Determination of Larval Instars of *Semanotus bifasciatus* (Coleoptera: Cerambycidae) Based on Frequency Distributions of Morphological Variables[1]

Na Li[3], Lei Wu[3], Yongxin Geng, Danfeng Wei, and Min Chen[2]

Beijing Key Laboratory for Forest Pest Control, Beijing Forestry University, No. 35, Tsinghua East Road, Haidian District, Beijing 100083, China

J. Entomol. Sci. 55(3): 405–415 (July 2020)

Abstract *Semanotus bifasciatus* Motschulsky (Coleoptera: Cerambycidae) is one of the most destructive pests of *Platycladus* trees in China. Morphological measurements, such as head capsule (HC) width, can be very useful and practical indicators for identifying larval instars of coleopteran species. In this study, six morphological variables, including HC width, pronotum width, mandible length and width, and body length and width were measured to determine the instars of field-collected larvae of *S. bifasciatus*. Both the HC width and pronotum width were reliable parameters for determining the instar and stage. Larvae of *S. bifasciatus* were divided into eight instars; we detected strong relationships between larval instar and both the HC width ($R^2 = 0.9640$) and pronotum width ($R^2 = 0.9549$). The ranges of body widths and lengths for each instar are provided as reference values for distinguishing among larval stages in field investigations.

Key Words *Semanotus bifasciatus*, instar, head capsule width, pronotum width

Semanotus bifasciatus Motschulsky (Coleoptera: Cerambycidae) is an important borer of cypress and pine trees (e.g., *Sabina* spp., *Chamaecyparis* spp., *Platycladus* spp., and *Cunninghamia lanceolata*) (Gao and Gong 2007; Zhu et al. 2018). It is widely distributed in China, Korea, and Japan (Kim and Park 1984; Zhang and Zhao 1996) and primarily attacks unhealthy trees, wilted trees, and newly cultivated woods. In most of its distribution, *S. bifasciatus* exhibits one or two generations per year and overwinters as an adult in the sapwood of tree trunks. In late February to early May of the following year, a large number of adults will feed on the bark of the trunk where they lay eggs 2 m above the soil surface. Initially, the larvae feed on the phloem and form an irregular tunnel on the surface of the xylem; they then feed on the xylem, causing damage. From mid-July to mid-October, larvae pupate, emerge as adults, and survive the winter from late August (Meng et al. 2016; Zhu et al. 2018). However, probably owing to its biology characteristics and the larvae remaining concealed in the trunks of host trees (Li et al. 2017), it is difficult to fully comprehend the biology and ecology of field populations, including the identification of larval instars according to the timing of molts. The accurate

[1]Received 31 May 2019; accepted for publication 18 October 2019.
[2]Corresponding author (email: minch@bjfu.edu.cn).
[3]These authors contributed equally to this work and should be considered co-first authors.

identification of the developmental stages of larvae can provide important information for pest management (Daly 1985; Gosik et al. 2019). The best-known model for determining larval instars of insects is Dyar's rule, which states that sclerotized structures of individual insects usually remain constant in size during any given stadia (Daly 1985). Frequency distributions of body measurements have been used to determine the number of instars (Bleiker and Régnière 2014; Daly 1985; Dyar 1890). The most suitable age structures are validated by mean difference analysis and regression analysis. Indexes of sclerotized body parts used to determine insect stages include antenna spacing, mandible width, head capsule (HC) width, ocellus spacing, and pronotum width (Cazado et al. 2014; Cen et al. 2015; Daly 1985; Dyar 1890; Luo et al. 2016; Morales-Ramos et al. 2015). Dyar's rule indicates that the HC width of insect larvae increases according to a certain geometric sequence; that is, the change in HC width is related to the larval instar and can, therefore, be used as the basis for the determination of instars (Daly 1985). Though body width and body length are often less reliable for determining insect instars, they are convenient indices of age in field investigations. Thus, the objectives of this study were to identify instars of field-collected larvae and to develop optimal morphometric variables for the determination of *S. bifasciatus* instars.

Materials and Methods

Larvae collection. To ensure the collection of first-instar larvae, adults were collected from the Summer Palace in Beijing (N 39.9°, E 116.6°) in early March 2016 and May 2018. Each pair of male and female adults was matched in a plastic cup for mating and oviposition. Then, eggs were collected and placed in plastic Petri dishes with moistened cotton until they hatched. Other instars were obtained from *Platycladus orientalis* (L.) Franco in the Summer Palace and the Temple of Heaven Park in Beijing. To cover all instar stages, larvae were collected from damaged trunks every 10–15 d from late March to August 2016 and early May to August 2018. All larvae were preserved in 70% ethyl alcohol.

