Original Article

SIMULATED CLIMATE WARMING INFLUENCED COLONY MICROCLIMATIC CONDITIONS AND GUT BACTERIAL ABUNDANCE OF HONEYBEE SUBSPECIES *APIS MELLIFERA LIGUSTICA* AND *A. MELLIFERA SINISXINYUAN*

Krouholé A. S. Coulibaly[1,2]
Muhammad Z. Majeed[1,3]*
Samy Sayed[4]
Kolo Yeo[5]

[1]Climate Change Biology Research Group, State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, China
[2]Institute of Apicultural Research, Chinese Academy of Agricultural Sciences, China
[3]Department of Entomology, University of Sargodha, Pakistan
[4]Department of Science and Technology, University College-Ranyah, Taif University, Saudi Arabia
[5]Station d'Écologie de Lamto, Université Nangui Abrogoua, Cote D'Ivoire (Ivory Coast)

*corresponding author: zeeshan.majeed@uos.edu.pk
Received: 03 May 2021; accepted: 08 February 2022

Abstract

Ectothermic organisms including insects are highly vulnerable to climate warming which not only influences their biology, ecology and physiology but also affects their symbiotic gut microbiota. This study determined the impact of ambient (control) and simulated warmer (heating) climatic conditions on the microclimate of brood nest and gut bacterial abundance of two *Apis mellifera* subspecies i.e. *A. mellifera ligustica* and *A. mellifera sinisxinyuan*. For both subspecies, brood nest temperature and relative humidity under the heating treatment were significantly different ($p \leq 0.001$) than those under the control treatment. Quantitative PCR data revealed that the abundance of gut bacteria (16*S r*RNA gene copy numbers) of *A. mellifera ligustica* and *A. mellifera sinisxinyuan* larvae was significantly higher ($P \leq 0.05$), 1.73 and 5.32 fold higher respectively, during the heating treatment than those in control conditions. Although gut bacterial abundance of *A. mellifera ligustica* (1.67×10^7 copies g^{-1} fw) and *A. mellifera sinisxinyuan* (1.7×10^7 copies g^{-1} fw) larvae was similar during the control treatment, *A. mellifera sinisxinyuan* larvae exhibited three times greater gut bacterial abundance than *A. mellifera ligustica* during the heating treatment. Similarly, adult *A. mellifera sinisxinyuan* bees harboured significantly greater bacterial abundance during the heating treatment than control. These findings elucidate that climate warming may significantly affect the honeybee colony microclimate and their gut bacterial abundance. However, further studies are needed to better understand how gut microbial community may influence the learning, physiological and behavioural mechanisms of the host bees in a climate warming scenario.

Keywords: *Apis mellifera ligustica, Apis mellifera sinisxinyuan,* brood microclimate, climate warming, gut bacterial abundance, 16*S r*RNA gene copy numbers

INTRODUCTION

Ectotherms such as reptiles, amphibians and insects are highly sensitive to temperature increases (Neven, 2000; Corn, 2005; Deutsch et al., 2008), and hence are vulnerable to the current scenario of climate warming (Huey et al., 2009; Huey et al., 2012). In the near future, population distributions of ectotherm animals can be affected by climate warming and many

species may become extinct (Johnson, 2010; Crotti et al., 2012; Nazzi et al., 2012; Bestion et al., 2015; Chen et al., 2016).

Adult honeybees (Apidae: Hymenoptera) usually show a greater tolerance to heat stress than their broods (Tautz et al., 2003; Groh et al., 2004; Jones et al., 2005; Human et al., 2006; Becher et al., 2009; Abou-Shaara et al., 2012). For normal honeybee brood development, the microclimate inside the brood nest is maintained in a strict temperature range between 33 and 36°C and relative humidity between 80 and 90% (Silva et al., 2009; Kaftanoglu et al., 2011). A slight deviation from this range, even by 1°C, negatively influences the brood's developmental period of (Tautz et al., 2003), wing morphology (Ken et al., 2005), learning ability (Tautz et al., 2003) and disease prevalence (Mardan & Kevan, 2002). Similarly, lower relative humidity inside the brood nest could increase the rate of larval mortality through desiccations (Ellis et al., 2008; Al-Ghamdi et al., 2016). To buffer the effect of such abiotic stresses as temperature fluctuations and relative humidity on the colony, honeybee workers invest considerable energy to maintain a brood nest microclimate independent of the climatic conditions outside of the hive (Human et al., 2006; Jones & Oldroyd, 2006). Although many previous studies have assessed the direct effects of temperature and relative humidity stresses on the worker honeybees and broods (Tautz et al., 2003; Jones et al., 2004; Ken et al., 2005; Jones & Oldroyd, 2006; Seeley, 2014), the indirect consequences of these abiotic stresses on gut symbiotic bacteria associated with honeybees remain largely unknown.

