Body size is one component of the female phenotype that has linked to variation in female reproductive success within species of mason bees. Size correlated positively with fitness through its effects on provisioning rate; provision mass, fecundity, and offspring size and sex ratio (Kim, 1997; Roulston and Cane, 2000; Bosch and Vicens, 2006; Rehan and Richards, 2010). Thus, factors that affect body size during development can affect reproductive success later in life. Heritability for body size is apparently zero or low for solitary bees (Tepedino *et al*., 1984; Frohlich and Tepedino, 1986; Owen and

Mcorquodale *et al*., 1994). Instead, body sizes attained by adult females are strongly influenced by environmental conditions during larval development, including the amount of pollen and nectar they received (Roulston and Cane, 2000; Radmacher and Strohm, 2010), Size and age at maturity are important life history traits. Body length is also one of the the defining traits of a species even though maximum species range little around a species characteristic an organism's length relates immediately to its shape and function (Schmidt-Neilson, 1984) and scaling research have end up increasingly critical in ecology and evolutionary biology (Peters, 1983; Calder, 1984; LaBarbera, 1989). Several variables have been used as an estimator of body size in bees, including body length (Gathmann and Tscharntke, 2002), radial cell length (Cane, 1987; Pouvreau, 1989; Bullock, 1999), and head width (van Nieuwstadt and Ruano Iraheta, 1996; Roulston and Cane, 2000). Larval age of larvae is distinguished

by the size of the head width (Dyar and Rhinebeck 1890; Sharifi and Mills 1971). This is because the larva grows exponentially when molting without growth (Nijhout, 2013), and the keratinized part of the head during the development of the larva grows in a discontinuous or stepwise manner in order that the larva grows exponentially (Gains and Campell, 1935). In addition, Bodenheimer (1927) stated that the larva's body length, body width, head height, tactile length, and leg length grow in stages like the head width, and have a linear relationship with the reiki. Yoon *et al.* (1997) determined to larvar instar of the mulberry longicorn beetle *Apriona germari* using head capsule width, weight and length, it is not easy to determine its age because the form of habitat is perforated and passes within the branches of the host plant. Each larva collected different places and different temperature.

This makes a study on the extent to which size can also have an effect on some of life-records traits impossible and information on size-weight relationships of living forms is more appropriate. Two measurements, head capsule width and larval length, have been used as a correlate of body weight in *O. cornifrons* larval stages. Kodaira (2009) demonstrated that the two measures among different types of worker bees found head widths to be a better indicator of body size. Our report indicates in three areas of size-weight relationships in the bee *O. cornifrons*: 1) Correlation between larval stage and head capsule width 2) Correlation between larval stage and weight 3) Correlatation between larval stage and body length. However, the characteristics of different larval stages of *O. cornifrons* commercialized in Korea remain unclear. In addition, the precise number of instars that *O. cornifrons* larvae go through at the optimal temperature of 25°C is almost not precisely known. Using body length, head capsule width and larval growth measurement to study *Osmia* spp length-weight relationship using simple linear measures, such as the head capsule width and body length. In order to verify our conclusions on growth and size regulation we developed a new strategy of the larval stage model that incorporates the new findings described in this paper and that accurately reproduces growth trajectories under a variety of temperatures and nutritional conditions. We characterized the developmental mechanisms shaping body size in the mason bee pollinator, *O. cornifrons*. This study manipulates larval development in mason bee to understand how developmental mechanism shape adult body size, we based our approach on the insect body size, weight, and length of head capsule width for each instar.

