Content list available at http://epubs.icar.org.in, http://www.icarneh.ernet.in; ISSN: 0970-6429

Indian Journal of Hill Farming

Special Issue 2023, Volume 36, Pages 188-202

Laboratory rearing of citrus trunk borer, *Pseudonemophas versteegii* (Coleoptera: Cerambycidae) to study the different developmental stages

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ARTICLE INFO

Article history:
Received: 10 January, 2023
Revision: 14 January, 2023
Accepted: 17 January, 2023

Key words: *Pseudonemophas versteegii, citrus trunk borer, rearing, larval length, larval weight, head capsule width*

DOI: 10.56678/iahf-spl36.2023.24

ABSTRACT

The citrus trunk borer (CTB), *Pseudonemophas versteegii*, under the group of longhorn beetles, is the most destructive pest in North-Eastern India. Studying these pest species in their natural environment is challenging as they develop inside the tree trunk. Since not much is known about this insect, we reared this insect in the laboratory and studied their development. CTB completed its life cycle in about 402 days. The adults were greyish white with rows of black spots. The freshly laid eggs were creamy white but changed to pale yellow after 2-3 days of incubation. The larvae hatched out after 10-14 days of incubation, and the larval duration lasted about 250 days with a total of nine larval instars. The larval body size varied from 5 - 35 mm, body weight from 6 - 550 mg, and head capsule width from 1.2 - 3.7 mm from the first to the last instar. The linear regression analyses showed that the head capsule width is highly dependent on larval body weight than the body size. Further, the larval weight and the head capsule width are more reliable tools to assess the larval stages. The morphometric studies and the relevant photographs of the different life stages of this insect as presented in the manuscript would help the entomologist to implement suitable and efficient control strategies for this pest, damaging hardwood trees, as the success of any control measure relies mainly on precise identification of the insect's life stages.

1. Introduction

The longhorn beetle, *Pseudonemophas versteegii* (Ritsema 1881) is a coleopteran insect belonging to the family Cerambycidae and is popularly known as citrus trunk borer (CTB) as it infests the plants mainly of the *Citrus* species in North-East India. Earlier, this taxon was documented as *Anoplophora versteegi*, but afterward, it was removed from *Anoplophora* and has been represented as a new combination of *Pseudonemophas versteegii* (Lingafelter and Hoebeke 2002; Kumawat et al. 2015; Kariyanna et al. 2017). This pest species is distributed in the eastern and north-eastern parts of India, China, Myanmar, Thailand, Laos, Nepal, Sumatra Island, and Vietnam and is regarded as the most destructive pest in the north-eastern region of India (Kumawat et al. 2015; Kariyanna et al. 2017). They are polyphagous and feed on various host plants of economic importance, mainly belonging to citrus groups such as *Citrus limon*, *C. reticulata*, *C. sinensis,* and other wild plants (Kumawat et al. 2015).

For numerous reasons, rearing wood-boring beetles and gathering morphometric data under natural conditions are challenging. Since the larvae develop deep into the trunk, it is practically impossible to make live observations as it would be difficult to identify and collect the larvae at certain specific stages from the live infected trees (Favaro et al. 2017). Moreover, the larval period is quite long to study in naturally infested trees. Thus, several attempts were made to rear the wood-boring beetles in the laboratory using artificial diets to collect scientific data. A low-cost artificial diet was developed for mass rearing of Asian longhorn beetles (ALB, *A. glabripennis*), the citrus longhorn beetle (CLB, *A. chinensis*), and the yellow-spotted longicorn beetle (YLB, *Psacothea hilaris*) (Favaro et al. 2017). These longhorn beetles were native to Asia but have also invaded North America and Europe (Haack et al. 2010). These are the most destructive pest as they can infest millions of healthy and

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stressed trees and finally lead to the trees' death after repeated years of attack (reviewed by Haack et al. 2010). The larvae of these insects are the most damaging ones as they can harbour inside the barks and the tree trunk and destroy the vascular tissues such as xylem and phloem (reviewed by Haack et al. 2010).

Like the ALB, CLB, and YLB larvae, the CTB larvae also harbours inside the bark and attack the soft tissues such as the xylem and phloem of host plants. The pupation takes place beneath the bark of the trunk and the adult emerges through the exit holes. In parts of North-East India, such as Meghalaya and Arunachal Pradesh, the adults usually emerge during March-May every year with the initiation of rainfall (Saikia et al. 2011a; Singh and Singh 2012). Afterward, as observed in Meghalaya, the heavy rain from the third week of May decreased the emergence of adults (Saikia et al. 2011a). The female adults, after successful copulation, cut the bark using their mandibles and deposit eggs beneath it (Kumawat et al. 2015). The female CTB prefers *C. reticulata* over *C. sinensis* for deposition of eggs as determined in single and multiple host tests in the laboratory (Singh and Singh 2012). Under the field conditions, CTB infests the *C. reticulata* plant most (Singh and Singh 2012).

The literature on the biology and morphometric data of this CTB insect is limited. Saikia et al. (2011b) successfully reared the CTB larvae in the laboratory using an artificial diet and studied the life history of this insect. Singh and Singh (2012) raised the CTB larvae using potted plants and studied the insect's life cycle and host preference. Saikia et al. (2012) also reported the length of the adult CTB and the length of the antennal segment. However, literature regarding the detailed rearing methodologies and the morphometric parameters concerning larval development is lacking. Considering the gap, we reared this insect in the laboratory and described the rearing techniques in detail in the present paper. We also studied the larval growth and development of CTB and described in detail various morphometric parameters such as larval body size, body weight, and the head capsule width throughout the larval development period. We recorded a total of nine instars for CTB and observed that the increase in larval body size and weight was relatively high during the early larval instars compared to late larval instars. Further, head capsule width was significantly related to larval weight than the larval length. This study thus would greatly enhance our knowledge in identifying the larval instars and the age of this wood-borer insect for its effective management.

2. Materials and Methods

Insect

The citrus trunk borer, *Pseudonemophas versteegii* (Ritsema 1881), was used in the present study.

Collection of the adult insect from the field

The adults of *P. versteegii* were obtained from the orange orchards in Pasighat, East Siang district, Arunachal Pradesh, a North-Eastern State in India. In Pasighat, the adult beetles usually emerge from April to June from the infected orange trees and live on the tree branches. In the present study, we collected the adult beetles by jerking the infected orange trees (Fig. 1A) in May 2018. We checked the presence of exit holes or frass to confirm the infestation of trees (Figs. 1B-C).

