Supercooling Point of Selected Developmental Stages of *Culex pipiens pallens* (Diptera: Culicidae)[1]

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Abstract *Culex pipiens pallens* Coquillet is an important public health pest and can spread various mosquito-borne infectious diseases. To assess the cold resistance of *Cx. pipiens pallens*, the supercooling points (SCPs) and freezing points (FPs) of *Cx. pipiens pallens*, including 1st through 4th larval instars, pupae, and male and female adults, were identified in the laboratory. SCPs of *Cx. pipiens pallens* ranged from −18.0353 to −7.9854°C. SCPs differed significantly among larvae, pupae, and adults ($F = 338.434$, df = 170, $P < 0.05$), but did not differ significantly between male (−17.9173 °C) and female (−18.0353 °C) adults. The mean SCPs of the imaginal stage were much lower than those of larvae and pupae. Unlike SCPs, the FPs of *Cx. pipiens pallens*, ranging from −12.8777°C to −5.5550°C, were not significantly different from 1st instars through the pupal stage, but were much higher than those of the imaginal stage ($F = 29.92$, df = 170, $P < 0.05$). These results suggest that *Cx. pipiens pallens* has the potential to spread farther northward into higher-latitude regions in China.

Key Words *Culex pipiens pallens*, supercooling point, freezing point, cold hardiness

Culex pipiens pallens Coquillet belongs to the *Cx. pipiens* complex and is the major vector of Bancroftian filariasis, the causative agent of West Nile virus disease, and Type B epidemic encephalitis. In China, *Cx. pipiens pallens* is the dominant mosquito in regions north of 33° latitude (Lu 1997). From the 1950s to the 1970s, these mosquitoes caused outbreaks of Bancroftian filariasis in Shandong Province. Currently, *Cx. pipiens pallens* still remains an important vector of epidemic encephalitis B (Cao et al. 1994, Li et al. 2014). *Culex pipiens quinquefasciatus* Say was reported to spread Zika virus (Guo et al. 2016). However, whether or not *Cx. pipiens pallens*, a subspecies of *Cx. pipiens quinquefasciatus*, can also spread the virus needs further research. As poikilothermic animals, insects have a poor adaptability to temperature. Temperature changes can seriously influence their reproduction and growth. When the temperature is lower than their minimum growth temperature, insects might suspend development and enter a diapause in different developmental life stages to live through the winter (Bale 1996, Denlinger and Lee 1988, Kawarasaki et al. 2014). *Culex pipiens pipiens* (L.) live through the winter

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from late October to mid-March of the next year as female adults living in cellars, caves, caliducts, and other habitats in China (Liu et al. 2016a). Obviously, their tolerance or resistance of cold temperatures determines their survival over the winter.

For many insects, low temperatures are not lethal unless their tissues freeze. Supercooling is the process of allowing cells to retain liquid water below its freezing point, due to the lack of a nucleation source. Therefore, supercooling points (SCPs) are important indicators of cold tolerance or resistance. Supercooling is a physiological adaptation to withstand low environmental temperatures, with the lower the SCP the greater the cold-resisting capacity of the insect (Abdelghany et al. 2015, Liu et al. 2016b). While SCPs have been defined for some insects (Ditrich and Boukal 2016, Pang et al. 2014), there is only limited available research on the SCPs of medically important mosquitoes (Hanson and Craig 1995,Wallace and Grimstad 2002). We, therefore, undertook this study to clarify the cold resistance of *Cx. pipiens pallens*. The SCPs and freezing points (FPs) of laboratory-reared larvae, pupae, and adults were measured to provide a basis for further research on the cold resistance or tolerance mechanisms of *Cx. pipiens pallens*.

Materials and Methods

Culex pipiens pallens individuals used in the study were obtained from a culture maintained at the Shandong Institute of Parasitic Diseases (Jining, Shandong Province, China) at a temperature of 26 ± 2°C, a relative humidity of 70–80%, and on a photoperiod of 14 L:10 D. Adult mosquitoes in the colony were fed 5% glucose, which was changed daily. Three to four days after emergence, adult mosquitoes were placed with laboratory rats to provide blood meals for the females. Larvae were fed on a 50:50 mixture of yeast and pork liver powder.

SCPs were measured using the thermistor method (Jing and Kang 2004). Instruments included the data collector, SUN-II smart supercooling point tester (jointly developed by the Institute of Plant Protection, Chinese Academy of Agricultural Sciences and by Pengcheng Electronics Co., Ltd., Beijing, China), and the low-temperature and constant-temperature groove (DCW-3506 type by Ningbo Haishu Tianheng Instrument Plant, Ningbo, China).

