

Cold hardiness of *Phaуда flammans* (Lepidoptera: Zygaenidae) larvae

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This study aimed to determine the cold hardiness of *Phaуда flammans* (Lepidoptera: Zygaenidae) larvae. Supercooling points of the 1st–6th instar larvae of *P. flammans* ranged from –7.7 to –13.0 °C. The lethal temperatures were –8 °C for 1st, –5 °C for 2nd, and –7 °C for 3rd–6th instars. Lethal times at the instar-specific lethal temperatures were 12 h for 1st, 14 h for 2nd, 15 h for 3rd, 17 h for 4th, and 18 h for 5th–6th instars. The times required for all larvae to die in an incubator at 5 °C were 30 d for 1st, 3rd, 4th, and 5th instars, and 25 d for 2nd and 6th instars. The findings suggest that *P. flammans* is a chill-intolerant species, and larvae will die if the air temperature decreases to –5 to –8 °C for 12–18 h or to 5 °C for 25–30 d. Such conditions are, however, unlikely to occur in southern China.

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1. Introduction

Ficus plants (Urticales: Moraceae) are the primary decorative trees used to line avenues in the urban landscapes of southern cities in China and Southeast Asian countries. *Ficus microcarpa* (L.) and *F. racemosa* (L.) are often extensively damaged by the defoliating pest *Phaуда flammans* (Walker) (Lepidoptera: Zygaenidae) in China (Liu *et al.* 2014, 2015a, 2015b) and India (Nageshchandra *et al.* 1972, Verma & Dogra 1982). Although outbreaks of *P. flammans* are becoming more frequent in China, there are no effective chemical control measures against this pest because of a conflict in the purpose of urban landscaping that aims to provide comfortable environment for pedestrians and residents.

Phaуда flammans develops 2–3 generations per year in Nanning City, Guangxi Zhuang Au-

tonomous Region, southern China. Adults fly around the food plants primarily in the daytime. Eggs are aggregated on leaves. Larval peaks of the first and second generations occur from mid-May to late June and early August to mid-October, respectively. Most larvae prefer to pupate near roots and tussocks exposed on the ground, and only few individuals pupate in the soil. Larvae of this species can be found on *F. microcarpa* leaves even during winter (Liu *et al.* 2015b). Although there have been some studies on this pest, including reports of its biological characteristics (Liu *et al.* 2014, 2015a, 2015b), occurrence (Nageshchandra *et al.* 1972, Verma & Dogra 1982), and control (Liu *et al.* 1985, Zheng *et al.* 2015), there has been no investigation concerning the cold hardiness of this species. Indeed, such results could provide useful information for understanding the population dynamics of this species

and for establishing effective population monitoring and outbreaks forecasting (Bale 2002).

Temperature plays a key role in the seasonal adaptation of insects. During seasonal cycles, many insect species are frequently exposed to stress from low temperatures that present a major challenge to their survival (Hance *et al.* 2007). Cold hardiness is defined as the capacity of a species to survive long or short term exposure to low temperatures (Lee 1991). Therefore, cold hardiness is a common insect strategy for surviving cold winters. Generally, cold hardiness is estimated using a supercooling point (SCP), lower lethal temperature, and chronic cold tolerance. First, the supercooling point is one predictor of cold hardiness in insects (Turnock & Fields 2005), although some researchers argue that the SCP is not a reliable index of cold hardiness because some insects die before their bodies freeze (Bale 1996). Second, the majority of insects undergo a greater risk of chilling injury and death than freezing injury and death in temperate climatic zones (Bale 2002), e.g., *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) (Zheng *et al.* 2011). Thus, lower lethal temperature and chronic cold tolerance are also considered as predictors of cold hardiness (Morey *et al.* 2012, Pang *et al.* 2014).

The objective of the present study was to gain insight into the cold hardiness of *P. flammans* larvae in temperate climatic zones. We assessed (1) the SCPs, (2) the lower lethal temperature after exposure of larvae to different subzero temperature for a constant period of time, (3) the lethal time after exposure of larvae to each stage-specific lower lethal temperature, and (4) the chronic cold tolerance of larvae after exposure to constant above-zero temperatures.