Maintenance of adult beetles in the laboratory

We brought the adult beetles to the laboratory in capped plastic bottles with holes for aeration. Inside each bottle, a piece of moist paper, approximately 10×10 cm^2, was kept to maintain the humidity and the moisture. The adults were kept separately in the individual plastic bottle since we observed that the males but not females tended to fight when kept in the same container and the proximity to each other. We have also avoided bringing males and females together in the same bottle to prevent unwanted copulation. The adults were acclimatized to the laboratory conditions under natural day length, with a temperature of 25±3°C and relative humidity of 70-90 %. Adults in each plastic bottle were fed with orange trees' fresh twigs (having leaves) (Figs. 1D-E).

Copulation and oviposition

Male and female beetles were kept together for copulation in a partitioned wooden cupboard (length: breadth: height- 120:50:193 in cms) with steel mesh on all four sides (Figs. 1F-H). Fresh logs (25cm long and 6-8cm in diameter), collected from the orange orchards in and around Shillong, were placed in the cupboard for the oviposition by the copulated females. The logs were replaced every three days.

Collection of the eggs

Using a blade and a pair of blunt-end forceps, we removed the bark at the region of the oviposition site, with minimum disturbance to the underlying eggs, and collected the eggs every day (Fig. 2). We harvested the eggs every day; otherwise, we observed they tend to stick to the bark, making it challenging to collect. The eggs were collected one by one using a thin piece of wood imitating a spatula (Saikia et al. 2011b; Favaro et al. 2017) and spanning from 15-May to 02-June 2018. We never observed the laid eggs in clusters. 10-15 eggs were kept over wet Whatman Filter Papers Grade-1 (125mm) on a plastic Petri dish. The Petri dishes, in turn, were kept in plastic trays, covered with a damp cloth to maintain humidity, and incubated in dark chamber for eggs to hatch (Fig. 1I; Saikia et al. 2011b).

Rearing of larvae

Larvae were reared individually in small plastic cups, covered with the lid and having holes for aeration of the developing larvae (Saikia et al. 2011b), as we observed that they tend to bite each other, causing injury or even death if kept together. The cups were covered with aluminium foil and were kept in a rack away from direct sunlight. The larvae were fed on a sawdust-based artificial diet, formulated (with modifications) as described (Lee and Lo 1998; Keena 2005; Saikia et al. 2011b) and composed mainly of sawdust of orange tree, agar-agar, ascorbic acid, methyl-paraben, and sorbic acid. We changed the diet once a week for the first two months, then once a month or as the need arises. The cup diet was checked once every 15 days for shedded head capsules and exuviae (Figs. 4A-C).

Maintenance of pupae

When pupation started, the pupae were transferred to a plastic cup without diet. For safety, the plastic cup with pupa was placed in a tight lid container with holes for aeration so that the adult, after emergence, does not fly out. The containers were kept over the rack in the dark until adults emerged.

Fecundity and development

A total of 164 adults were collected from the field and were brought to the laboratory. On reaching the laboratory, ten adults died, and out of the remaining adults, there were 103 females and 51 males. The male and female adults were identified based on antennae. The male beetles have long antennae compared to females (Fig. 4F). For copulation, we set free 100 females and 50 males (2 females:1 male) in netted enclosures having fresh logs of orange trees and noted the oviposition from 15-May to 2-June 2018. We recorded the total number of larvae hatched out from the incubated eggs in the laboratory and calculated the hatching percentage as the total number of larvae hatched/total number of incubated eggs. Afterward, we randomly selected thirty larvae from one hatched batch and studied them for further development. The beginning of each larval instar was assessed by looking for the presence of the shedded head capsule and the exuviae in the rearing cups (Figs. 4A-C). The pupation percentage was calculated as the total of pupae that emerged / the total number of larvae. Similarly, the adult emergence rate was calculated as the number of adults that emerged/the total number of pupae.

Larval weight, body length, and width of head capsule

The larval weight was measured using a weighing balance (Sartorius, Model No. B223S) every week or fifteen days. Using a scale, we measured the larval body length from the head to the tip of the last body segment. The width of the larval head capsule was measured using a digital Vernier Caliper (Tiny Deal Digital Caliper 150 mm 6-inch with Display). Each larva was held firmly with its head capsule under the Calipers while taking the measurements.

Statistical analyses

The data on larval length, weight, and head capsule width was presented as a Scattered plot, showing all the data as dots with the vertical line as mean values and error bars (Mean ± SD). A repeated measure analysis of variance (RM one-way ANOVA) was used to analyze the data, and Tukey's multiple comparisons test was performed to compare the mean values of the data. Linear regression analyses were done to evaluate the relationship between variables such as larval length, weight, head capsule width, and the larval instars.

3. Results

Rearing of the insect in the laboratory condition and its development

We recorded two thousand and twenty-one eggs laid by one hundred females over a period spanning from 15-May to 02-June 2018. All the eggs were laid individually and not in clusters. Each egg, when freshly laid, was creamy white in colour and, after 2-3 days became pale yellow in colour. The eggs were spindle-shaped, having a soft and leathery shell (Fig. 2). The incubation period of the eggs varied from 10-14 days, and the larval hatching rate was 81 % (Table 1).

We observed nine instars for the larval stage as judged by the presence of shedded-head capsules and exuviae in the diet cup (Fig. 4A-C). The larvae used to bore into the supplied diet. The larval mortality rate was negligible, and almost all the larvae underwent successful moulting. The larval duration was approximately 250 days beginning from 1^{st} to 9^{th} instar.

Pupal duration lasted for approximately 52 days and the number of days between the first adult emergence and the survival of the last adult beetle was about 86 days. Male adults survived (approx. 80 days) longer than females (approx. 70 days), and their antennae were longer than females. The entire life cycle thus lasted for approximately 402 days in the laboratory condition, beginning from the day of oviposition till the last day of adult survival (Fig. 5).