Measurement of six morphological variables. Six morphological variables were measured: HC width, pronotum width, mandible length and width, and body length and width (Fig. 1). All measurements were made with the aid of a fluorescence stereo zoom microscope (M205FA; Leica, Xi'an, Shaanxi, China).

Statistical analyses. A frequency distribution analysis of the six morphological variables was performed using SPSS (Statistical Package for the Social Sciences, Version 21.0, Chicago, IL), and Origin (2018, www.originlab.com) was used to plot a histogram of the frequency distributions (Fig. 1) (Zhou et al. 2015). Based on these analyses, the lowest point between two adjacent peaks and the separation points between instars can be determined (Li et al. 2015). Curves were generated based on measurements of the sclerotized structures to evaluate the relationship between larval stages and each variable (SPSS 21.0 for Windows). The mean, standard error (SE), and coefficient of variation (CV) can be calculated for each variable. The exponential growth model was used to analyze the relationship between the HC width, pronotum width, and instar of *S. bifasciatus* larvae based on Dyar's rule (Crosby 1973), Brooks' ratios (Gaines and Campbell 1935), and linear regression

Fig. 1. Schematic diagram of the measurements for *Semanotus bifasciatus*: Y1, mandible width; Y2, head capsule width; Y3, body width; Y4, pronotum width; Y5, mandible length; Y6, body length.

(Loerch and Cameron 1983; Logan et al. 1998). According to the Crosby growth rule, a difference of 10% or less between Brooks' ratios indicates the correct grouping (Loerch and Cameron 1983). Based on the Crosby growth rule and regression analysis, differences in the six morphological variables between successive instars were evaluated and the best criteria for instar division were determined (Crosby 1973). A Crosby index of greater than 10% indicated that the grouping of age-dependent indicators was not reasonable.

Results and Discussion

We measured 589 larvae. The frequency histograms for HC width and pronotum width showed eight distinct peaks, indicating that the larval development of *S. bifasciatus* can be divided into eight instars (Figs. 2, 3). However, the frequency histograms for the other four variables failed to show conspicuous peaks and regular variation (Figs. 4–7), indicating that body length, body width, mandible length, and mandible width were not reliable indicators for identifying larval instars of *S. bifasciatus*.

We calculated the Crosby ratios to evaluate the reliability of the results (Table 1). The Crosby ratios and CV (%) for the two traits were less than 10%, and the Brooks' ratio ranged from 1.1649 to 1.6804, indicating that HC width and pronotum width effectively group the *S. bifasciatus* larvae into eight instars. Linear regression indicated significant relationships for HC width and pronotum width ($P < 0.0001$, $R^2 > 0.95$). This was consistent with the exponential relationship based on the Brooks' ratios, further validating the division of larvae into eight instars. The regression equation for the relationship between the HC width and the instar number was $Y = 0.5919^{0.2360x}$ ($R^2 = 0.9640$, $P < 0.0001$, regression coefficient $=$ 0.9691) and that for the relationship between pronotum width and instar number was $Y = 0.5930^{0.2442x}$ ($R^2 = 0.9549$, $P < 0.0001$, regression coefficient $= 0.9614$) (Table 2). Accordingly, both the HC width and pronotum width are the best variables for larval instar determination (Figs. 8, 9).

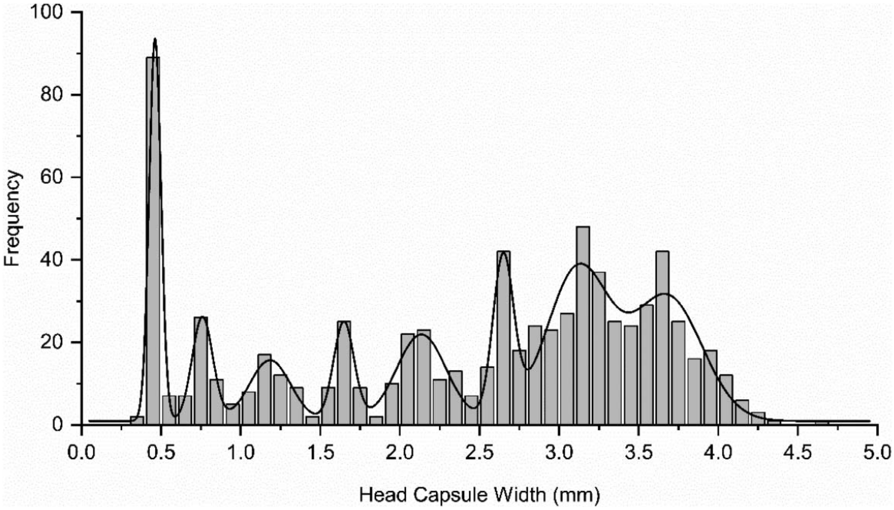

Fig. 2. Frequency distribution of the head capsule width of *S. bifasciatus* larvae.

