Proboscis Extension Reflex in *Apis florea* (Hymenoptera: Apidae) in Response to Temperature[1]

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Abstract Increased atmospheric temperatures may negatively affect the ecology, biology, and physiology of insect pollinators by increasing asynchrony between pollinator foraging and flowering of angiosperms. *Apis florea* F. (Hymentoptera: Apidae) is an important pollinator of vegetables and spice plants in India and, compared to other honeybee species native to Asia, tolerates higher temperatures. We tested the effects of three temperatures (25°C ± 0.5°C, 35°C ± 0.5°C, and 42°C ± 0.5°C) on changes in proboscis extension reflex (PER) in *A. florea* in response to increasing sucrose concentrations (3%, 10%, 30%, 40%, 50%, and 70% w/v). Across the six sucrose concentrations, the mean %PER scores of *A. florea* exposed to 25°C ± 0.5°C were significantly higher than those at 35°C ± 0.5°C and 42°C ± 0.5°C, although the mean %PER scores at 35°C ± 0.5°C and 42°C ± 0.5°C did not vary significantly. This result suggests a possible reduction in *A. florea* feeding motivation at temperatures above 25°C, which may negatively affect their winter foraging patterns. This could be especially problematic with rising minimum air temperatures in the semiarid lateritic belts of West Bengal, India.

Key Words proboscis extension reflex, *Apis florea*, heat tolerance, feeding motivation

Apis florea F. (Hymenoptera: Apidae) is a wild-nesting, eusocial bee that forms single exposed combs, usually on tree branches. These bees are absent in cold climates (Hepburn and Radloff 2011) and are primarily restricted to the warmer areas of the Asian continent. Along with *Apis andreniformis* Smith, these bees constitute the subgenus *Micrapis* and are the most primitive of all living species under the genus *Apis. A. florea* has a mild temperament (Oldroyd and Wongsiri 2006) but is usually not human managed because of the poor yield of honey from individual combs. The honey, however, is commonly used in traditional medicine systems (www.icimod.org/?q=1519). Although these bees are tiny (average worker size is about 7–9 mm), they are significant pollinators of many tropical plants (Aluri et al. 2003, Abrol 2010, Suwannapong et al. 2011). *Apis florea* is usually prevalent in hot subtropical climates (Haddad et al. 2008, Hepburn and Radloff 2011) and, although the exact colony temperature of this bee species is not known, usually the temperature of most *Apis* colonies is maintained at 30°C–36°C (Suwannapong et al.

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2011). At lower temperatures, the eusocial *Apis* bees use metabolic heat to raise the temperature of the colony and consume more food in the process (Suwannapong et al. 2011). In a study conducted on *A. florea* foraging on onion, researchers found the greatest percentage of bees foraging when the ambient temperature was approximately 26°C (Abrol 2010). This is an indication that foraging of these bees is increased with reduced temperature, possibly to address the increased metabolic demand of the colony to cope with the decreased atmospheric and colony temperatures. To the contrary, with rising ambient temperatures, colonies of bees maintain the internal colony temperature at the optimum level by fanning and spraying water (Stabentheiner et al. 2010).

Apis florea is ubiquitous in the hot, semiarid, lateritic district of West Midnapore in the western part of the state of West Bengal in India. However, recent observations have suggested a possible decline in the frequency of locally occurring colonies of this bee compared with that observed in the last decade (Bhattacharyya et al. 2017). There may be several causal factors responsible for the suggested decline, including an increase in ambient air temperature that has been well documented in this region (Dolui et al. 2014). According to that report, the mean annual temperature of this district increased from 21.69°C ± 1.9°C in 2001 to 27.78°C ± 1.5°C in 2011. Such increases in ambient temperature may have an impact on *A. florea* feeding motivation, as manifested through its proboscis extension reflex (PER).