Larval size

The 1^{st} instar larvae were tiny, measuring about 5.8±1.1 mm in size (Figs. 3, 6). The size afterward increased significantly ($p<0.0001$) to 11.4±2.1 and 14.4±1.7 mm in the 2^{nd} and 3^{rd} instar, respectively. The larvae increased ($p<0.0001$) their size to 17.7±2.3 in the early 4^{th} instar, 21.8±2.3 in the late 4^{th} instar, and 25±3.3 in the 5^{th} instar. In

the 6th instar, the size increased significantly to 31.4±3.8 mm. However, from the 7th to 9th instar, the size didn't rise much and it was maintained on an average between 30-35 mm (Figs. 3, 6).

Larval weight

At the first instar, the larvae had a body weight of 6.2±1.5 mg, which increased ($p<0.001$) to 13.44±1.8 mg in the 2nd instar. A significantly sharper increase in the body weight to 92.2±3.6 mg was observed when larvae entered the 3rd instar. At the beginning of the 4th and 5th larval instar, the weight was recorded as 160.1±36.9 mg and 216.5±77.6 mg, respectively. The body weight further increased to 244.4±115.3 mg at the beginning of the 6th instar, and by the end, the body weight reached 379.6±107.6 mg. Towards the end of the 7th instar, the body weight reached 536.4±101.4 mg. Afterward, the larval weight didn't increase much and maintained almost the same weight till the end of the 9th instar (Fig. 7).

Larval head capsule width

The head capsule width increased significantly ($p<0.001$) progressively from 1.2±0.2 mm in the 1st instar to 1.4±0.2 mm in the 2nd instar and 1.7±0.2 mm in the 3rd instar. At the beginning of the 4th instar, the head capsule width increased significantly to 2.0±0.2 mm, and towards the end of the 5th and 6th instar, the head capsule width increased to 2.4±0.4 mm and 2.9±.3 mm respectively. At the end of the 7th instar, the head capsule width increased to 3.4±0.3 mm. However, afterward and till the end of the 9th instar, the head capsule width didn't increase (Fig. 8).

Relationship between larval weight, larval length, and head capsule width

A linear regression study showed that the larval body size is dependent on the larval body weight, and the variations in the head capsule width are more closely dependent on the larval body weight ($R^2= 0.98$) than on larval body size ($R^2= 0.87$) (Fig. 9). Further, the larval body weight and head capsule width is highly dependent on larval instars (Fig. 10).

4. Discussion

The present study described the laboratory rearing methods of CTB beetles in detail and measured the developmental time, larval body size, body weight, and head capsule width. In the laboratory, we maintained the rearing temperature at 25±3 °C since the environmental temperature dramatically influences the larval development and total developmental time of longhorn beetles (Adachi 1994; Keena and Moore 2010). Under the laboratory rearing conditions in the artificial diet for larvae, we observed that CTB completes its life cycle in about 402 days. Singh and Singh (2012) reported the total developmental period as 308-330 days when CTB larvae were reared in potted plants. The other longhorn beetles, like ALB and CLB, complete their life cycle between 1 and 3 years (Faccoli et al. 2015; Straw et al. 2015). In ALB, a long developmental time affects larval survival negatively and causes much mortality, as larvae tend to molt repetitively and avoid pupation (Favaro et al. 2017). Keena (2006) observed that a chilling period for the eggs at 10±1°C and 85±10% relative humidity for up to 12 weeks is beneficial for reducing the developmental time in ALB.

The fecundity of CTB, as observed in the present study, was relatively less and was only 20 eggs/female compared to 170.6 ± 57.46 eggs/female, as observed by Saikia et al. (2011b). In the present study, the total eggs were collected only for 20 days; afterward, the adult collected from the field died. Since the normal life span of emerged adults is about 70-80 days (Saikia et al. 2011b; the present study), we speculate that the adults we collected from the field were of a late age, and thus the average number of eggs collected were only 20 per female. A low hatching rate of 81% in the present study as compared to 90-100%, as reported by Saikia et al. (2011b), might be because of the high humidity that prevailed in the present rearing environment.

The CTB larvae were pale yellow in colour, and a majority of them completed their larval growth in about 250 days and pupated after that. However, few larvae did not enter pupation and continued to moult and survive in the larval forms beyond their average life span. As observed in the present study, the larval duration is on par with the earlier studies, where the larval period was between 240 and 310 days (Saikia et al. 2011b; Singh and Singh 2012).

The CTB adults measured about 20 mm in length and were greyish white with rows of black spots. On the other hand, ALB and CLB adults were black with white spots (Haack et al. 2010). The male antennae of CTB adults are much longer, pointed, and show the alternate banding pattern of black and white stripes, unlike the male antennae of ALB and CLB beetles (Haack et al. 2010). The antennae in females were shorter than in males, as also observed by Saikia et al. (2012).

The present study showed the adult beetles that emerged in the laboratory developed perfectly and had similar sizes and morphology to field-collected individuals. These adult beetles feed on fresh twigs and leave like field-collected ones, and survive for several months after emergence. The adults that emerged under laboratory conditions were fertile, the females laid viable eggs, and the hatching was normal. We noticed an increased number of emergences for female adults compared to males. In laboratory rearing, an increased emergence of females over males is preferred since a few males can mate

with many females. In the present study, we also observed that males survive longer than females, an added advantage since males, fewer in number than females, become available to mate with late-emerging females.

We reported nine larval instars for CTB and showed the measurements of larval body size, body weight, and head capsule width throughout all the nine larval instars. The linear regression analyses showed that the head capsule width is more closely related to larval weight than larval body size, as the R-square value, a goodness-of-fit measure for linear regression equation, is larger for head capsule width (dependent variable) versus larval weight (R^2=0.9785) than larval size (R^2=0.8687) (independent variables). Further, large R-square values for head capsule width (R^2=0.9288) and weight (R^2=0.9380) versus larval instars than larval body size (R^2=0.7985) suggest that the larval weight and the head capsule width are more reliable tools to assess the larval stages. The data from other coleopteran insects such as *Heilipus lauri, Monochamus alternatus* also emphasize the usefulness of head capsule width in determining the larval instars (Castañeda-Vildózola et al. 2016; Go et al. 2019). Although head capsule width or body weight can predict the larval instars of CTB, these morphometric parameters cannot identify and classify the late larval instars since the larvae increased their body size, weight, and head capsule width only until the 7th instar. Afterward, the larvae didn't significantly increase their body size, weight, and head capsule width.