Statistical techniques based on the normal distribution are adopted in most studies of instar determination for insects (Hunt and Chapman 2001; Lionel et al. 2010; Logan et al. 1998). HC width was considered the most reliable indicator to determine larval instar, but various additional characters are used to identify instars (McClelan and Logan 1994; Velásquez and Valoria 2010). In most Cerambycidae species, HC width is the best morphological variable for larval instar identification. For *Dorysthenes granulosus* (Thomson) and *Monochamus alternatus* Hope, the HC

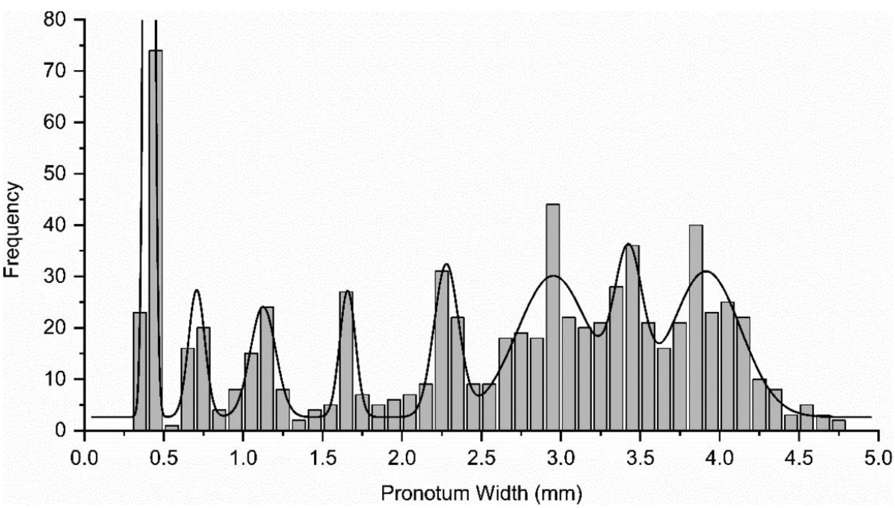

Fig. 3. Frequency distribution of the pronotum width of *S. bifasciatus* larvae.

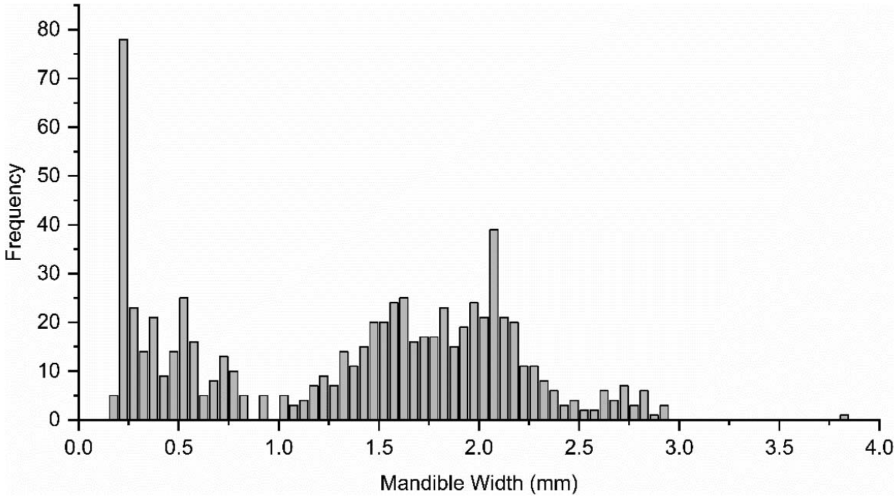

Fig. 4. Frequency distribution of the mandible width of *S. bifasciatus* larvae.

width, with the most significant regression result, is the best indicator of larval instars (Yu et al. 2012). In *Arhopalus rusticus* (L.), *Apriona germari* (Hope), and *Xylotrechus rusticus* (L.), besides the HC width, the pronotum width can also be used as an indicator for larval instar determination (Go et al. 2019; Li et al. 2012; Pan et al. 2015). For other coleopterans, and potentially for all insects, instars are determined based on the HC width, pronotum width and length, mandible length and width, and body length (Cazado et al. 2014; Loerch and Cameron 1983; Luo et al. 2016;, Novák et al. 2018). Although the body length and width were not stable