Recently, symbiont bacteria are receiving attention as strong and effective immune-modulators of insects (Ryu et al., 2008; McFall-Ngai et al., 2012). Gut-harboring bacterial communities greatly influence the resistance of their hosts to biotic and abiotic stresses (Crotti et al., 2012; Engel & Moran, 2013), which suggests their crucial contribution to hosts' adaptation and evolution (Rosenberg & Zilber-Rosenberg, 2011). Recent studies have shown that gut bacteria could change ectotherms' population composition or metabolism to fight with low temperature stress by (Chevalier et al., 2015; Hylander & Repasky, 2019; Raza et al., 2020). However, during high temperature stress could reduce the gut bacterial abundance of reptile and amphibian ectotherms (Bestion et al., 2017; Li et al., 2020). A more comprehensive analysis of gut bacteria within a host under a simulated climate-warming pattern is of a great interest to predict the consequences of climate warming on the ecology of symbiotic microbes and their hosts.

An understanding of how abiotic factors affect host-microbiota interactions would improve our knowledge of honeybees' response to climate warming. In this study, we investigated the impact of simulated climate-warming on the brood nest microclimate and gut bacterial populations of honeybees. We hypothesized that temperature increase may affect directly the incubation temperature inside the brood nest and indirectly mediate the gut bacterial abundance of *Apis mellifera.* To conduct this study, we used two subspecies of *A. mellifera* i.e. *A. mellifera ligustica* and *A. mellifera sinisxinyuan* to demonstrate how the response of gut bacteria might differ according to the evolution of a species. *A. mellifera ligustica* is a common honeybee dispersed in temperate and tropical climates, while *A. mellifera sinisxinyuan* is strictly an endemic subspecies in temperate climates particularly in north-east China.

MATERIAL AND METHODS

Honeybee colonies

The study was carried out from April to August 2017 at the Institute of Apicultural Research (40°00'12.8"N; 116°12'29.5"E), Beijing, China. The study population of each honeybee subspecies (*A. mellifera ligustica* and *A. mellifera sinisxinyuan*) was comprised of eight colonies. Each colony was comprised of approximately 6,000 worker bees at the beginning of the experiment and all queen bees were of the same age (~18 months) and descendants from different mother bees. There were eight frames in each bee hive.

Treatments

During the experiment, eight housed bee hives as the control were kept outdoors and shaded with ceramic tiles under ambient climatic conditions and eight hives for the heating treatment were maintained at 2-3°C higher than ambient temperature conditions. For each honeybee subspecies used in this study, there were four replications (four bee hives) in the control and four replications in the heating treatment. Under the heating treatment, heat was supplied to honeybee hives with a ceramic infrared radiator heater (FTE-L10-Y; 1000 W, 240 V; 60 mm long × 245 mm wide) the hives. Each hive was heated up with two such heaters set at a height of 190 cm at the front and behind of the hive to make the ambient climatic condition warmer. The heating treatment was designed according to the global warming prediction of IPCC (2014).

Monitoring of brood nest temperature and relative humidity

In order to monitor the brood nest microclimate conditions, one probe (Onset® HOBO® Pro V2 logger; Onset Computer Corporation, MA, USA) was introduced in each hive through the lateral holes (Ø=10 mm). The probe was installed in the brood area at 0.3 cm from the comb surface. The probes were wrapped in a plastic protective membrane to impede honeybee workers from covering the sensor with water, nectar, wax or propolis. Data regarding hive temperature and relative humidity were recorded and stored automatically by the logger in each hive at regular 10 min intervals throughout the duration of the experiment (24 hrs in a day) (Fig. 1). At the end of experiment, data collected by the loggers were downloaded to a laptop through an optic coupler (2-E) using the Onset Optical Base Station (BASE-U-4) software.