5. Conclusion

The present study on the rearing and development of this insect along with its morphometric study would help us identify and classify the early larval instars of CTB and implement suitable and efficient control strategies for this wood-borer pest, as the success of any control measures relies mainly on precise identification of the life stages of the insect.

6. Acknowledgments

We sincerely extend our thanks to Professor M. M. Kumawat (College of Agriculture, Agriculture University, Jodhpur, Rajasthan) for providing information and help in acquiring adult beetles, Tek Bahadur (College of Horticulture and Forestry, Central Agriculture University, Pasighat), Creminar D. Shira, RodesonThangkhiew, Khirod S. Das (Department of Zoology, North-Eastern Hill University) for help in collection and part of rearing. The necessary support provided by the Department of Zoology, North-Eastern Hill University (NEHU), Shillong, is greatly acknowledged. K. Malakar also thanks UGC for providing a National Fellowship for SC to carry out research work. The authors declare that they have no conflict of interest within. themselves and others including the funding agency and the agency where the research was carried out. Furthermore, she thanks her father, (L) Damodar Malakar, for accompanying and helping her in collecting insects

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Table 1. The pattern of eggs laid for a period, spanning from 15-May to 02-June 2018 by the female beetles (100 in numbers) of *P. versteegii* and the hatching of the larvae afterwards.

Date	**No. of eggs laid**	**No. of larvae hatched**
15-May	41	No Hatching
16-May	449	
17-May	134	
18-May	123	
19-May	87	
21-May	136	
22-May	238	
23-May	110	
24-May	83	
25-May	321	3
27-May		1
29-May	271	8
30-May		150
31-May		201
01-Jun		167
02-Jun	28	97
03-Jun	All adults died	113
04-Jun		121
05-Jun		150
06-Jun		177
07-Jun		150
08-Jun		54
11-Jun		221
12-Jun		9
13-Jun		7
14-Jun		1
TOTAL	**2021**	**1630**

Figure 1. Adults of *P. versteegii* collected from orange orchards and their rearing and maintenance under laboratory conditions A. Jerking of the infected tree branches for collection of adults B. Exit hole in the trunk of an infected tree in an orchard C. Frass coming out from the hole where the larva is boring into the trunk D. Maintenance of adult beetles individually in capped plastic bottles with holes and having orange trees twigs E. Close-up view of adult beetle maintenance in the plastic bottle F. Meshed cupboard (Open view) used for copulation and egg-laying G: Closed Meshed cupboard (closed view) used for copulation egg-laying, ensuring beetles do not fly out H. Copulating adults I. Incubation chamber for eggs

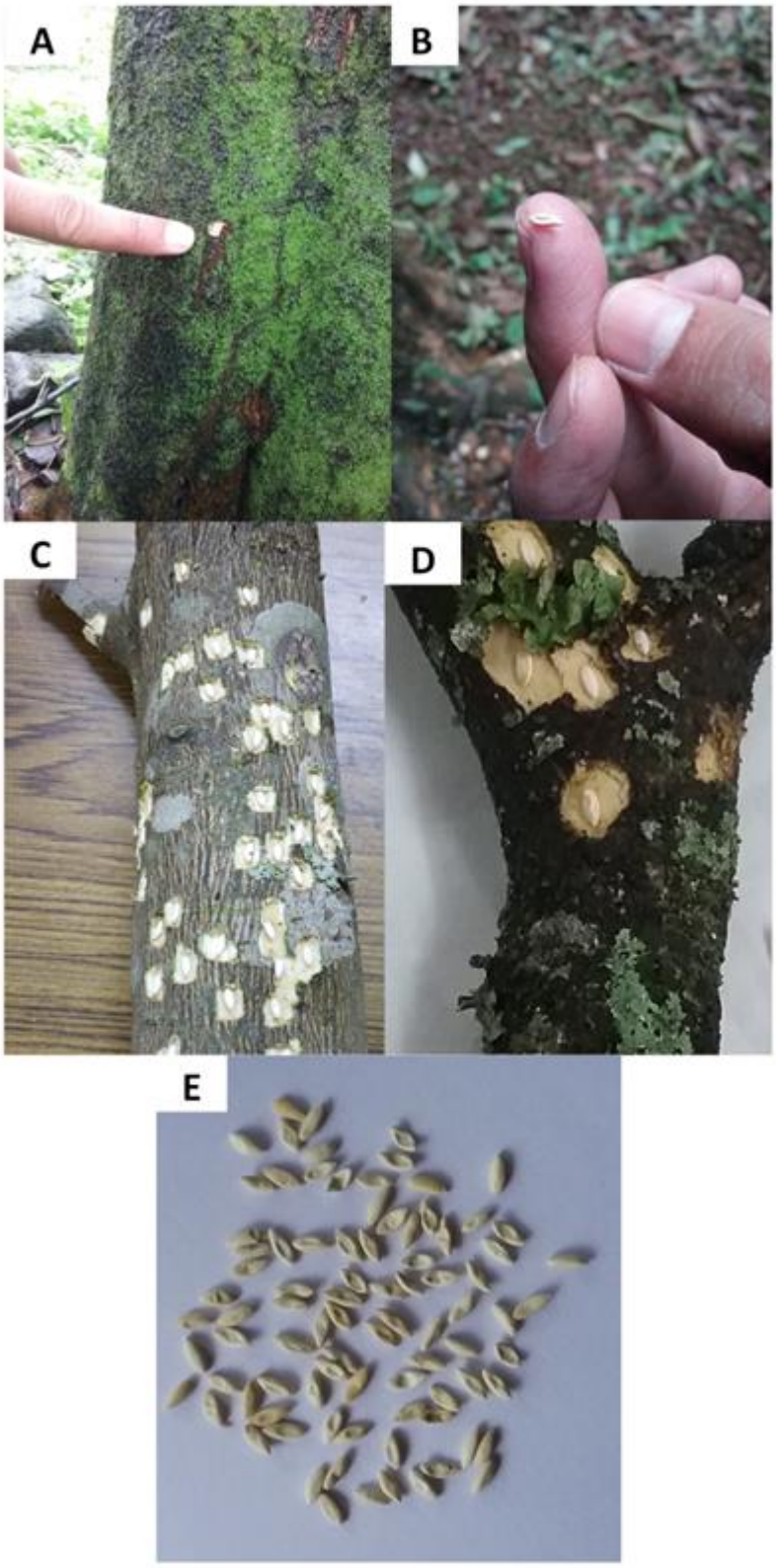


Figure 2. Eggs of *P. versteegii* A. An egg, seen under the bark of an infected tree trunk found in orchards B. A single egg, taken out from an infected tree's bark in orchards C-D. Eggs laid under bark (partially peeled out for observation) in the log in the laboratory E. Eggs harvested from logs in the laboratory