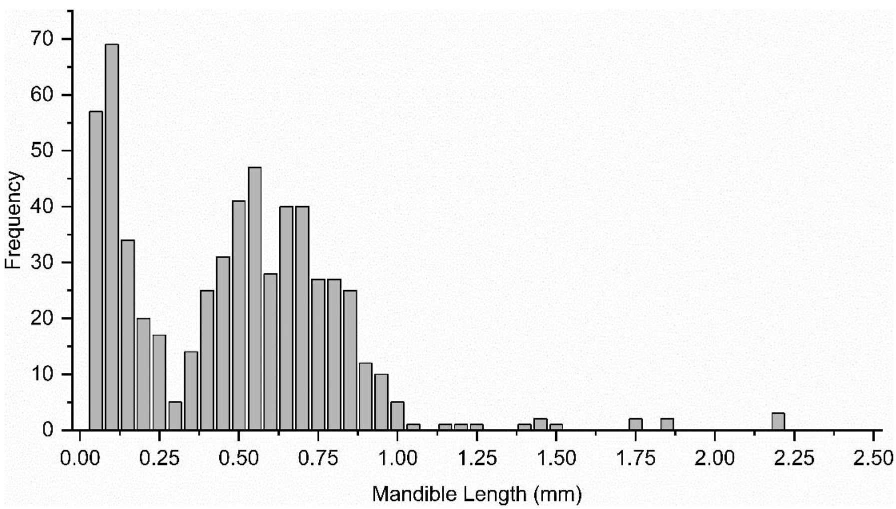

Fig. 5. Frequency distribution of the mandible length of *S. bifasciatus* larvae.

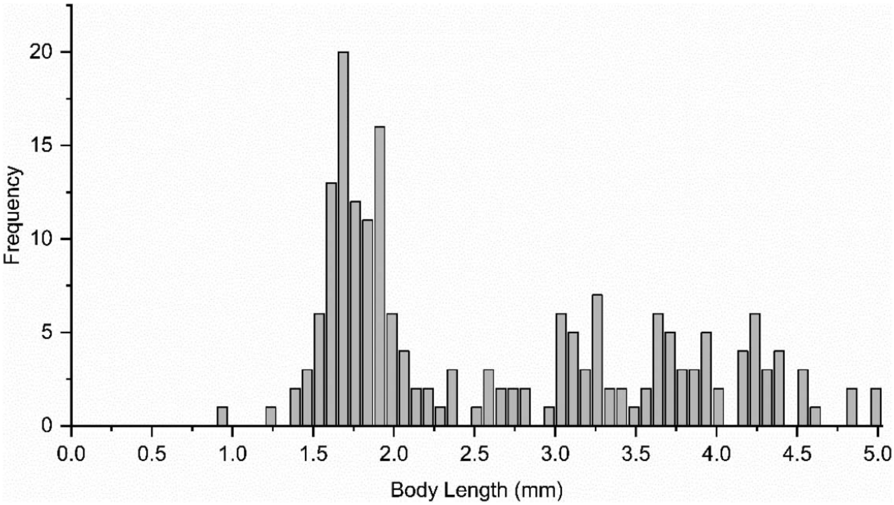

Fig. 6. Frequency distribution of the body length of *S. bifasciatus* larvae.

parameters for determining instars, we provide ranges and average values of these traits for each instar (Table 3) to serve as a reference for field observations.

The determination of the larval instar number can provide a basis for further understanding the species. The number of instars is often considered to be constant within species (Esperk et al. 2007). However, various factors, such as the growth environment (e.g., temperature, humidity, photoperiod, food quality, and host species) and genetic factors, impact the number of instars in insects (Dyar 1890; Langor et al. 1990; Morales-Ramos et al. 2015). Guo et al (2016) reported