2. Materials and methods

2.1. Colony maintenance

Larvae were collected from Guangxi University (108°29'E, 22°85'N) on *F. microcarpa* 'Golden leaves' (Urticales: Moraceae) in Nanning City, Guangxi Zhuang Autonomous Region, southern China during November and December of 2014 and January of 2015. Larvae of 1st to 6th instar

were housed in plastic containers (R = 15.0 cm, H = 7.5 cm) and chosen as experimental insects to measure the SCPs. Larvae collected in January of 2015 during the lowest air temperatures of the winter were used to determine the lower lethal temperature, time, and chronic cold tolerance. Larval instars were determined according to Liu *et al.* (2015b).

2.2. Measurement of supercooling points

Supercooling points were measured using the approach developed by Zheng *et al.* (2011). A larva was fixed on a copper-constantan thermocouple linked to an automatic multichannel temperature recorder (SUN-V, Beijing Pengcheng Electronic Technology CO., LTD, Beijing, China). The thermocouple was placed into a water bath (BCD-301 W, Haier Group, Shandong, China) that was cooled from room temperature with a nonlinear cooling rate of approximately 0.5 °C min⁻¹ from 0 to -25 °C. Larval body temperatures were recorded at 1 s intervals. We terminated each measurement 20 min after seeing a freezing exotherm. Sample size was between 10 and 31 for each treatment, and 392 larvae in total.

2.3. Determination of lower lethal temperature and time

To examine the lower lethal temperature, 1st to 6th instar larvae were incubated in a low temperature incubator (IN602 W, Yamato Scientific Co., Ltd., Tokyo, Japan) for 24 h at a constant temperature ranging from 0 to -8 °C at 1 °C increments. The cooling rate was 0.5 °C min⁻¹ from 26 °C to each constant temperature mentioned above. After the designated exposure, temperature of the low temperature incubator was set at 26 °C with 60–80% RH and a 12 h photoperiod for recovery of larvae. The heating rate was 0.5 °C min⁻¹ from each constant temperature to 26 °C. Larval survival at each stage was assessed 24 h later. If larvae were able to move when they were prodded with forceps once each in the head, thorax, and abdomen (Kim & Song 2000a), they were considered alive. Lethal times for each developmental stage were also measured. The survival rates were measured for each instar at exposure times from 1 to 18 h with 1

Table 1. Results of two-way ANOVA showing effects of instar, month, and their interaction on supercooling point of *Phaуда flammans* larvae.

Source	df	Mean square	F	P
Intercept	1	35459.2	7134	<0.0001
Instar	5	83.9	16.9	<0.001
Month	2	0.50	0.11	0.90
Instar × Month	10	10.5	2.1	0.022
Error	378	5.0		

h intervals at each instar-specific lower lethal temperature. The survival standards after warming were the same as those described for lower lethal temperature. Three replications were performed and the sample size was 30 specimens per temperature / exposure time treatment, 4,860 larvae in total.

2.4. Determination of chronic cold tolerance

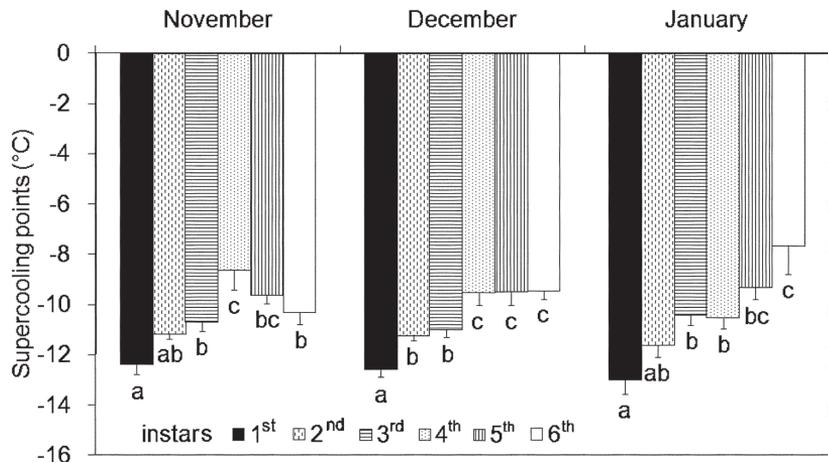
The average minimum air temperature during the winter is approximately 5 °C in Nanning City according to the meteorological data provided by the China Meteorological Administration. To investigate the chronic cold tolerance, 1st to 6th instar larvae were incubated in the low temperature of 5 °C for 5, 10, 15, 20, 25, and 30 days in the incubator. After the designated exposure, the specimens were removed from the incubator to another setting at 26 °C with 60–80% RH and a 12 h photoperiod. Larval survival was assessed at each stage 24 h later. If the larvae were able to move when they were prodded with forceps once each in the head, thorax, and abdomen, they were