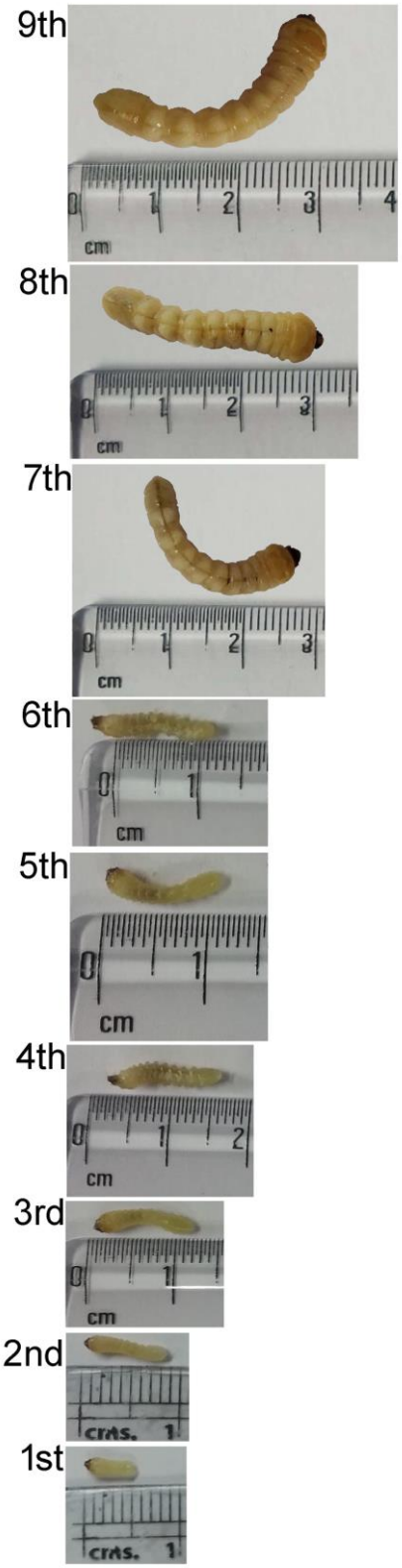


Figure 3. The larvae from the first to the ninth instar of *P. versteegii*, showing their size in centimeter-scale

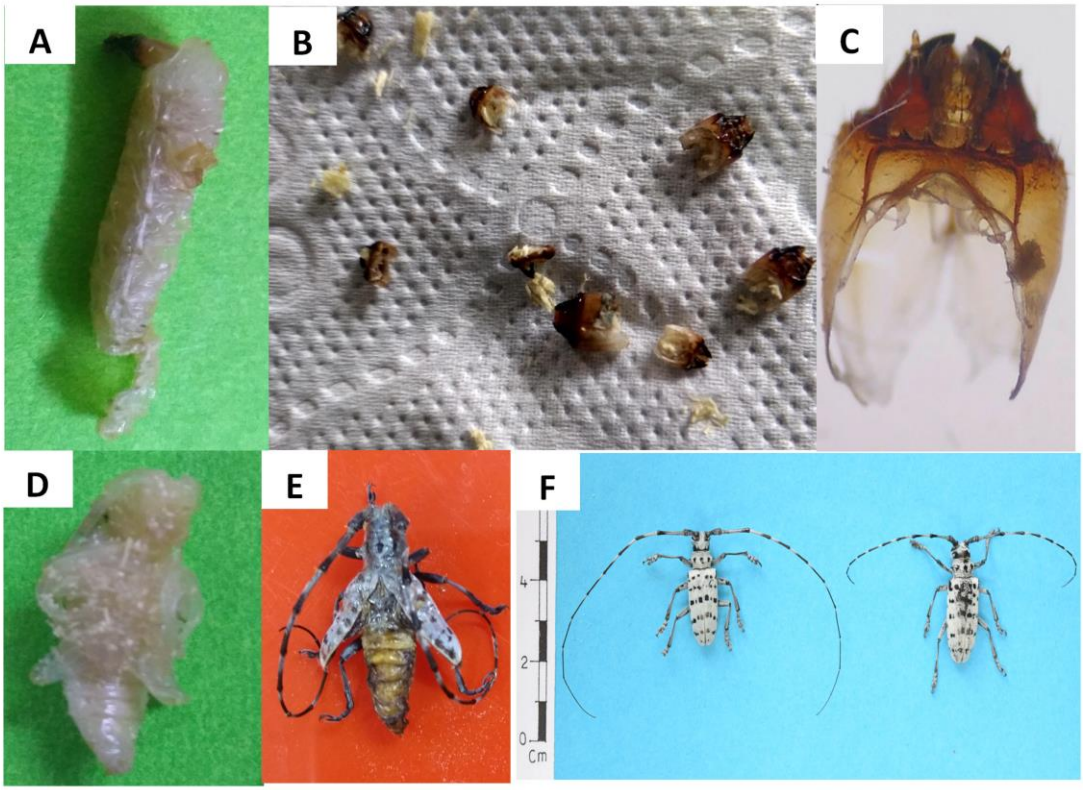


Figure 4. Development of *P. versteegii* A. Larva showing exuvia B. Shedded head capsules by larvae. C. Closed view of shedded head capsule D. Young pupa E. Unsuccessful adult emergence when pupa is stuck in the diet F: Male having longer antennae (left) and female having shorter antennae (right)