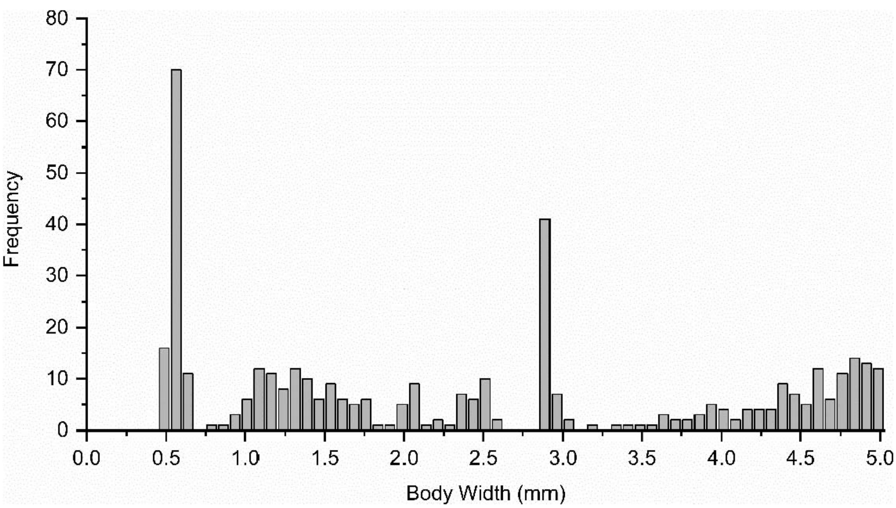

Fig. 7. Frequency distribution of the body width of *S. bifasciatus* larvae.

Table 1. Measurements and summary statistics for head capsule width and pronotum width of *Semanotus bifasciatus* larvae for the determination of larval instars.[A]

Variable	Instar	Number of Samples	Mean (mm) ± SE	Range (mm)	CV (%)	Brooks' Ratio	Crosby's Ratio (%)
Head capsule width (Y2)	1	98	0.4570 ± 0.0294	0.3918–0.5977	6.4279		
	2	44	0.7561 ± 0.0569	0.6330–0.8695	7.5208	0.2991	
	3	40	1.1342 ± 0.0812	0.9764–1.2550	7.1631	0.3781	−0.0932
	4	55	1.5838 ± 0.1568	1.2940–1.9160	9.8985	0.4496	−0.0691
	5	59	2.1211 ± 0.1040	1.9630–2.3720	4.9025	0.5372	−0.0410
	6	66	2.6931 ± 0.0925	2.5220–2.9290	3.4366	0.5720	−0.0519
	7	90	3.1797 ± 0.0881	2.9820–3.3490	2.7700	0.4866	−0.0701
	8	137	3.6808 ± 0.2201	3.3580–4.6310	5.9788	0.5012	−0.0195
Pronotum width (Y1)	1	98	0.4216 ± 0.0308	0.3540–0.5109	7.3017		
	2	44	0.7533 ± 0.0697	0.6809–0.9898	9.2531	0.3317	
	3	40	1.1072 ± 0.0790	0.9535–1.2989	7.1392	0.3539	−0.1774
	4	55	1.5988 ± 0.1531	1.2952–1.9464	9.5764	0.4916	−0.0175
	5	59	2.2771 ± 0.1798	1.7850–3.0591	7.8969	0.6784	−0.0136
	6	66	2.8868 ± 0.2815	1.8773–3.5513	9.7515	0.6097	−0.1099
	7	90	3.4002 ± 0.2600	2.9193–4.0512	7.6471	0.5134	−0.0709
	8	137	3.9028 ± 0.3129	2.8432–4.7713	8.0161	0.5026	−0.0255

[A] SE, standard deviation; CV, coefficient of variation.

Table 2. Linear regression results for the number of larval instars and phenotypic variables for *Semanotus bifasciatus*.

Variables	Regression Equation	*P*-Value	Regression Coefficient	R^2
Pronotum width (Y1)	$Y = 0.5930e^{0.2442x}$	<0.0001	0.9641	0.9549
Head capsule width (Y2)	$Y = 0.5919e^{0.2360x}$	<0.0001	0.9691	0.9640

that larvae of the long-horned beetle *Anoplophora glabripennis* (Motschulsky) can be grouped into five instars whereas Keena and Moore (2010) detected nine instars. *Opisina arenosella* Walker has 5 larval instars in field populations and 5–8 larval instars under laboratory conditions (Yang et al. 2015). However, *M. alternatus* larvae sampled from Zhejiang province and Sichuan province were both identified as having five instars (Huang et al. 2018; Xu et al. 2009). This situation also happened in *Dendroctonus ponderosae* Hopkins; larvae samples from southern and northwestern British Columbia and northcentral Alberta were identically four instars (Bleiker and Régnière 2014). Because of the hidden life of the larvae in Cerambycidae species, the larval instars within species could be most affected by the host plant rather than other environment factors.