considered alive. Three replications were performed and the sample size was 30 specimens in each treatment condition, 1,080 larvae in total.

2.5. Statistical analyses

The normality and homoscedasticity of all data were tested prior to analysis using Kolmogorov-Smirnov and Levene’s test, respectively. The effects of instar, month and their interaction on larval SCPs were analyzed using two-way ANOVA. The data were further analyzed using one-way ANOVA followed by Tukey’s HSD test when the two-way ANOVA results showed that instar had a significant effect for SCPs. The lower lethal temperature (°C) and time (h) and chronic cold tolerance (d) were calculated from the survival fraction of each larval instar using Kaplan-Meier test. The significance of the differences of survival curves among all instars in each experiment was tested by Log-rank (Caesar 2003). The level of P < 0.05 was accepted as statistically significant. Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, Illinois, U.S.A.).

Fig. 1. Mean supercooling points (± SEM) of 1st to 6th instar larvae of *Phaуда flammans* from November to January. Values followed by different lowercase letters for the same month are significantly different by Tukey’s HSD test (P < 0.05). Sample size was between 10 and 31 for each treatment, and 392 larvae in total.



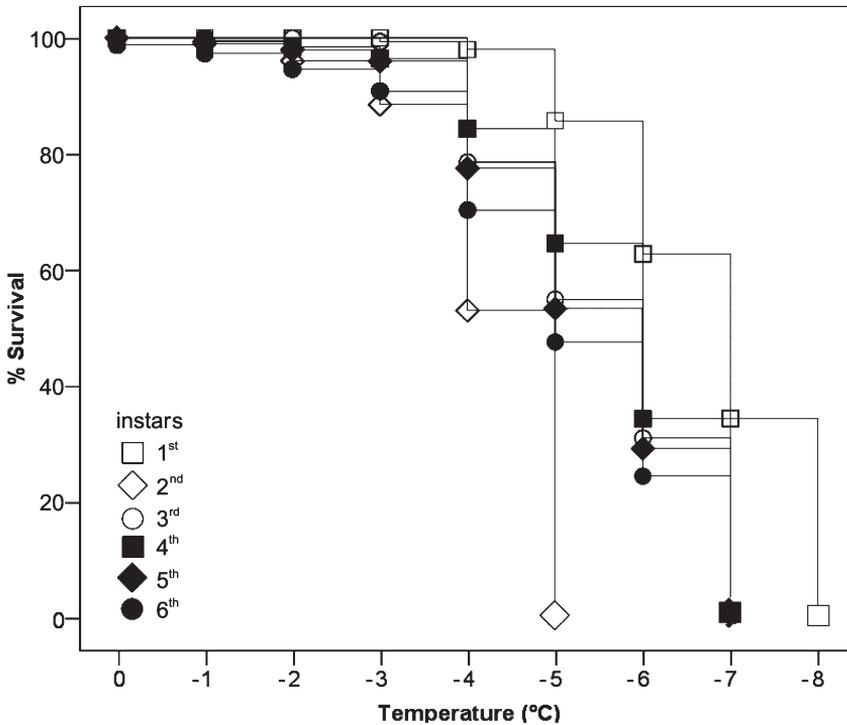


Fig. 2. Lower lethal temperature of 1st to 6th instar larvae of *Phaeda flammans* after 24 h exposure to sub-zero temperatures from 0 to -8 °C in 1 °C increments. Sample size was 30 specimens for each temperature, 1,620 larvae in total.