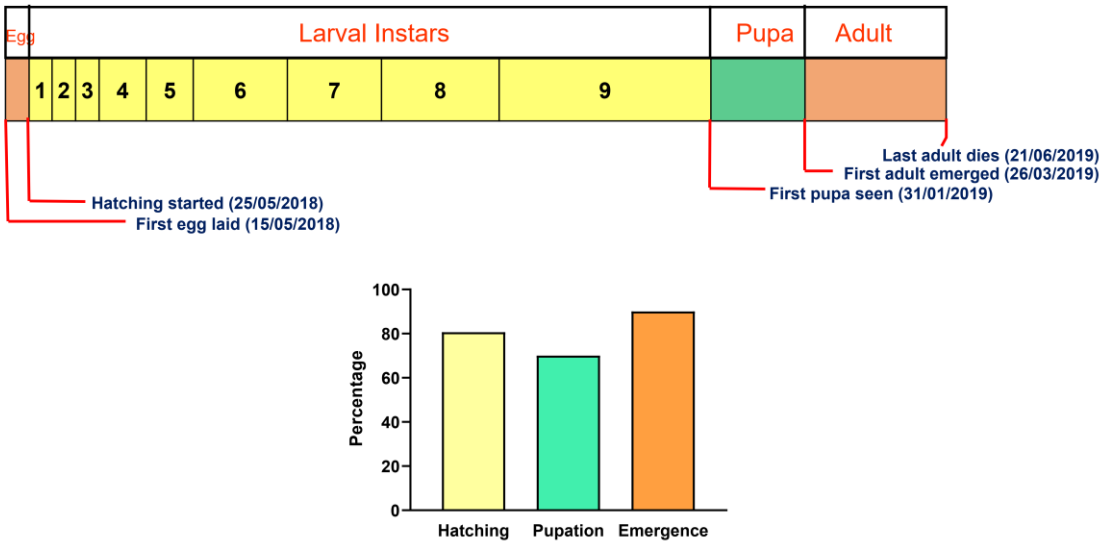
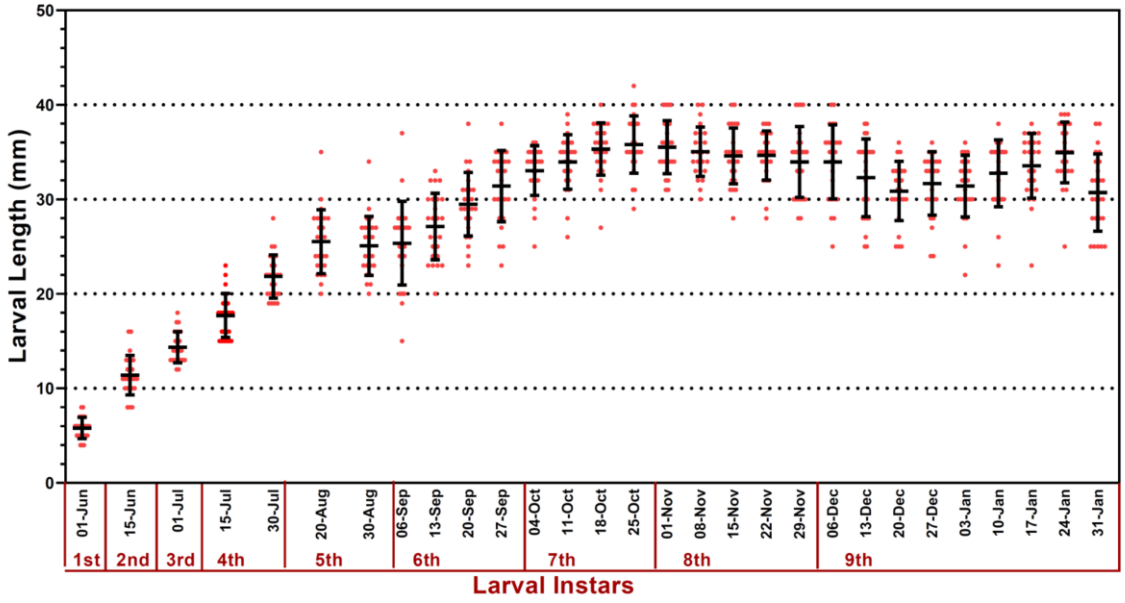


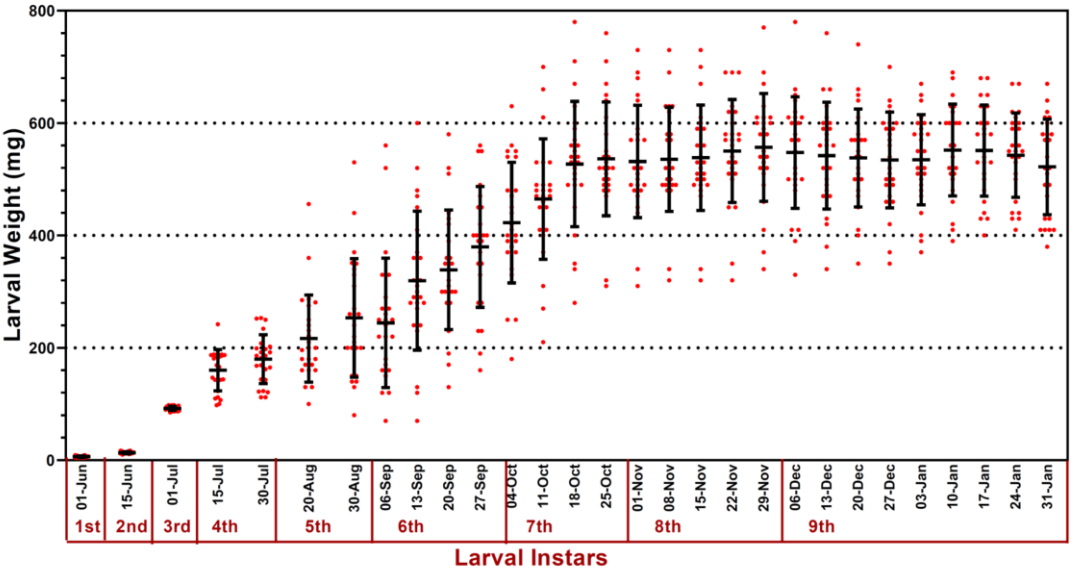
Figure 5. Diagrammatic representation of the life span of *P. versteegii*. The adults were brought from the infected orange orchards during 2018-2019, and the life cycle was studied under laboratory conditions. One hundred females and 50 males were allowed to copulate, and the total number of eggs laid was counted. A total of two thousand and twenty-one eggs were laid, and a total of one thousand and six hundred thirty larvae were hatched. The larvae were maintained in separate plastic cups and were fed the artificial diet. Thirty larvae were selected randomly from a batch and studied for further development. Hatching=Number of larvae hatched/Total number of eggs. Pupation= Number of pupae/ Total number of larvae reared. Emergence= Number of adult emerged/ Total number of pupae. Male adults survived (approx. 80 days) longer than females (approx. 70 days).