In this study, the results indicate that *S. bifasciatus* larvae sampled in Beijing has eight instars. Though *S. bifasciatus* is widely distributed in China (Yang et al. 2015),

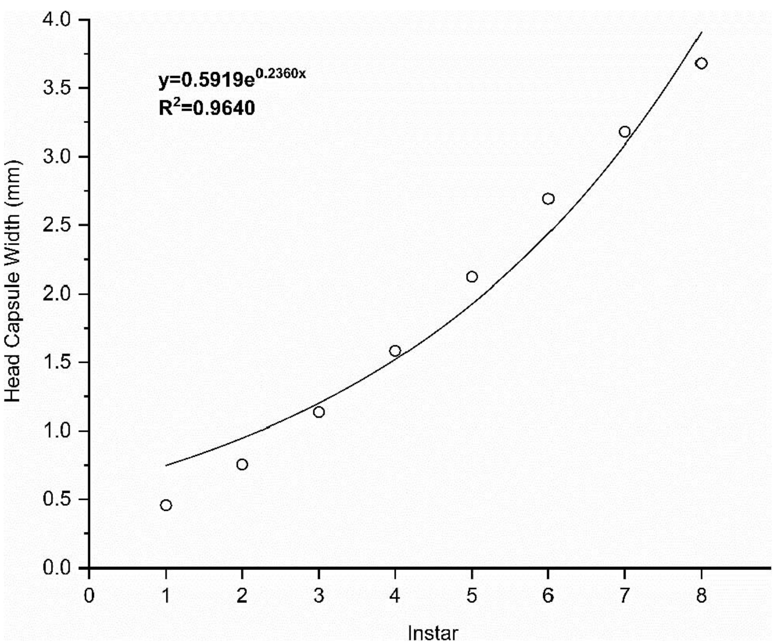

Fig. 8. Relationship between the head capsule width and instar number.

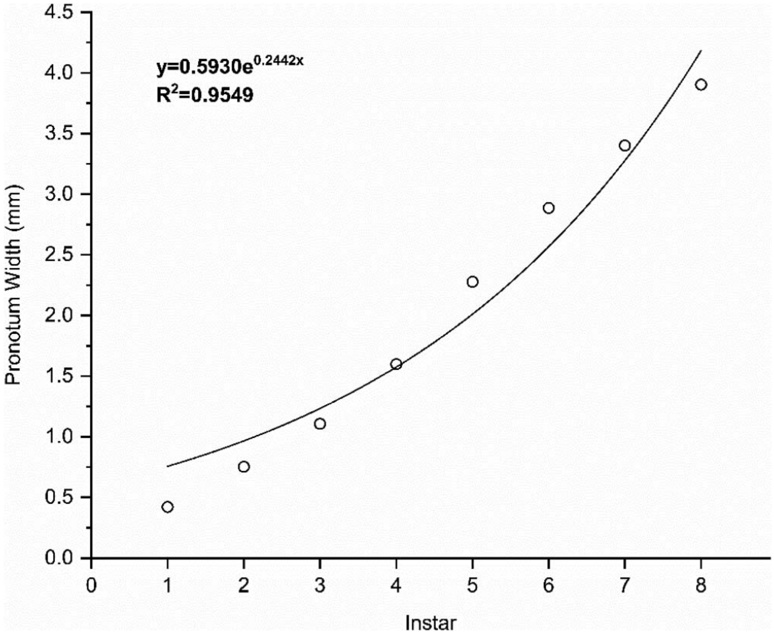

Fig. 9. Relationship between the pronotum width and instar number.

it has a narrow host range: mainly hosted on *Platycladus orientalis* (L.) Franco and occasionally feeding on other cypress trees such as trees in genera of *Sabina* and *Chamaecyparis* (Liang et al. 2002). Moreover, the life history of *S. bifasciatus*, almost one generation per year in China, is relatively short compared with other Cerambycidae species**.** Based on its biology and ecology traits we infer that the

Table 3. Mean values for body length and width of *S. bifasciatus* larvae at different ages.

Instar	Body Length (mm) ± SE	Range (mm)	Body Width (mm) ± SE	Range (mm)
1	1.9263 ± 0.0606	0.9670–6.3400	0.6147 ± 0.0161	0.4517–1.2240
2	3.2905 ± 0.0996	2.2260–4.3810	1.1901 ± 0.0261	0.9112–1.6090
3	4.3687 ± 0.1986	2.3830–10.9400	1.6097 ± 0.0675	1.1780–4.2240
4	6.5714 ± 0.2130	3.7330–11.9030	2.2921 ± 0.0385	1.7560–3.0670
5	11.5799 ± 0.2048	6.2840–20.1810	3.0328 ± 0.0591	2.3660–4.6780
6	14.8496 ± 0.2651	9.7820–20.1970	4.3323 ± 0.0638	3.4420–5.3680
7	17.2123 ± 0.1861	10.0300–25.6150	5.1667 ± 0.0502	3.8540–6.5990
8	20.2015 ± 0.2048	13.6200–25.4800	6.0959 ± 0.0515	4.5390–7.1520

larvae instars of this species may be identical in different geographic populations. However, it should be tested by collecting samples from different distributions if factors such as hosts or geographic conditions can impact on the number of larvae instar of *S. bifasciatus*.