MATERIALS AND METHODS

1. Experimental insect

Mason bee eggs and larvae, used as experimental insects, were collected by trap nesting from an insect garden in the middle eastern part of the Korean Peninsula in Yeongwol (37°12′55″N, 128°21′46″E), Jeongseon (37°16′04″N, 128°44′31″E) and Jecheon (37°04′03″N, 128°09′51″E) of the Republic of Korea, in the early to late 2017. Periodically, we collected a trap-nest straw we plugged with a mud and then brought into the rearing room at 25°C, 65% relative humidity (RH), and continuous darkness (Lee *et al.*, 2016). Harvested bamboo nest straws were sliced longitudinally along one side to allow access to the nest contents for parasite removal; then, we collected egg and larvae from the nest straws. The collected eggs and larva were moved into transparent 24-well cell culture plates marked to indicate. Egg and larvae were reared in the rearing room to investigate the species growth rate, length and head width and when the larvae become cocoons; we sorted the species of *O. cornifrons* from the cocoons of *Osmia* spp. using the manuscript by Maeta (1978) and book by Yoon *et al.* (2015).

2. Measurement of head width capsule, length, and weight of *O. cornifrons* larvae

To determine the growth rate for *O. cornifrons*, larvae we investigated the period and sizes of head width, body length, and weight of each instar. The sizes of head widths, body lengths, and weights were measured using a HVC-2000A (Cisvision, Korea) digital inspection microscope. The growth ratio for larval development was determined on the basis of head width, body weight and body length of 1st to 5th after molting. Widths of the molted head and body lengths were measured using the HySCALER software for image analysis (HySCALER software; version 1.4; Cisvision, Korea). Larval weight
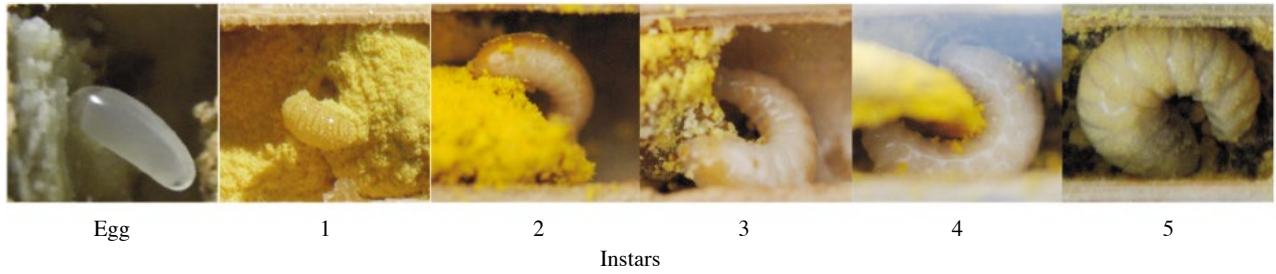
Fig. 1. Eggs and larval stages of *Osmia* spp. on the bamboo trap nests.

was recorded using an electronic balance (Scaltec, Germany). Additionally, the larval period of each instar was monitored.

3. Statistical analysis

The statistical analyses were conducted using the one-way ANOVA test (followed by the post-hoc Tukey HSD test) and regression analysis. In particular, the Welch's ANOVA was performed if the data were not of equal variance. The one-way ANOVA test was used to investigate differences in the average head width, body length, body weight and duration in each larval instar. The relationships between larval size (head capsule width, weight, and body length) and larval stage were confirmed using a regression analysis, and each regression equation was derived if a significant regression model was confirmed. In addition, fitness was confirmed by substituting measurement data of head width capsule, length, and weight into respectively derived regression equations. All data were statistically processed after normality was tested using the Shaprio-Wilks test. All statistical analyses were performed using the SPSS PASW 22.0 package for Windows (IBM, Chicago, IL, USA).