ANOVA table	Sum of Squares	DF	Mean Square	F (DFn, DFd)	Significance
Between groups	42752	28	1527	F (3.972, 95.33) = 205.9	P<0.0001
Between Individuals	1965	24	81.88	F (24, 672) = 11.04	P<0.0001
Residual (random)	4984	672	7.416		
Total	49701	724			

P<0.0001 is considered statistically significant

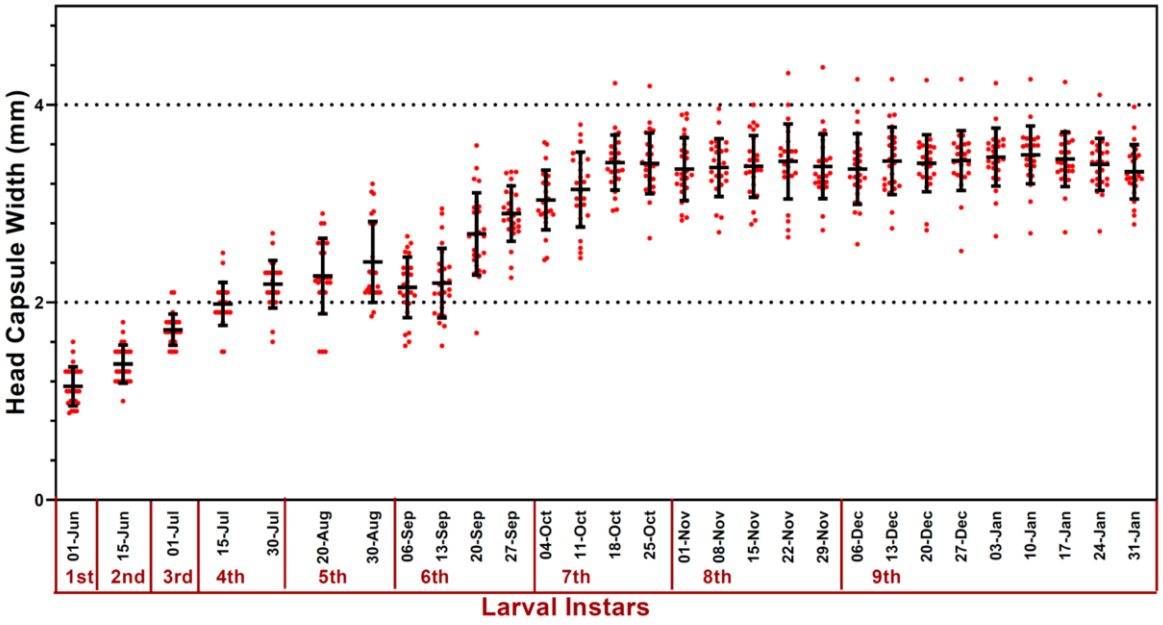
Figure 6. The body length of *P. versteegii* larvae from the first to the ninth instar. The scattered plot diagram shows all the data as dots with the vertical line as mean values and error bars (Mean ± SD). n=25



ANOVA table	Sum of Squares	DF	Mean Square	F (DFn, DFd)	Significance
Between groups	22869203	28	816757	F (3.958, 95.00) = 204.7	P<0.0001
Between Individuals	2812392	24	117183	F (24, 672) = 29.37	P<0.0001
Residual (random)	2681546	672	3990		
Total	28363141	724			

P<0.0001 is considered statistically significant

Figure 7. The body weight of *P. versteegii* larvae from the first to the ninth instar. The scattered plot diagram shows all the data as dots with the vertical line as mean values and error bars (Mean ± SD). n=25



ANOVA table	*Sum of Squares*	*DF*	*Mean Square*	*F (DFn, DFd)*	*Significance*
Between groups	*351.2*	*28*	*12.54*	*F (6.156, 147.8) = 239.2*	*P<0.0001*
Between Individuals	*30.81*	*24*	*1.284*	*F (24, 672) = 24.48*	*P<0.0001*
Residual (random)	*35.24*	*672*	*0.05244*		
Total	*417.2*	*724*			

P<0.0001 is considered statistically significant

Figure 8. The head capsule width of *P. versteegii* larvae from the first to the ninth instar. The scattered plot diagram shows all the data as dots with the vertical line as mean values and error bars (Mean ± SD). n=25