3. Results

3.1. Supercooling points

The SCPs of different instar larvae in November, December, and January followed a normal distribution ($P > 0.05$). The two-way ANOVA indicated that the SCPs of larvae were significantly affected by the instar and the interaction between instar and month (Table 1).

According to also one-way ANOVAs, instar had a significant influence on the SCPs of larvae that were collected in November ($F = 6.10$, $df = 5$, 131 , $P < 0.001$), December ($F = 10.98$, $df = 5$, 176 , $P < 0.001$), and January ($F = 5.79$, $df = 5$, 82 , $P < 0.001$). The specific differences among instars according to Tukey's HSD tests are shown in Fig. 1. The SCPs did not differ significantly among the months in the two-way ANOVA (Table 1).

3.2. Lower lethal temperature and time

Fig. 2 illustrates the responses of 1st to 6th instar larvae of *P. flammans* following 24 h exposure to

different low temperatures ranging from 0 to -8 °C. The survival curves of lower lethal temperature of 1st to 6th instar larvae differed significantly from each other ($\chi^2 = 199.8$, $df = 5$, $P < 0.001$). The lethal temperature was -8 °C for 1st instar larvae, -5 °C for 2nd instar larvae, and -7 °C for 3rd to 6th instar larvae (Fig. 2).

The 50%, 95% and 100% lethal times at the stage-specific lower lethal temperature were 7.3 h, 10 h and 12 h for 1st instar larvae, 5.2 h, 12 h and 14 h for 2nd instar larvae, 7.7 h, 13.8 h and 15 h for 3rd instar larvae, 7.4 h, 15.4 h and 17 h for 4th instar larvae, 9.7 h, 16.9 h and 18 h for 5th instar larvae, and 9.1 h, and 16.5 h and 18 h for 6th instar larvae (Fig. 3).

The survival curves of lower lethal time of 1st to 6th instar larvae were significantly different from each other ($\chi^2 = 476.6$, $df = 5$, $P < 0.001$).

3.3. Chronic cold tolerance

The survival rate of each instar larvae were negatively associated with the chronic cold tolerance at 5 °C (Fig. 4) but the survival curves differed

Fig. 3. Lower lethal time of 1st to 6th instar larvae of *Phaуда flammans* exposed at instar-specific temperatures for 1 to 18 h with 1 h intervals. Sample size was 30 specimens for each exposure time, 3,240 larvae in total.