RESULTS

1. Determination of growth rate by head capsule widths instar-wise in larvae

More than 1000 larvae were collected from 16 April 2017 to August 2017. Among these, 90% larvae were of mason bee *O. cornifrons* passes through larval instars to become a pupa. The head capsule widths of *O. cornifrons* ranged from 0.7 to 0.1 mm. The head capsule widths of *O. cornifrons* larvae reared individually

Table 1. Head capsule width and growth ratio of each instar in *O. cornifrons* larvae

Instar	n	Head width (mm)	Coefficient variation (%)	Growth ratio
1	35	0.7±0.1	16.4	–
2	35	0.9±0.1	8.5	1.3±0.3a
3	30	1.0±0.1	7.3	1.1±0.1b
4	30	1.1±0.1	6.9	1.1±0.1b
5	30	1.3±0.1	8.0	1.1±0.1b

1) n mean number of larva surveyed.
2) There was significant difference in growth ratio over each instar (oneway ANOVA test $F_{3,120} = 9.208$, $p = 0.0001$).

through all five instars under laboratory conditions were in the range of 0.7±0.1 mm, 0.9±0.1 mm, 1.0±0.1 mm, 0.1±0.1 mm, and 1.3±0.1 mm, 1st to 5th instar respectively (Table 1). There were significant differences among the mean head capsule widths of different instars (one-way ANOVA test $F_{3,120} = 9.208$, $p = 0.0001$). Growth peaked in the 2nd instar. The growth ratio of each instar in head capsule width was significantly highest between 1st and 2nd instars. The coefficient variation (CV) in the head width was highest (16.4%) duration the 1st instar, and gradually decreased until the 4th instar. When the larval head capsule width was plotted against the number of instars, the calculated simple linear regression model was $y = 0.146x + 0.551$ ($R^2 = 0.838$, ANOVA test: $F_{1,164} = 849.587$, $p = 0.0001$, DW = 1.625), (Fig. 2). The fitness to the linear regression model for the larval head width shows a relatively poor fitness (97%), but using the 1st instar compared with better fitness than when based on the other instars (Table 2). The fitness for the larval head width from the total instars was 98.5%. However, the fitness difference between the observed and theoretical values was clear.
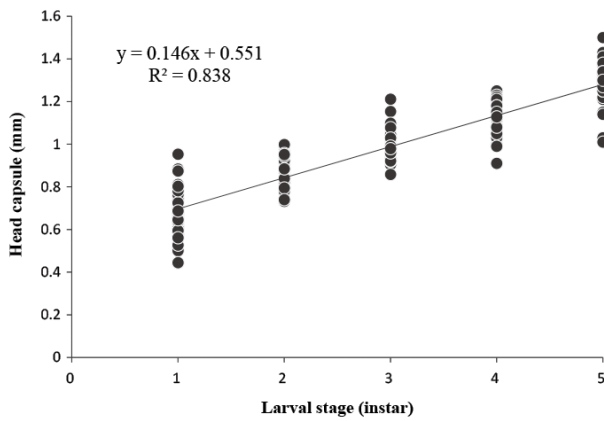
Fig. 2. Correlation between larval stage and head capsule during lrava period in the laboratory condition. The reared condition was maintained 25°C, 60% (RH). Average head width ($\pm$ SD) and applied the exponential regression model ($R^2 = 0.838$, $p = 0.0001$, $y = 0.146x + 0.551$).
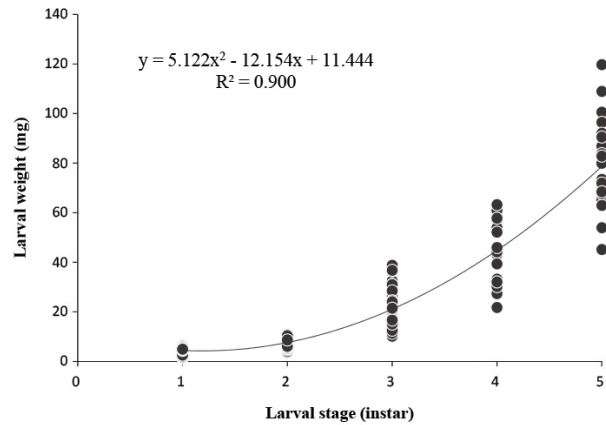

Fig. 3. Correlation between larval stage and weight during lrava period in the laboratory condition. The reared condition was maintained 25°C, 60% (RH). Average weight ($\pm$ SD) and estimated exponential curve model ($R^2 = 0.900$, $p = 0.0001$, $y = 5.122x^2 - 12.154x + 11.444$) in regression analysis.