PER is a behavior of any insect with an extendable proboscis (e.g., a honey bee) in response to stimulation of its antennae with a sucrose solution of sufficient concentration and is manifested by the extension of its proboscis in anticipation of a sugar reward (Pankiw and Page 2003). In nature, insects display PER when they find nectar in a flower. In the laboratory, insects display the same behavior when their antennae are stimulated artificially with a sucrose solution of sufficient concentration, usually 0.1%, 0.3%, 1%, 3%, 10%, and 30% (Matsumoto et al. 2012).

A PER bioassay is frequently used as part of a conditioning protocol to investigate the perception of stimuli by bees (Smith and Burden 2014) and is regarded, as such, as a classical Pavlovian conditioning (Takeda 1961, Bitterman et al. 1983). This response behavior can be interpreted to explore learning and memory in honey bees under different treatment conditions, which are designed to explore the behavioral and neural mechanisms underlying such learning (Menzel and Giurfa 2006, Menzel 2012). Coupled with electrophysiology and molecular genetics, it can be used to test hypotheses on the roles of specific components in the nervous system (Hammer and Menzel 1995). PER protocols have also provided a reliable means to evaluate the sublethal effects of environmental conditions as well as toxins on the health and foraging efficiency of honey bees (Hladun et al. 2012) and are adopted widely to test the physiology of bees under different treatment conditions because of their high efficiency and relative low costs (Mayack 2012). Besides conventional stressors such as parasitic load and toxins, other factors such as laboratory handling and hormones can also affect bee PER (Pankiw and Page 2003). Through these studies, it has been established that PER is a robust response behavior to an array of physiological conditions to which a bee may be subjected, and the absence of such response can be recorded as an indicator of stress in the case of the tested bees (Smith and Burden 2014).

In PER bioassays, the responsiveness of honey bees to sucrose is recorded as dichotomous data and is measured as positive (01), indicating proboscis extension upon stimulation, or negative (00) when there is no extension (Matsumoto et al. 2012). The positive responses of bees to a specific sucrose concentration are summed as a PER score (Matsumoto et. al. 2012). PER scores are variable and influenced by several factors, including genotype, starvation state, foraging experience, and physiological state of the test bees (Pankiw et al. 2001, Frost et al. 2012). A more refined approach to documenting the responsiveness of a bee to a sucrose solution is to express the PER score as a percentage, that is as %PER scores, in which the number of bees responding to a specific sucrose solution through proboscis extension is divided by the total number of bees tested for that solution, the result being expressed as a percentage (Yang et al. 2013).

The present study was undertaken to compare the PER scores (as %PER) of three groups of *A. florea* exposed to three different temperatures under laboratory conditions, as a manifestation of their physiological state under varying degrees of heat stress. Significant differences in %PER scores would indicate significant differences in the feeding motivations of the three groups of bees. The implications of reduced PER at higher temperatures could include reduced foraging when ambient temperatures are higher than usual, which may have a possible negative effect on the pollination success of *A. florea*-pollinated plants, such as mustard and sesame that are cultivated during winter in the semiarid lateritic belt of West Bengal (Shekhawat et al. 2012), where this study is based.

Materials and Methods

Two *A. florea* colonies were located in the wild, and the foragers leaving the colony early in the morning were captured using nets, stunned on ice in ice boxes, caged in temporary plastic cages (1,000 ml), and transferred to the laboratory. In the laboratory, the bees were transferred to bee cages constructed of sturdy, transparent plastic cups (250 ml) placed on dry filter paper beds on petri dishes. Food in the form of 50**%** sucrose solution was provided to the bees through 2-ml injection syringes (minus the needles and the narrow ends of the syringes excised) inserted halfway into the cages through their inverted bases. Air ventilation in the cages was facilitated with 10 to 12 small holes located equidistantly around the circumference of the cup, approximately 5 cm above the lower margin. A single cup (bee cage) housed 15–20 bees. The bees were maintained at room temperature (i.e., normal ambient daytime temperature at which the colonies were found in the wild at that time), which was 25.5°C $\pm$ 1.0°C, for a maximum of 48 h prior to experimentation, under approximately 12:12 h light:dark conditions.