Molecular analysis

For the characterization of the gut bacterial community of each honeybee subspecies, i.e. *A. mellifera ligustica* and *A. mellifera sinisxinyuan*, ten 5-days old larvae and ten teneral worker bee imagoes were randomly collected from each of the sixteen hives (including 4 heated and 4 control hives for each subspecies) once at the end of the field experiment. Insects collected from each bee hive were surface-sterilized with 70% ethanol, rinsed thoroughly with sterilized double-distilled water, dried over a lint-free laboratory wipe and weighed with a digital balance (accuracy 0.001 g, Shimadzu Corporation, Japan). All sampled specimens were preserved at -20°C in 95% ethanol for the dissection of their guts.

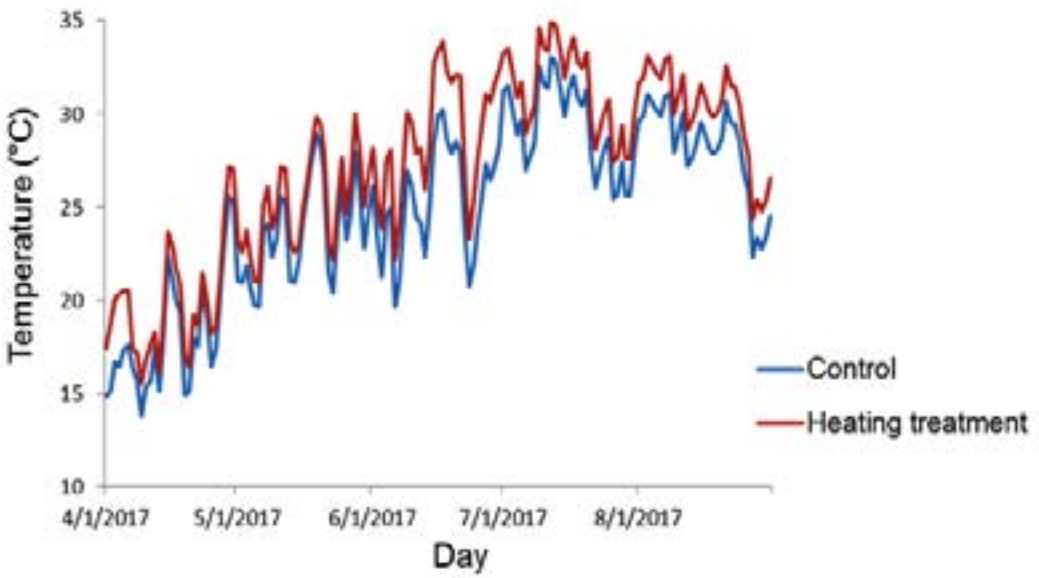


Fig. 1. Mean daily temperature (°C) recorded from April to August, 2017 under two temperature conditions. Blue and red lines indicate temperature fluctuations for the control and heating treatments, respectively.

DNA extraction

Total genomic DNA was extracted from the ileums and rectums of individual honeybee larvae and adults with the use TIANamp® genomic DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's protocol. Purified DNA was eluted from each sample using 200 µl of AE buffer provided in the extraction kit. Extracted DNA quality was verified by gel electrophoresis through staining 1.5% (w/v) agarose gel with ethidium bromide and visualizing it under UV-transillumination system and then was quantified with NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Extracted DNA samples were preserved at -20°C until downstream molecular analysis. Final DNA concentrations in all samples were normalized to 10 ng/µl with nuclease-free water.

Abundance of total symbiotic bacterial community

Total bacterial symbionts harboured by the honeybee adults and larvae of *A. mellifera ligustica* and *A. mellifera sinisxinyuan* were estimated through the amplification and quantification of the 16*S r*RNA gene copy numbers with a set of universal primers i.e. 341F (5'-CCT ACG GGA GGC AGC AG-3') and 515R (5'-ATT CCG CGG CTG GCA-3') targeting 174 bp region of eubacterial gene.