Table 2. Fitness to regression model for head width of *O. cornifrons* larvae

Instar	Observed	Theoretical	Fitness (%)
1	0.676	0.697	97.0
2	0.865	0.843	97.4
3	0.995	0.989	99.4
4	1.128	1.135	99.4
5	1.275	1.281	99.5

1) Fitness to regression model was calculate by '(observed value / theoretical value) $\times$ 100'. It was calculated by '1 + (1 − (observed value / theoretical value)) $\times$ 100', if the theoretical value was smaller than the observed value.

Table 3. Weight and growth ratio of each instar in *O. cornifrons* larvae

Instar	n	Weight (mg)	Coefficient variation (%)	Growth ratio
1	55	3.9 $\pm$ 1.1	27.1	−
2	55	7.6 $\pm$ 1.7	22.7	2.1 $\pm$ 0.9ab
3	31	18.0 $\pm$ 4.8	26.4	2.5 $\pm$ 0.9a
4	30	46 $\pm$ 12.6	27.0	2.5 $\pm$ 0.9a
5	30	78.3 $\pm$ 16.1	20.2	1.8 $\pm$ 0.5b

1) n mean number of larva surveyed.
2) There was significant difference in growth ratio over each instar (oneway ANOVA test $F_{3,167} = 5.474$, $p = 0.001$).

2. Determination of growth rate by body weight instar-wise in larvae

The larval body weights of *O. cornifrons* were in the range of 3.9 $\pm$ 1.1 mm to 78.3 $\pm$ 16.1 mm (Fig. 1 and Table 3). There were significant differences among the mean larval body weight of the different instars. The growth ratio for larval weight was based on 1st and 5th instars, and no significant difference in growth ratio over each instar (one-way ANOVA test $F_{3,167} = 5.474$, $p = 0.001$) was apparent. The growth ratio of larval weight was highest between the 3rd and 5th instars. The growth ratio was higher in the 3rd and 4th instar, whereas that of the 5th instar exhibited a decrease. The average larval weight CV was 24.7% higher than comparative percentages related to head capsule width and length. The calculated exponential curve model

Table 4. Fitness to regression model for weight of *O. cornifrons* larvae

Instar	Observed	Theoretical	Fitness (%)
1	4.475	4.121	98.6
2	7.591	8.768	99.6
3	20.795	18.655	98.6
4	45.980	39.692	97.3
5	78.253	84.448	99.4

1) Fitness to regression model was calculate by '(observed value / theoretical value) $\times$ 100'. It was calculated by '1 + (1 − (observed value / theoretical value)) $\times$ 100', if the theoretical value was smaller than the observed value.

was $y = 5.122x^2 - 12.154x + 11.444$ and R^2 was 0.900 (ANOVA test: $F_{2,290} = 1299.383$, $p = 0.0001$) (Fig. 3). The fitness to the exponential curve model for the larval weight from total instars was 98.7% (Table 4), showing

Table 5. Body length and growth ratio of each instar in *O. cornifrons* larvae

Instar	n	Body length (mm)	Coefficient variation (%)	Growth ratio
1	35	3.7 ± 0.6	15.7	−
2	35	5.2 ± 0.5	9.9	1.4 ± 0.2b
3	30	7.6 ± 1.1	14.1	1.5 ± 0.3b
4	30	11.3 ± 1.1	9.8	3.8 ± 0.4a
5	30	13.6 ± 1.3	9.3	1.2 ± 0.2c

1) n mean number of larva surveyed.
2) There was significant difference in growth ratio over each instar (oneway ANOVA test $F_{3,121} = 9.614$, $p = 0.000$).