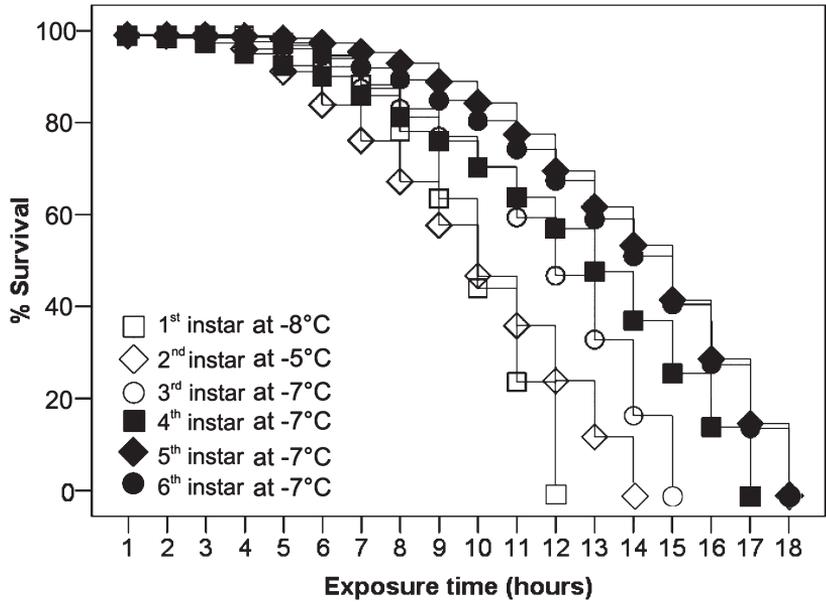
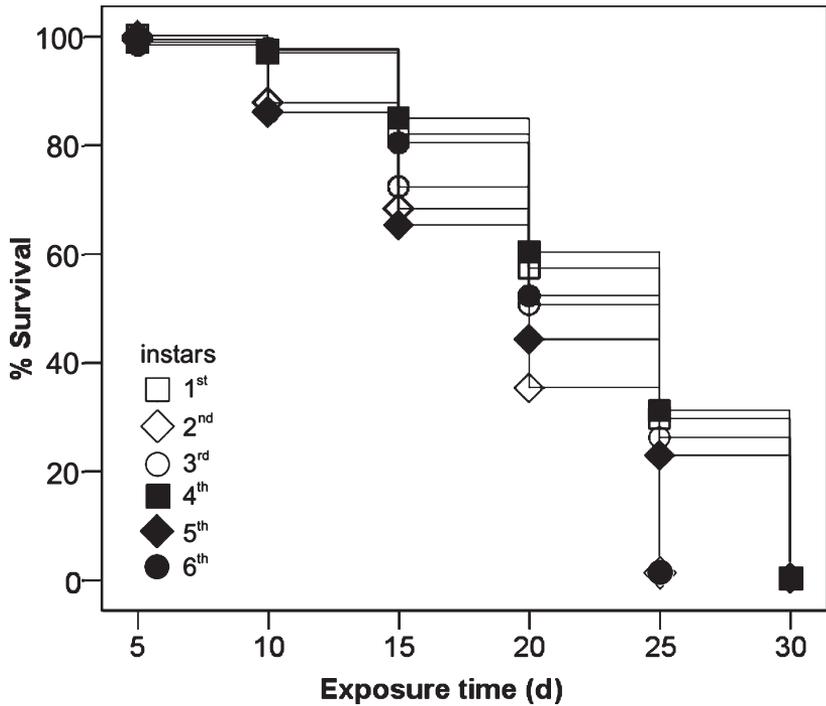


Fig. 4. Chronic cold tolerance of 1st to 6th instar larvae of *Phaуда flammans* exposed to 5 °C in 5-d increments. Sample size was 30 specimens for each exposure time, 1,080 larvae in total.



significantly among the instars ($\chi^2 = 39.0$, $df = 5$, $P < 0.001$). The times taken by 95% and 100% to die were 26 d and 30 d for 1st instar larvae, 21 d and 25 d for 2nd instar larvae, 20 d and 30 d for 3rd instar larvae, 26 d and 30 d for 4th instar larvae, 17 d and 30 d for 5th instar larvae, and 22 d and 25 d for 6th instar larvae.

4. Discussion

Animals in nature are exposed to temperatures that fluctuate on both seasonal and diel time scales. The downward trend of seasonal air temperatures in the autumn could enhance cold hardiness by decreasing the SCPs, as e.g. in *Phyllo-*

Table 2. Air temperatures from November to January in Nanning City, southern China 1951–2016^a.

Month	Longest daily below 5 °C (d)	Longest duration continuously below 5 °C (d)	Lowest daily minimum temperature (°C)
November	4 (1971)	4 (1971)	0.7 (2007)
December	19 (1975)	14 (2013)	-1.9 (1999)
January	12 (2009)	9 (2003)	-2.1 (1955)

a) Air temperature data provided by Climate Data Centre, China Meteorological Administration.

treta undulata (Kutschera) (Hiiesaar *et al.* 2009). In general, the ability to acclimate is considered an adaptive response to changing environmental conditions (Hoffmann 1995). In the current study, the seasonal fluctuation of air temperature did not significantly decrease the SCPs of larvae. Air temperature is in a constant state of flux, and can include sudden cooling or warming. Rapid environmental cooling, as often occurs during diurnal cycles, induces a corresponding increase in the exposed insect's cold hardiness. Later, as environmental temperature rises, the insect's cold tolerance decreases as it readjusts to higher temperatures. Therefore, fluctuations of diel temperature during winter in Nanning City could not enhance the cold hardiness of *P. flammans* larvae. We suggest that this stems from a conflict between the induction mechanisms for sudden cooling and warming, but further studies are needed to confirm this.