Individual adult male and female mosquitoes from the colony were attached to the thermistor probe using Vaseline. Larvae (1st through 4th instars) and pupae collected from the colony were first dried using filter paper and then attached to the thermistor probe using Vaseline. Thirty specimens of each developmental stage were tested. The temperature-sensing probe with the insect attached was inserted into a 1-ml centrifuge tube. Cotton was used in each tube to stabilize the probe and to avoid direct contact of the probe and insect with the tube wall. Each centrifuge tube containing an insect and a probe was placed separately in a test tube (15 cm high and 1.5 cm in diameter) that was inserted in the low-temperature groove. Temperature was decreased at a rate of 0.5°C per minute as per methods of Lee et al. (1987). Temperatures of the insect were recorded by a computer using the supercooling system software (developed by Pengcheng Electronics Co., Ltd.).

Data collected were subjected to analysis using SPSS 11.5 (IBM, New York, NY). The single-sample Kolmogorov–Smirnov test (Justel et al. 1997) analyzed the frequency distribution characteristics of the SCPs of the developmental stages. Analysis of variance was carried out to test the effect of developmental stages on the supercooling capacity of *Cx. pipiens pallens*. Means were compared using Tukey's multiple comparison test (Sokal and Rohlf 1995).

Results

The SCPs of larvae, pupae, and adults of *Cx. pipiens pallens* were normally distributed and ranged from −18.0353 to −7.9854°C (Fig. 1). The mean SCP values decreased with progressively later life stages. The SCPs differed significantly among the life stages of larva, pupa, and adult ($F = 338.434$, df = 170, $P < 0.05$), but did not differ significantly between male (−17.9173°C) and female (−18.0353°C) adults. The mean SCPs of the imaginal stage were much lower than those of larval and pupal stages. However, Tukey's multiple comparison suggested no significant differences of the SCPs between the 1st instar and 2nd instar and the 2nd instar and 3rd instar (Table 1).

The FPs of the various developmental stages of *Cx. pipiens pallens* ranged from −12.8777 to −5.5550°C. Mean FP values decreased with progressive development. FPs differed significantly among the larval, pupal, and adult stages, but no significance was detected among the larval instars to the pupal stage, and FPs were much higher than those of the imaginal stage ($F = 29.92$, df = 170, $P < 0.05$) (Table 2).

Discussion

It was found that the SCPs and the FPs of adult *Cx. pipiens pallens* mosquitoes from laboratory colonies were lower than those of larval instars and pupae. For most insects, the SCP values can indicate cold tolerance or resistance, with the SCPs of insect stages that live through the winter being, in general, lower than that of other stages that do not overwinter (Hanson and Craig 1995, Liu et al. 2016b). It has been postulated that this is due to the lower water content of diapausing stages (Benoit et al. 2010, Kawarasaki et al. 2014). Our results, therefore, may help in further research of the overwintering potential of various developmental stages of *Cx. pipiens pallens*.

Differences between the SCPs and FPs at each development stage increased and then decreased with successive developmental stages (Table 1), with the greatest differences between SCPs and FPs observed in pupae and adults. This is likely related to the resistance of cells to ice crystals.

SCPs and FPs were not affected by gender of adult *Cx. pipiens pallens*. In temperate climates, only female adult *Cx. pipiens pallens* overwinter (Liu et al. 2016a). Although male *Cx. pipiens pallens* adults also have low SCPs and FPs, they do not survive over the winter, which is possibly related to nutritional or physiological status. Our finding of an SCP of −18°C for female *Cx. pipiens pallens* adults was lower than that reported by Rinehart et al. (2006) (−16°C). This could be due to variations in geographical populations studied as well as our use of a laboratory colony for our study. A similar phenomenon was observed in other insects (Andreadis et al. 2014). Indeed, cold hardiness of mosquitoes in each