Table 6. Fitness to regression model for body length of *O. cornifrons* larvae

Instar	Observed	Theoretical	Fitness (%)
1	3.710	3.067	79.0
2	5.107	5.662	90.2
3	7.619	8.257	92.3
4	11.332	10.852	95.6
5	13.579	13.447	99.0

1) Fitness to regression model was calculate by '(observed value / theoretical value) × 100'. It was calculated by '1 + (1 − (observed value / theoretical value)) × 100', if the theoretical value was smaller than the observed value.
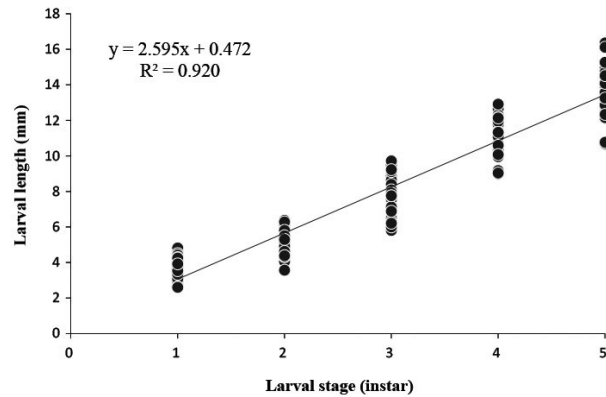

Fig. 4. Correlation between larval stage and body length during lrava period in the laboratory condition. The reared condition was maintained 25°C, 60% (RH). Average body length (± SD) was applied the simple liner regression model ($R^2 = 0.920$, $p = 0.0001$, $y = 2.595x + 0.472$).

a relatively poorer relationship than for the larval head width.

3. Determination of growth rates by body length instar-wise in larvae

The larval lengths ranged from 3.7 ± 0.6 mm to 13.6 ± 1.3 mm. The body length all five instars of *O. cornifrons* larvae were measured (Fig. 1), and significant differences in connection with the growth ratio for each instar (one-way ANOVA test $F_{3,121} = 9.614$, $p = 0.0001$) (Table 5). The growth ratio of larval length was highest between the 3rd and the 4th instars, whereas that after the 4th instar showed a decrease. The coefficient variation in the larval length was highest with 15.7% in the 1st instar. The correlation between larval

stage and body length obeyed the simple linear regression model ($y = 2.595x + 0.472$, $R^2 = 0.920$, ANOVA test: $F_{1,164} = 1887.908$, $p = 0.0001$, $DW = 1.235$) (Fig. 4). The fitness to 1st linear regression model covering larval lengths of all instars was 91.2% (Table 6).

DISCUSSION

We confirmed the characteristics of potential correlations of instar and larval stage of *O. cornifrons* larvae. However, 1st and 5th, showed smaller significant differences head capsule width and 2nd instar showed an increased growth ratio. Thus, it agreement with studies involving lepidopteran species (Calvo and Molina, 2008), this study recorded a variable number of molts for *S. panda* larvae. A variable number of instars is well recorded in the literature for many insect species. This is largely attributable to the nutritive value of the food of the larvae, which is affected by environmental factors (Gaines and Campbell, 1935; Fogal and Kwain, 1972; Schmidt *et al.*, 1977). Temperature was constant in this study; thus, the main source of variation for both head capsule width and instar number is likely to be food type (namely, plant species) and quality. Regression models have been employed by many researchers for determining the number of instars in several species worldwide (Hansen *et al.*, 1981; Cave and Smith, 1983; Logan *et al.*, 1998; Chen and Seybold, 2013; Cazado *et al.*, 2014; Castaneda-Vildozola *et al.*, 2016; Chen *et al.*, 2017). Panzavolta (2007) used the Gaines and Campbell method (the linear regression). In the present study, growth ratios were calculated both

for the observed and theoretical data on instar-wise head capsule widths of *O. cornifrons*. Furthermore, the linear relationship between the natural logarithm of mean head capsule widths and their corresponding instars indicated perfect geometric progression in the growth of the *O. cornifrons* larvae. This supports, which reiterates the geometric growth of head capsule widths through the successive instars. The highly significant linear regression equation ($P \leq 0.0001$; $R^2 \geq 0.838$) for the head capsule widths of successive instars indicates a representation of all instars.