Prior to experimentation, the bees were starved for 8 h based on prior observations that ascertained that *A. florea* were suitably starved only after a minimum of 8 h of food denial. This was in contrast to the starvation times usually adopted for *Apis mellifera* L. (Human et al. 2013) and *Apis cerana* F. (Chakrabarti et al. 2015), as well as contrary to that adopted for *A. florea* in the single existing study on this species (Kaspi and Shafir 2013). Two hours before the initiation of each experiment, the cages of bees were placed inside refrigerators to slow bee activity. The lethargic bees were removed from refrigeration and placed in individual

Eppendorf tubes on cold water to maintain the lethargy. Next, each bee was harnessed in individual plastic straws (the lower half of which was slit and cut to form a trough) such that only the head and forelegs of the bee extended out of the straw and remained movable, while the rest of its body remained inside and stationary. This is the accepted technique of harnessing bees for PER experiments (Human et al. 2013), and only slight modifications have been adopted in this case to suit the requirements of the study.

Bees harnessed in this way were allowed to adjust to the harnessed condition for approximately 30 min, and then their antennae were touched with wooden toothpicks soaked in 50% sucrose solution to check their responsiveness. Only positively responding bees (indicating sufficient hunger) were selected to perform the temperature experiments.

Summer temperatures in the lateritic, semiarid western region of West Bengal, where this study is based, frequently exceeds 40°C, staying high for prolonged periods. Previous studies based in this region have explored the effects of high temperatures, both in the air and water, on the biology and physiology of different groups of animals (Dey at al. 2015, Maiti-Dutta et al. 2018). As such, based on the information in these previous studies, as well as direct observation of air temperatures over the last several years, three different temperatures were selected as representative temperatures in this study: 25°C ± 0.5°C, 35°C ± 0.5°C, and 42°C ± 0.5°C. The bees were kept in environmental chambers maintained at the respective temperatures for 30 min. This exposure time was selected because preliminary observations showed that harnessed bees exposed to a temperature of 42°C ± 0.5°C exhibited increased restlessness and eventually died after approximately 75 min. Furthermore, *A. mellifera* reportedly fly up to a distance of between 45 to 5,000 m (Hagler et al. 2011, as cited by Abou-Shaara 2014) and typically fly at a speed of approximately 7 to 7.8 m/s in neutral wind (Wenner 1963). Therefore, if bees fly the maximum flight distance of 5,000 m at that speed, they should typically take 10 to 12 min to cover this distance. A period of 30 min should, therefore, be considered as adequate for a bee to visit a flower 5,000 m away from its hive, process it, and then return with the pollen and/or nectar load to the conditioned atmosphere within the hive. As such, in this study, 30 min was selected as a conservative and suitable estimate of exposure time of *A. florea* to the selected temperatures in the laboratory, which was expected to closely approximate their exposure time to ambient temperatures outside the hive under real conditions.

After 30 min of exposure, with the bees remaining in the chamber and the chamber door open, the antennae of the bees were touched with wooden toothpicks soaked in distilled water to check PER in response to water. If a bee responded, it was fed water to satiation. Next, following a gap of 10 min, the bees were then tested for PER by reaching inside the chamber and stimulating antennae with sucrose solutions of ~3% (w/v) (actual value 2.982%), 10% (w/v) (actual value 9.94%), 30% (w/v) (actual value 29.82%), 40% (w/v) (actual value 39.76%), 50% (w/v) (actual value 49.7%), and 70% (w/v) (actual value 69.58%) concentrations, prepared with purified organic sugar (99.4% sucrose, Food Safety and Standards Authority of India certified) in distilled water. With *A. florea* used in this study, no PER could be elicited with sucrose solutions of concentrations <3% at any temperature, indicating a greater sucrose threshold for these bees. Hence, after trial tests were repeated with bees from two colonies, a 3% w/v sucrose solution was

used as the starting concentration. The gap between two sucrose concentration stimulations was 2 min; between subsequent stimulations, the antennae of the bees were wiped gently with a water-soaked cotton swab to reduce possible sensory sensitization to antennal touch (Bitterman et al. 1983).