Acknowledgment

We thank the National Major Project of GMO New Species Cultivation (2018ZX08020002) for support.

References Cited

Bleiker, K.P. and J. Régnière. 2014. Determining the instar of mountain pine beetle (Coleoptera: Curculionidae) larvae by the width of their head capsules. Can. Entomol. 147: 635–640.

Cazado, L.E., Ga.V. Nieuwenhove, C.W. Aposbrien, G.A. Gastaminza and M.G. Murua. 2014. Determination of number of instars of *Rhyssomatus subtilis* (Coleoptera: Curculionidae) based on head capsule widths. Florida Entomol. 97: 639–643.

Cen, G., Y. Yu, X. Zeng, X. Long, D. Wei and X. Gao. 2015. An adaptive kernel smoothing method for classifying *Austrosimulium tillyardianum* (Diptera: Simuliidae) larval instars. J. Insect Sci. 15: 159–166.

Crosby, T.K. 1973. Dyar's Rule predicted by Brooks' Rule. N. Z. Entomol. 5: 175–176.

Daly, H.V. 1985. Insect morphometrics. Entomol. 30: 415–438.

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

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

Gaines, J.C. and F.L. Campbell. 1935. Dyar's Rule as related to the number of instars of the corn earworm, *Heliothis obsoleta* (Fab.), collected in the field. Ann. Entomol. Soc. Am. 28: 445–461.

Gao, S., Z. Xu and X. Gong. 2007. Progress in research on *Semanotus bifasciatus*. Forest Pest Dis. 26: 19–22.

Go, M.S., S.H. Kwon, S.B. Kim and D.S. Kim. 2019. The developmental characteristics for the head capsule width of *Monochamus alternatus* (Coleoptera: Cerambycidae) larvae and determination of the number of instars. J. Insect Sci. 19. doi: 10.1093/jisesa/iez010.

Gosik, R., P. Sprick and M.G. Morris. 2019. Descriptions of immature stages of four species of the genera *Graptus*, *Peritelus*, *Philopedon*, and *Tanymecus* and larval instar determination in *Tanymecus* (Coleoptera, Curculionidae, Entiminae). Zookeys 813: 111–150.

Guo, W., P. Gao, F. Wang and Z. Hu. 2016. Occurrence and control techniques of *Anoplophora glabripennis* and *Anoplophora chinensis*. Modern Gardening 8: 53.

Huang, D., R.J. Wang, P. Tang, G.Z. Li, J.Y. Peng, S.F. Wang and L.X. Zhu. 2018. Study on the age division of *Monochamus alternatus* in Panzhihua City and the regularity of each age. Sichuan Agric. Sci. Tech. 373: 31–33.

Hunt, G. and R.E. Chapman. 2001. Evaluating hypotheses of instar-grouping in arthropods: A maximum likelihood approach. Paleobiology 27: 466–484.

Keena, M.A. and P.M. Moore. 2010. Effects of temperature on *Anoplophora glabripennis* (Coleoptera: Cerambycidae) larvae and pupae. Environ. Entomol. 39: 1323–1335.

Kim, K.C. and J.D. Park. 1984. Studies on ecology and injury characteristics of Japanese Juniperus bark borer, *Semanotus bifasciatus* Motschulsky. Korean J. Plant Prot. 23(2): 109–115.

Langor, D.W., J.R. Spence and G.R. Pohl. 1990. Host effects on fertility and reproductive success of *Dendroctonus ponderosae* Hopkins (Coleoptera: Scolytidae). Evolution 44: 609–618.

Li, B., B. Ji, S. Liu, G. Wang, K. Zhang and H. Wu. 2012. Study on larvae instar number and division criterion of *Apriona germari*. J. For. Eng. 26: 38–41.

Li, J.L., Z. Wang, P. Chang, J. Shi, X. Jin and Z. Yao. 2017. Inter-forest trapping monitoring of *Semanotus bifasiatus* Motschulsky (Coleoptera: Cerambycidae) in the Songshan area of Beijing. Beijing Garden 2: 50–52.