Larval development proceeds through five instars and we collected larva from different places and different temperature (Torchio, 1989) and takes approximately a month under controlled conditions (Bosch and Kemp, 2000). Maeta (1978) reported that larval development involved 5th instars. Temperature thresholds for egg and larval development have been estimated at 10~14°C and 7~14°C, respectively (Maeta, 1978, 2006). In *O. lignaria* and *O. cornifrons*, egg and larval developmental rates increase with increasing temperatures from 18°C to 26°C, and then stabilize at 29~30°C (Bosch and Kemp, 2000; Maeta *et al.*, 2006). On consuming the pollen-nectar provision and completing defaecation, the fifth instar spins a thick multilayered cocoon with secretions originating from its salivary glands (Torchil, 1989). The larval developmental at 26°C took 26 days in *O. lignaria* (Bosch and Kemp, 2000; Sgolastra, 2007).

Hubner (1820) has considered a pest of several ornamental and fruit plants in the Mediterranean area (Hemiptera Coccoidea) (Balachowsky, 1966; Zhang, 1994; Molina and Calvo, 2005), however, information about this insect is scarce. There are no published data on either instar numbers, or head capsule width as a function of the instar in our results. Brief anatomical descriptions of larval stages are available for this species (Amphibian) (Huertas, 1980; Bogner, 1999; Gomez and Aizpurua, 2002; Calvo and Molina, 2008), but no morphometric studies have been published (Molina, 2004). An increment of instar numbers mainly due to adverse developmental conditions can be found in other (Lasiocampidae) lepidopteran species, such as Forest tent caterpillars (Malacosoma disstria), Huber (Esperk *et al.*, 2007). Developmental time (measured as number of instars) seems to increase head capsule width over-

laps and misclassification probabilities, and must be related with the feeding strategies adopted by this species (Malacosoma neustrium) along its life cycle.

The mason bee (*O. cornifrons*) appeared to be highly related in the width of larval head capsule, weight and length. A thorough understanding of its biology and unambiguous identification of larval instars, in particular, are pre-requisites for managing this mason bee successfully by any appropriate means. Based on the studies carried out during the sixties measurement of larval stage, it was concluded Maeta (1978), that *O. cornifrons* pollinator undergoes four or five molts. Among several morphological variables tested, head capsule width, body weight, and length were observed to be the most appropriate features in determining the actual number of instars in *O. cornifrons* by regression analysis (Yoon *et al.*, 2015). We have now determined the number of instars in *O. cornifrons* after quite a long period. This is the first report of instar determination based on the regression analysis of head capsule width, larval weight and length. The regression analysis of fixing the instars has also been validated by counting the number of molts upon individually rearing all instars until pupation under laboratory condition. The mean growth ratios for both instar-derived and theoretical data from the regression analysis were the same. The mean growth ratios of 1.1~1.3 indicate that the number of instars in *O. cornifrons* is five. The linear regression with R^2 values of more than 0.838 between the larval stage (instar) numbers and their corresponding mean head capsule widths reaffirms that no instar has been missed or added in the larva. It is thus, evident from the study that the instar determination by regression analysis is appropriate and reliable. Ours is the first report that the regression analysis to determine the number of instars in *O. cornifrons*. Furthermore, after scanning through the available literatures thoroughly, to the best of our knowledge, this is probably the detailed study, which proves that the mason bee of *O. cornifrons* through five larval instars to reach its pupal stages.

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