To determine whether there was any difference between the bees of the two colonies in terms of antennae lengths and tongue lengths, morphometric analysis of these body parts of some bees from the colonies was conducted using high-resolution photographs and ImageJ software (Tschinkel 2013). There were no significant differences between the lengths of antennae ($t = 0.645$, df $= 20$, $P \geq 0.05$) and tongues ($t = 1.603$, df $= 20$, $P \geq 0.05$) of the bees from two colonies.

Because there also were no significant differences in the **%**PER scores between the two colonies in response to all six sucrose concentrations, the response scores from two colonies after each experiment were pooled for statistical analyses. The total numbers of bees from the two colonies used in five replicate experiments at each temperature were 38 for 25°C ± 0.5°C, 44 for 35°C ± 0.5°C, and 53 for 42°C ± 0.5°C. Experiments were conducted in the months of October and November in 2015.

All data were tested for normality using the Shapiro-Wilks test and found to be normally distributed. Data were also tested for homogeneity of variance using the modified Levene's test, which uses the median instead of mean (Brown-Forsythe-Levene test) and found to have homogeneity of variance. This modified Levene's test has been shown to be more robust than the standard Levene's test that uses the data mean for comparison and gives accurate error rates even when the deviation of the scores from normal distribution is significant (Olejnik and Algina 1987). Two-way mixed analysis of variance (ANOVA) using the **%**PER scores as the dependent variable and each of the six sucrose concentrations as the repeated measures factor (within subjects factor) and the three temperatures as the other independent variable (between subjects factor) was performed with *post hoc* tests for the between subjects variable. The main effects of sucrose concentrations on **%**PER scores were compared using Bonferroni correction to restrict the family-wise error rate to an overall 5**%**. To further compare mean differences in **%**PER scores of the three temperature groups at each sucrose concentration (in the event of a nonsignificant interaction), one-way ANOVA using Bonferroni correction was performed. An alpha value of 0.05 was taken for estimating significance. All analyses were performed using SPSS (version 16.0; 2007).

Results

Mauchly's test of sphericity indicated that the assumption of sphericity had not been violated in this case ($\chi^2 = 20.113$, df $= 14$, $P = .136$). A significant main effect of sucrose concentration was observed, suggesting that higher sucrose concentrations tended to increase PER responses in bees irrespective of the temperature to which the bees were exposed (main effect of sucrose concentration: $F = 52.648$; df $= 5, 60$; $P \leq 0.001$). Sucrose concentrations had a significant effect on **%**PER scores but interaction of sucrose concentration and temperature did not ($F = 1.807$; df $= 10, 60$; $P = 0.079$).