Quantitative PCR assay

The real-time quantitative PCR assays were performed using a C1000™ thermo-cycler (CFX96®, BioRad, Hercules, California). A final reaction mixture of 20 µl contained 0.6 µl of each 10 µM 16*S r*RNA primer, 10 µl of SYBR Green PCR mix having HotStar Taq™ DNA Polymerase, SYBR Green PCR Buffer, dNTP mix, SYBR Green I, ROX and 5 mM $MgCl_2$ (FastFire qPCR PreMix; Tiangen®, Beijing, P.R. China), 1 µl of template DNA corresponding to 10 ng of total DNA and 7.8 µl of DNase- and RNase-free distilled deionized water. Thermal protocol for 16*S r*RNA amplification reactions were comprised of 900 s at 95°C for enzyme activation followed by forty cycles of 15 s at 95°C for denaturation, 30 s at 60°C for annealing and 30 s at 72°C for extension steps. At the end of each reaction, melting curve analysis for data acquisition involved an additional step of heating from 65°C to 95°C (@ 0.2°C/s). Specificity and purity of the amplified qPCR products were confirmed by melt-curve analysis and by observing the band of expected fragment size in 1.5% (wt/v) agarose gel stained by GelRed® 10,000X (Coollab, Beijing, P.R. China). The standard curve for 16*S r*RNA gene quantification was worked out through the plotting of threshold cycle (Ct) values as a function of $\log^{10}$ of the copy numbers of target gene fragment. To this end, amplified target gene fragments were cloned in Top10® (*Escherichia coli*) Competent Cells (Tiangen®, Beijing, P.R. China) using pGM-T Blunt Cloning Kit (Tiangen®, Beijing, P.R. China) according to the manufacturer's instructions. Cloned plasmids carrying a standard target sequence were linearized through incubation at 37°C for 1 hr along with the 10 U of *Sal*I restriction enzyme. Standards were prepared by tenfold serial dilution of these linearized plasmids containing 10^2 to 10^9 16*S r*RNA gene copies, calculated from the concentration of extracted plasmids.

Statistical analysis

Using mean daily temperature and relative humidity in the brood nest of each hive, we calculated the mean brood nest temperature and relative humidity under the two temperature treatments. For statistical analyses, samples were grouped by treatment categories for each subspecies. Data regarding brood temperature and relative humidity were analysed with the use of one-way analysis of variance (ANOVA), followed by Fisher's Least Significant Difference (LSD) test to assess the difference between the means of treatment groups and honeybees subspecies at standard probability level ($p \leq 0.05$). Gut bacterial gene abundances for both treatments and subspecies were analysed using two-sample Student's *t* test at α=0.05. Data analysis was carried out with IBM® SPSS Statistics V. 20 (IBM Corp., NY, USA).

RESULTS

Effects of simulated climate warming on the brood nest microclimate

For both *A. mellifera* subspecies, the brood nest mean temperature under heating treatment significantly differed ($p \leq 0.001$) from that under the control treatment (Fig. 2 and Fig. S1). The mean brood nest temperatures of *A. mellifera ligustica* and *A. mellifera sinisxinyuan* during the heating treatment was recorded as 34.90 and 34.54°C, respectively, and both were significantly higher than the brood temperatures of 33.51 and 34.14°C, recorded respectively for the control treatments. Moreover, the brood nest temperature during heating treatment did