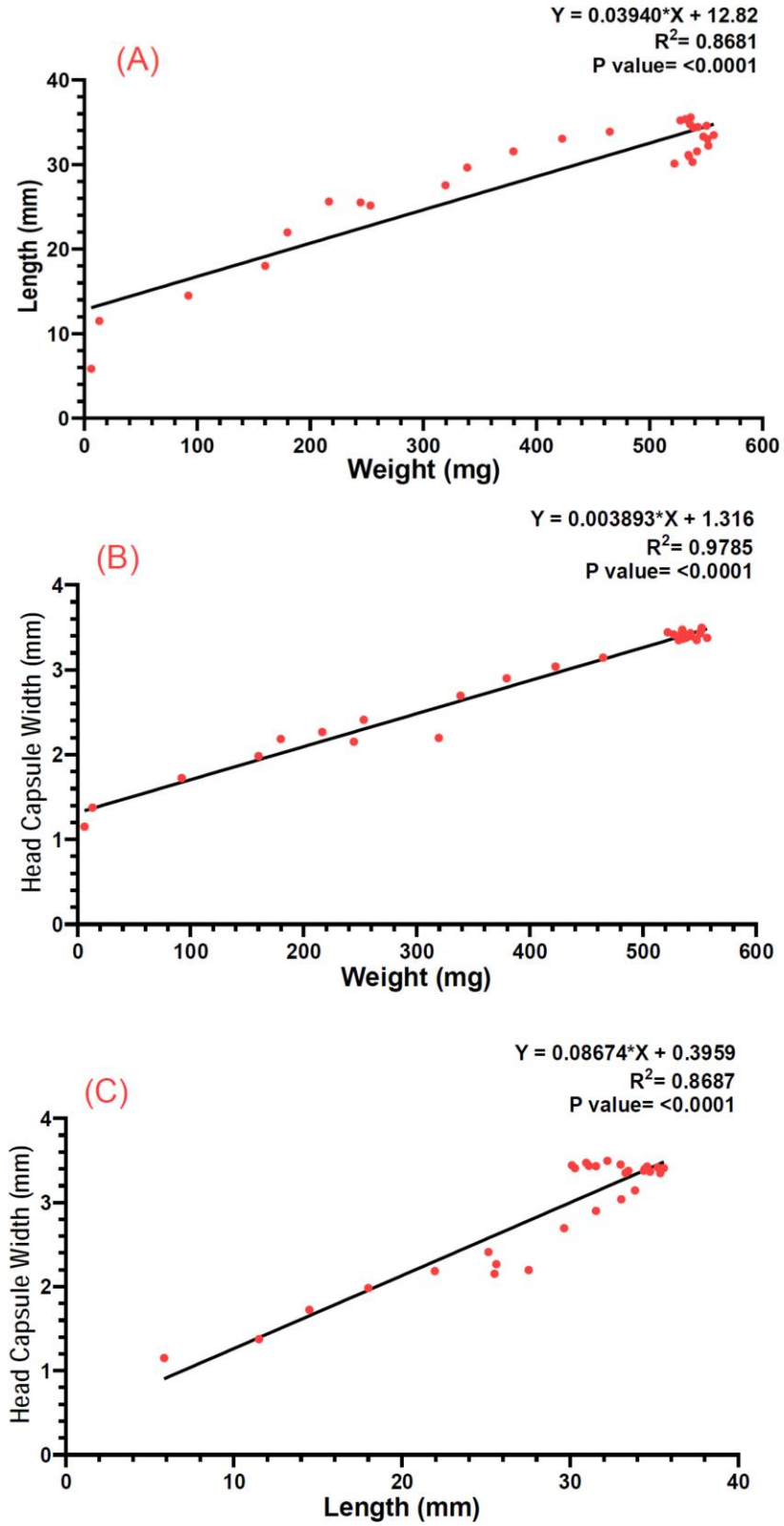


Figure 9. Linear regression analyses showing the relationship between A. Larval length vs. larval weight B. Head capsule width vs. larval weight C. Head capsule width vs. larval size of P. *versteegii*.

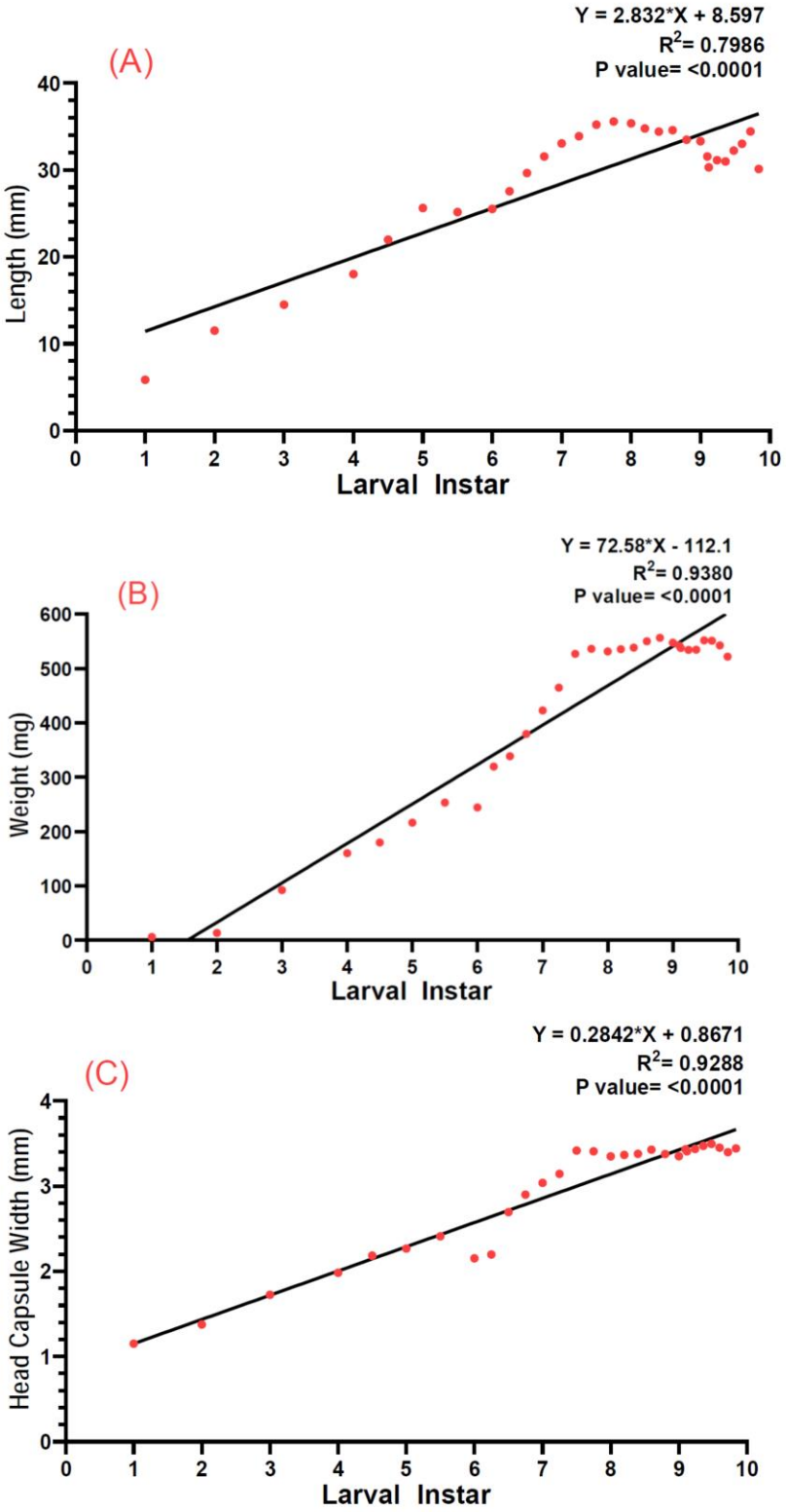


Figure 10. Linear regression analyses showing the relationship between A. Larval size vs. larval instar B. Larval weight vs. larval instar C. Head capsule width vs. larval instar of *P. versteegii*.