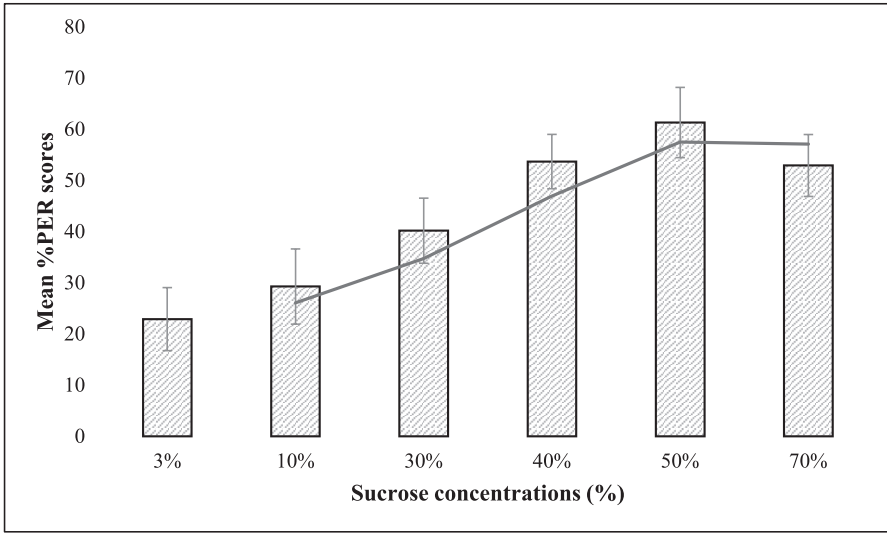

Fig. 1. Mean %PER scores of *Apis florea* at different sucrose concentrations.

In general, %PER scores tended to increase with increasing sucrose concentrations from 3% to 50% but decreased at 70% (Fig. 1). This increase was not significant across all possible pairwise combinations of sucrose concentrations (averaging over all three temperature groups). There was a significant difference between the mean %PER scores of bees in response to subsequent higher sucrose concentrations than an immediately preceding lower concentration ($P \leq 0.05$), except between the concentrations 3% and 10%, 40% and 50%, and 50% and 70% ($P \geq 0.1$). Table 1 details the pairwise comparisons for all possible sucrose concentrations.

There was a significant main effect of temperature on the %PER responses ($F = 49.935$; df = 2, 12; $P < 0.001$). The %PER scores of bees exposed to 25°C ± 0.5°C (mean ± SD, 73.64 ± 14.4) was found to be significantly higher than the %PER scores of bees exposed to 35°C ± 0.5°C (mean ± SD, 29.44 ± 20.5) and 42°C ± 0.5°C (mean ± SD, 27.09 ± 17.6) (Table 2). However, the %PER scores of the bees exposed to 35°C ± 0.5°C did not vary significantly from the %PER scores of bees exposed to 42°C ± 0.5°C. This finding was also validated by the result of a separate one-way ANOVA analysis computed using the Bonferroni test to compare the mean %PER scores of the three different temperature groups in response to each of the six sucrose concentrations (Fig. 2).

Discussion

Sucrose responsiveness, as indicated by the PER, of bees or any insect with an extendable tongue is usually associated with the feeding motivation of the study insect (Dethier 1976, Ozaki et al. 2003, Maeda et al. 2015) and is indicative of its physiological state. Higher sugar concentrations are expected to increase sucrose

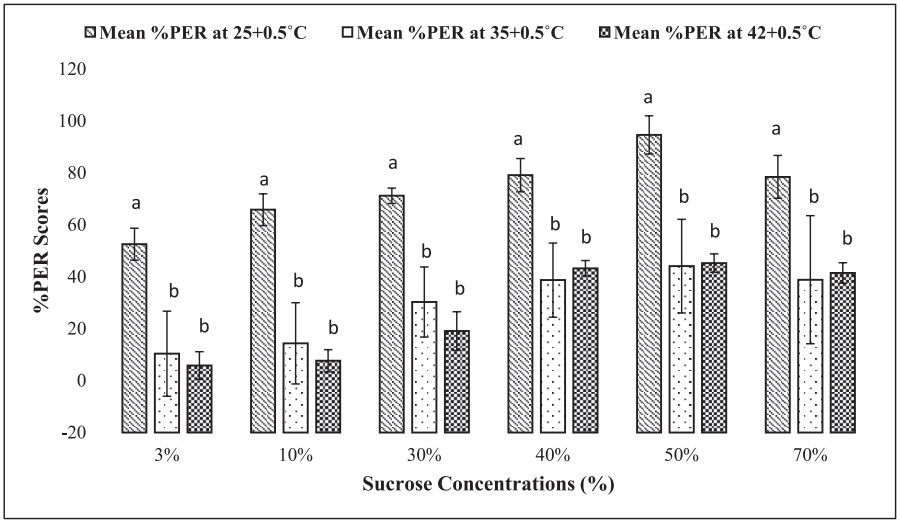

Fig. 2. Mean %PER scores of *Apis florea* exposed to three different temperatures, in response to six different sucrose concentrations. Bars, indicating mean responses at each sucrose concentration, with different lowercase letters on top, are significantly different from each other ($P \leq 0.05$).