Li, Z.W., H.E. Li, J. Xia, L. Ma and A.P. Zeng. 2015. Determination of larval instars of the camellia weevil, *Curculio chinensis* (Coleoptera: Curculionidae). Acta Entomol. Sinica 58: 181–189.

Liang, X.S., M. Zhang, G.Y. Xu and D.H. Xiong. 2002. Study on biological characteristics of *Semanotus bifasciatus* (Matsch). J. Anhui Agric. Univ. 29(1): 20–24.

Lionel, D., L. Patrice and T. Denis. 2010. Larval instars determination for the European grapevine moth (Lepidoptera: Tortricidae) based on the frequency distribution of head-capsule widths. Crop Prot. 29: 623–630.

Loerch, C.R. and E.A. Cameron. 1983. Determination of larval instars of the bronze birch borer, *Agrilus anxius* (Coleoptera: Buprestidae). Ann. Entomol. Soc. Am. 76: 948–952.

Logan, J.A., B.J. Bentz, J.C. Vandygriff and D.L. Turner. 1998. General program for determining instar distributions from head capsule widths: Example analysis of mountain pine beetle (Coleoptera: Scolytide) data. Environ. Entomol. 27: 555–563.

Luo, W., Y.C. Ji and J.B. Wen. 2016. Application of a frequency distribution method for determining instars of *Eucryptorrhynchus brandti* (Coleoptera: Curculionidae) from several morphological variables. Biocontrol Sci. Tech. 26: 1–12.

McClellan, Q.C. and J.A. Logan. 1994. Instar determination for the gypsy moth (Lepidoptera: Lymantriidae) based on the frequency distribution of head capsule widths. Environ. Entomol. 23: 248–253.

Meng, Q., X. Wang., Y. Liu, S. Hou and X. Miao. 2016. Investigation on the occurrence regularity of *Semanotus bifasciatus* (Coleoptera: Cerambycidae) and screening test of control agents. Modern Agric. Tech. 22: 104–106.

Morales-Ramos, J.A., S. Kay, M.G. Rojas, D.I. Shapiro-Ilan and W.L. Tedders. 2015. Morphometric analysis of instar variation in *Tenebrio molitor* (Coleoptera: Tenebrionidae). Ann. Entomol. Soc. Am. 108: 146–159.

Novák, M., P. Jakubec, J. Qubaiová, H. Šuláková and J. Růžička. 2018. Revisited larval morphology of *Thanatophilus rugosus* (Coleoptera: Silphidae). Int. J. Legal Med. 132: 939–954.

Pan, L., Y. Li and Y.Q. Luo. 2015. Determining larval instars in grey tiger longicorn beetle, *Xylotrechus rusticus* (L.) (Coleoptera: Cerambycidae). J. Northeast For. Univ. 43(1): 110–113.

Velásquez, Y.L. and Á. Viloria. 2010. Instar determination of the Neotropical beetle *Oxelytrum discicolle* (Coleoptera: Silphidae). J. Med. Entomol. 47: 723–726.

Xu, J.H., X.F. Huang, H.C. Xu, J.M. Cheng, Y. Jiang and W.J. Fang. 2009. Rearing and biological properties of *Monochamus alternatus*. J. Zhejiang For. Sci. Tech. 29: 86–88.

Yang, C., Y. Wei, C. Li, S. Huang, L. Li and Z. Ma. 2015. Division of larval instars of the coconut black-headed caterpillar, *Opisina arenosella*. Plant Prot. 41(2): 70–74.

Yu, Y., G. Cen, D. Wei, X. Zeng and T. Zeng. 2012. Division of larval instars of *Dorysthenes granulosus* based on Crosby growth rule. J. South. Agric. 43: 1485–1489.

Zhang, S. and Y. Zhao. 1996. The geographical distribution of agricultural and forest insects in China. Agriculture Press, Beijing, China.

Zhou, B., G.Y. Zhou, Q. Yang, W.T. Dong, X.M. Li and S. Zhou. 2015. Biological characteristics and larval instar determination of *Orvasca subnotata* (Lepidoptera: Lymantriida), a defoliator of *Dalbergia odorifera* (Leguminosae). Acta Entomol. Sinica 58: 1253–1261.

Zhu, X.H., J.X. Zeng, B.G. Luo and J.H. Miao. 2018. Study on the control methods of common diseases and insect pests of *Cunninghamia lanceolate* (Lamb.) Hook. South China Agric. 12(3): 81–82.