Some research has shown that the SCP is not a suitable index for cold hardiness, because mortality can occur at temperatures above the SCP (Renault *et al.* 2002, Hiiesaar *et al.* 2011). In contrast, other researchers have reported that there is a good correlation between low temperature survival and SCP in several species and have suggested that the SCP is reliable as an indicator of cold hardiness (Lee & Denlinger 1985, Pang *et al.* 2014). In the present study, the SCP results show that *P. flammans* larvae could resist cold temperatures (-7.7 to -13.0 °C). However, they succumbed above their SCPs when subjected to low temperatures for the longer period of 24 h (Fig. 2). Therefore, the SCP of *P. flammans* larvae might not be a good indicator of their cold hardiness. According to Lee (2010), if most mortality occurs at the SCP, the insect is freeze-intolerant, below the SCP it is freeze-tolerant, and above the

SCP it is chill-intolerant. As in this study the lower lethal temperatures of larvae were above the SCP, *P. flammans* can be classified as a chill-intolerant species.

It is well known that chronic cold tolerance (non-lethal temperature) cannot be neglected when insect survival needs to be accurately evaluated under natural conditions. Many studies have confirmed that long-term exposure to subfreezing non-lethal low temperatures also decreases insect survival. For example, in the beet armyworm, *S. exigua*, indirect chilling injuries occur, such as low egg hatchability, retarded larval development and low pupation rates, which are caused by long-term exposure to the low temperature of 5 °C (Kim & Song 2000b). In Nanning City, the January air temperature was the lowest of the year: the average minimum air temperature was approximately 5 °C. In January 2015, there were three days below 5 °C.

In this study, no *P. flammans* larvae exposed to non-lethal low temperature (5 °C) survived beyond 25–30 days (Fig. 4). From this point of view, shorter durations of non-lethal temperatures could be beneficial in the overwintering of this species.

In conclusion, the findings of our study indicate that *P. flammans* is a chill-intolerant species, and its larvae will die, if the air temperature decreases to -5 to -8 °C for 12–18 h or to 5 °C for 25–30 days during winter. Such conditions are, however, not likely to occur in Nanning City in southern China (Table 2). It is therefore clear that cold weather in winter is not an important mortality factor of *P. flammans* in Nanning. For this reason, management of the overwintering population should be particularly important from an integrated pest management (IPM) perspective (Bale 2010).