responsiveness in bees, and our results corroborated that the %PER scores of bees exposed to three different temperatures increased steadily as the concentration of sucrose increased from 3% to 50% but decreased thereafter. A reason for the decrease in the %PER scores in bees in response to 70% sucrose stimulation may be attributed to the aversion of honeybees to viscous solutions, as reported by Nicolson et al. (2013). Honey bees (*Apis* spp.) and bumble bees, (*Bombus* spp.), in general, have been shown to prefer sucrose concentrations between 50% and 60% (Woodrow 1968) and 30% and 40% (Pouvreau 1974), respectively. In our study, *A. florea* exposed to three different temperatures appeared to prefer sucrose concentrations of 50%.

Our results also clearly demonstrated that, at lower temperatures in controlled laboratory settings, bees displayed greater proboscis extension in response to increasing sucrose concentrations than that at higher temperatures. These results, therefore, suggest that bees exposed to temperatures around 25°C ± 0.5°C have a greater feeding motivation than bees exposed to temperatures at and above 35°C ± 0.5°C. Studies have shown that bees of the genus *Apis* tend to use metabolic heat to increase colony temperatures when the ambient temperature drops below a defined threshold, usually 30°C (Suwannapong et al. 2011). This might be a shared behavior across one or more species under the genus *Apis,* leading to greater food consumption for generating metabolic heat, which was indicated in our study through the increased PER of *A. florea* exposed to the lower temperature. At temperatures ≥30°C, there no longer remains the necessity for generating

Table 1. Variations in mean %PER scores of *Apis florea* in response to increasing sucrose concentrations.

Sucrose Concentration	Sucrose Concentration	Mean Difference	Standard Error	*P* Value	95% Confidence Interval for Difference	
					Lower Limit	Upper Limit
3%	10%	−6.371	2.364	0.262	−14.716	1.975
	30%	−17.281	2.560	0.000**	−26.316	−8.246
	40%	−30.798	2.321	0.000**	−38.989	−22.608
	50%	−38.428	2.594	0.000**	−47.582	−29.273
	70%	−30.033	3.176	0.000**	−41.245	−18.822
10%	3%	6.371	2.364	0.262	−1.975	14.716
	30%	−10.910	2.738	0.020*	−20.575	−1.245
	40%	−24.428	3.516	0.000**	−36.840	−12.016
	50%	−32.057	3.000	0.000**	−42.644	−21.470
	70%	−23.663	4.131	0.001**	−38.243	−9.082
30%	3%	17.281	2.560	0.000**	8.246	26.316
	10%	10.910	2.738	0.020*	1.245	20.575
	40%	−13.517	2.843	0.005*	−23.553	−3.482
	50%	−21.147	3.044	0.000**	−31.890	−10.403
	70%	−12.752	4.351	0.164	−28.110	2.606
40%	3%	30.798	2.321	0.000**	22.608	38.989
	10%	24.428	3.516	0.000**	12.016	36.840
	30%	13.517	2.843	0.005*	3.482	23.553
	50%	−7.629	2.664	0.188	−17.033	1.774
	70%	0.765	3.772	1.000	−12.548	14.079
50%	3%	38.428	2.594	0.000**	29.273	47.582
	10%	32.057	3.000	0.000**	21.470	42.644
	30%	21.147	3.044	0.000**	10.403	31.890
	40%	7.629	2.664	0.188	−1.774	17.033
	70%	8.394	2.847	0.159	−1.655	18.444
70%	3%	30.033	3.176	0.000**	18.822	41.245
	10%	23.663	4.131	0.000**	9.082	38.243

Table 1. Continued.