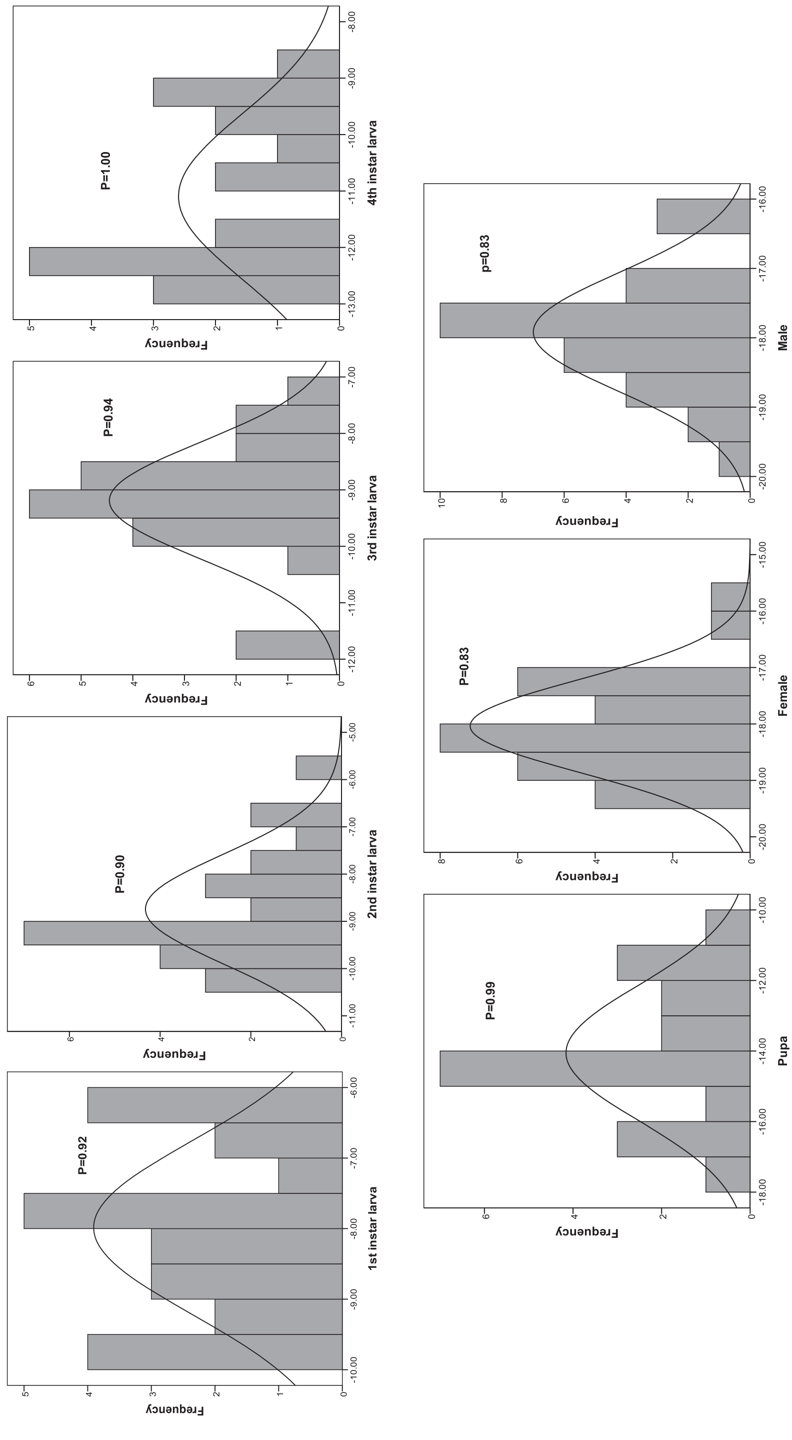

Fig. 1. Frequency distribution chart of the supercooling point of *Culex pipiens pallens*.

Table 1. The mean supercooling point (SCP) of *Culex pipiens pallens* of different developmental stages.

Developmental Stage	Mean SCP (°C ± SE)*	95% Confidence Interval for Mean	
		Lower Bound	Upper Bound
1st-instar larvae	−7.9854 ± 0.25018 a	−8.5030	−7.4679
2nd-instar larvae	−8.7396 ± 0.23061 ab	−9.2156	−8.2636
3rd-instar larvae	−9.1896 ± 0.21481 b	−9.6350	−8.7441
4th-instar larvae	−11.0968 ± 0.33495 c	−11.8006	−10.3931
Pupae	−14.0525 ± 0.42913 d	−14.9507	−13.1543
Female	−18.0353 ± 0.15120 e	−18.3446	−17.7261
Male	−17.9173 ± 0.15621 e	−18.2368	−17.5979

* Means within a column followed by the same letter are not significantly different ($P > 0.05$, Tukey test).

climatic zone differs (Mogi 2011). Therefore, integrating the research of the SCPs to that of geographical distribution of *Cx. pipiens pallens* can contribute to a better understanding of the cold resistance of *Cx. pipiens pallens*.

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Table 2. The mean freezing point (FP) of *Culex pipiens pallens* of different developmental stages.

Developmental Stage	Mean FP (°C ± SE)*	95% Confidence Interval for Mean	
		Lower Bound	Upper Bound
1st-instar larvae	−5.5550 ± 0.3038 a	−6.1835	−4.9265
2nd-instar larvae	−6.2660 ± 0.3165 a	−6.9192	−5.6128
3rd-instar larvae	−6.7387 ± 0.3320 a	−7.4271	−6.0503
4th-instar larvae	−7.4247 ± 0.8343 a	−9.1775	−5.6720
Pupae	−7.9175 ± 0.4120 a	−8.7799	−7.0551
Female	−12.6143 ± 0.3543 b	−13.3390	−11.8896
Male	−12.8777 ± 0.9526 b	−14.8260	−10.9293

* Means within a column followed by the same letter are not significantly different ($P > 0.05$, Tukey test).

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