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References

- Bale, J. S. 1996: Insect cold hardiness: a matter of life and death. — *European Journal of Entomology* 93: 369–382.
- Bale, J. S. 2002: Insects and low temperatures: from molecular biology to distributions and abundance. — *Philosophical Transactions of the Royal Society of London B* 357: 849–862.
- Bale, J. S. 2010: Implications of cold-tolerance for pest management. — In: Denlinger, D. L. & Lee, R. E. (eds), *Low Temperature Biology of Insects*: 342–373. Cambridge University Press, Cambridge. 406 pp.
- Caesar, A. J. 2003: Synergistic interaction of soilborne plant pathogens and root-attacking insects in classical biological control of an exotic rangeland weed. — *Biological Control* 28: 144–153.
- Hance, T., Van Baaren, J., Vernon, P. & Boivin, G. 2007: Impact of extreme temperature on parasitoids in a climate change perspective. — *Annual Review of Entomology* 52: 107–126.
- Hiisaar, K., Williams, I., Luik, A., Metspalu, L., Muljar, R., Jõgar, K., Karise, R., Mänd, M., Švilponis, E. & Ploomi, A. 2009: Factors affecting cold hardiness in the small striped flea beetle, *Phyllotreta undulata*. — *Entomologia Experimentalis et Applicata* 131: 278–285.
- Hiisaar, K., Williams, I. H., Mänd, M., Luik, A., Jõgar, K., Metspalu, L., Švilponis, E., Ploomi, A. & Kivimägi, I. 2011: Supercooling ability and cold hardiness of the pollen beetle *Meligethes aeneus*. — *Entomologia Experimentalis et Applicata* 138: 117–127.
- Hoffmann, A. A. 1995: Acclimation: increasing survival at a cost. — *Trends in Ecology & Evolution* 10: 1–2.
- Kim, Y. & Song, W. 2000a: Effect of thermoperiod and photoperiod on cold tolerance of *Spodoptera exigua* (Lepidoptera: Noctuidae). — *Environmental Entomology* 29: 868–873.
- Kim, Y. & Song, W. 2000b: Indirect chilling injury of *Spodoptera exigua* in response to long-term exposure to sublethal low temperature. — *Journal of Asia-Pacific Entomology* 3: 49–53.
- Lee, R. E. 1991: Principles of insect low temperature tolerance. — In: Lee, R. E. & Denlinger, D. L. (eds), *Insects at low temperature*: 7–46. Chapman & Hall, New York. 514 pp.
- Lee, R. E. 2010: A primer on insect cold-tolerance. — In: Denlinger, D. L. & Lee, R. E. (eds), *Low temperature biology of insects*: 3–34. Cambridge University Press, Cambridge. 406 pp.
- Lee, R. E. & Denlinger, D. L. 1985: Cold tolerance in diapausing and nondiapausing stage of the flesh fly, *Sarcophaga crassipalpis*. — *Physiological Entomology* 10: 309–315.
- Liu, J. Y., He, Q. L., Su, S., Wei, H., Yang, J., Lu, W. & Zheng, X. L. 2014: Investigated method of *Phauda flammans* pupae. — *China Plant Protection* 34: 51–53. [In Chinese with English summary.]
- Liu, J. Y., He, Q. L., Wei, H., Yang, J., Li, J., Lu, W. & Zheng, X. L. 2015a: Developmental duration, threshold temperature and effective accumulated temperature of *Phauda flammans* under natural temperature indoor. — *Plant Protection* 41: 137–140. [In Chinese with English summary.]
- Liu, J. Y., He, Q. L., Wei, H., Yang, J., Li, J., Lu, W. & Zheng, X. L. 2015b: Studies on the biological characteristics of *Phauda flammans* (Lepidoptera: Zygaenidae). — *Plant Protection* 41: 188–192. [In Chinese with English summary.]
- Liu, Q. L., Wu, R. G. & Zeng, C. X. 1985: Nuclear polyhedrosis virus collected from *Phauda flammans*. — *Guangdong Forest Science and Technology* 14: 29–30. [In Chinese with English summary.]
- Morey, A. C., Hutchison, W. D., Venette, R. C. & Burkness, E. C. 2012: Cold hardiness of *Helicoverpa zea* (Lepidoptera: Noctuidae) pupae. — *Environmental Entomology* 41: 172–179.
- Nageshchandra, B. K., Rajagopal, B. K. & Balasubramanian, R. 1972: Occurrence of slug caterpillar *Phauda flammans* Wlk. (Lepidoptera: Zygaenidae) on *Ficus racemosa* L. in South India. — *Mysore Journal of Agricultural Science* 6: 186–189.
- Pang, B. P., Li, N. & Zhou, X. R. 2014: Supercooling capacity and cold hardiness of band-winged grasshopper eggs (Orthoptera: Acrididae). — *Journal of Insect Science* 14: article 289.
- Renault, D., Salin, C., Vannier, G. & Vernon, P. 2002: Survival at low temperatures in insects: what is the significance of the supercooling point. — *CryoLetters* 23: 217–228.
- Turnock, W. J. & Fields, P. G. 2005: Winter climate and coldhardiness in terrestrial insects. — *European Journal of Entomology* 102: 561–576.
- Verma, T. D. & Dogra, G. S. 1982: Occurrence of *Phauda flammans* Wlk. (Lepidoptera: Zygaenidae) on *Ficus* species in Himachal Pradesh. — *Journal of Tree Science* 1: 130–132.
- Zheng, X. L., Cheng, W. J., Wang, X. P. & Lei, C. L. 2011: Enhancement of supercooling capacity and survival by cold acclimation, rapid cold and heat hardening in *Spodoptera exigua*. — *Cryobiology* 63: 164–169.
- Zheng, X. L., Li, J., Su, L., Liu, J. Y., Meng, L. Y., Lin, M. Y., Zhang, J. & Lu, W. 2015: Ecological and morphological characteristics of parasitoids in *Phauda flammans* (Lepidoptera: Zygaenidae). — *Parasite* 22: 36.