Sucrose Concentration	Sucrose Concentration	Mean Difference	Standard Error	*P* Value	95% Confidence Interval for Difference	
					Lower Limit	Upper Limit
	30%	12.752	4.351	0.164	−2.606	28.110
	40%	−0.765	3.772	1.000	−14.079	12.548
	50%	−8.394	2.847	0.158	−18.444	1.655

Adjustments for multiple comparisons: Bonferroni. * Significant at $P < 0.05$; ** Significant at $P < 0.01$.

additional metabolic heat to maintain colony temperatures and, thus, the feeding motivation appeared to have decreased significantly.

Studies on insect herbivory indicate that the consumption rate of insects increases with temperatures rising from 20°C to 30°C but does not increase further (Lemoine et al. 2014). It can be assumed that the ambient temperature is instrumental in determining feeding motivation and food consumption in ectothermic animals, including insects. In this study, it has been demonstrated that, at the lower temperature, the motivation of a starved bee to extend its proboscis in anticipation of food is higher than the motivation of equally starved bees kept at higher temperatures, across several possible food reward levels.

It is expected that, as the results show here, greater food consumption would occur when the ambient temperature is low, compelling the bees to use metabolic heat to raise colony temperatures, thereby requiring greater foraging on part of the

Table 2. Differences in mean %PER scores of *Apis florea* at different temperatures.

Temperature Groups	Temperature Groups	Mean Difference	Std. Error	*P* Value	95% Confidence Interval	
					Lower	Upper
25°C	35°C	44.195	5.247	0.000**	29.609	58.781
	42°C	46.546	5.247	0.000**	31.960	61.132
35°C	25°C	−44.195	5.247	0.000**	−58.781	−29.609
	42°C	2.350	5.247	1.000	−12.234	16.936
42°C	25°C	−46.546	5.247	0.000**	−61.132	−31.960
	35°C	−2.3509	5.24767	1.000	−16.9367	12.2348

Mean Square(Error) = 68.845. ** Significant at $P \leq 0.01$.

workers. Observations suggest there are greater abundances of *A. florea* colonies in winter than in any other season in this district. However, temperature may not be the only determining factor of this greater abundance of bees in winter. Besides temperature, availability of floral resources and light intensity have a strong effect on the foraging behavior of *A. florea* (Abrol 2010). Several studies suggest that insect feeding responses to temperature gradients can be highly variable (Lemoine et al. 2014), often markedly in natural settings and artificial, controlled environments (Harrison and Fewell 1995). Further investigations are needed to supplement and corroborate our findings and conclusions with this and other bee species found in this region of India.

The effect of rising air temperatures on terrestrial insects is most likely to be manifested through alterations in insect life-history parameters, physiology, behavior, and ecological roles, as well as intra- and interspecific interactions (Cornelissen 2011). Also, indirect effects of atmospheric warming on insect host plants and natural enemies are likely to affect insect populations (Cornelissen 2011). Pollinators, such as bees, are expected to be negatively affected, in general, because of the asynchrony in the flowering of angiosperms with their foraging activities (Memmott et. al. 2007). There is, therefore, a possibility that future temperature change will negatively affect bee populations, and a change in feeding motivation, as observed within the limited scope of this study, will perhaps be one of the many changes occurring simultaneously in bees. With reports of increasing minimum temperatures in India, bees such as *A. florea* might be less motivated to forage for nectar during the colder months, thereby possibly affecting pollination of several important oilseed crops (e.g., mustard and sesame) and winter vegetables. A recent report by India's Meteorological Department suggests that the average minimum air temperature during the winter months in the state of West Bengal, where the study area is located, has increased significantly in the last five decades (Rathore et al. 2013). Further studies are necessary, therefore, to ascertain how extensively rising temperatures might affect these crucial ecosystem service providers, especially in developing countries like India, where the economy is still largely agriculture reliant.

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