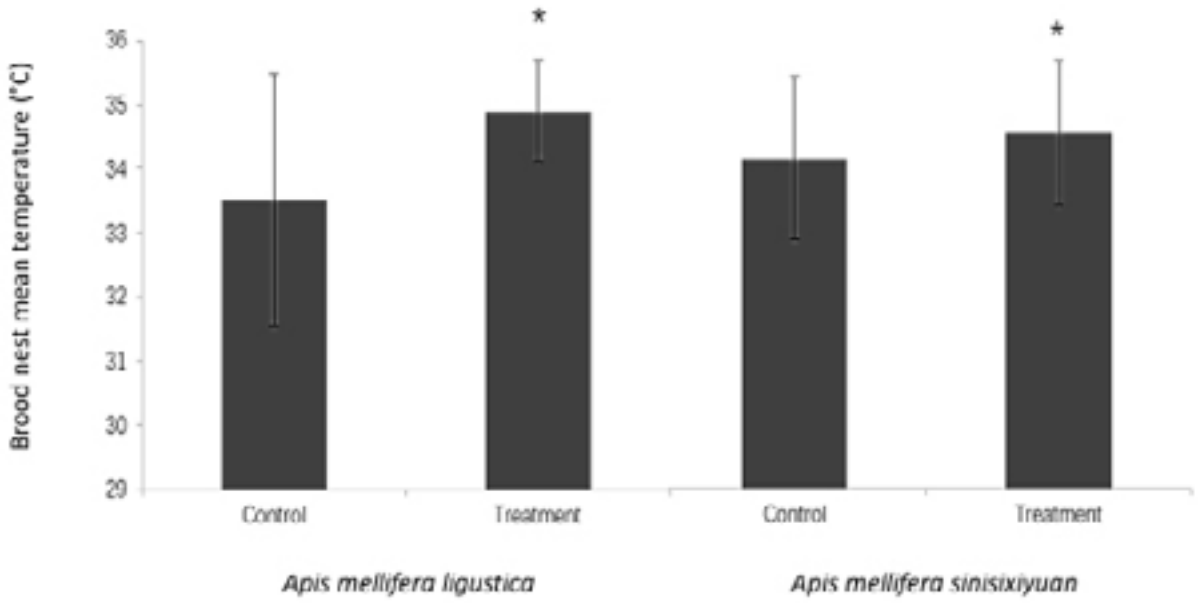


Fig. 2. Brood nest temperature (mean±SD; n=8) of *A. mellifera ligustica* and *A. mellifera sinisxinyuan* recorded during the control and heating treatments from April to August 2017. Cumulative data of eight bee hives is presented for each treatment. * indicates significant difference between treatments for each subspecies (two-sample Student's t-test; $p \leq 0.05$).

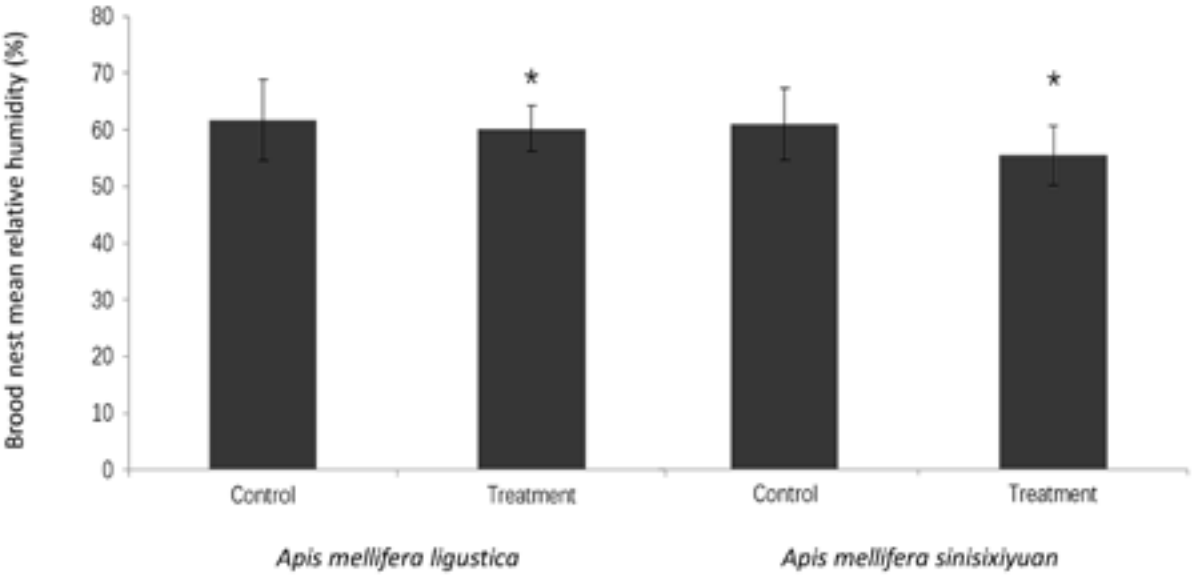


Fig. 3. Brood nest relative humidity (mean±SD; n=8) of *A. mellifera ligustica* and *A. mellifera sinisxinyuan* recorded during control and heating treatments from April to August 2017. Cumulative data of eight bee hives is presented for each treatment. * indicates significant difference between treatments for each subspecies (two-sample Student's t-test; $p \leq 0.05$).

not significantly differ between both honeybee subspecies ($p \geq 0.05$), while for the control treatment, the brood nest temperature of *A. mellifera sinisxinyuan* was significantly higher than that of *A. mellifera ligustica* ($p \leq 0.05$).

Similarly, the mean relative humidity in both *A. mellifera* subspecies brood nests during the control and heating treatments was significantly ($p \leq 0.001$) different (Fig. 3 and Fig. S2). Mean brood nest relative humidity values of *A. mellifera ligustica* (60.14%) and *A. mellifera sinisxinyuan* (55.63%) during the heating treatment were significantly lower than those recorded during the control treatment i.e. 61.71 and 61.21%, respectively (Fig. 3). Under the control treatment, mean brood nest relative humidity for both subspecies was statistically similar ($p \geq 0.05$). However, the brood nest relative humidity of *A. mellifera ligustica* was significantly higher than that of *A. mellifera sinisxinyuan* ($p \leq 0.05$) during the heating treatment.

Impact of simulated climate warming on the honeybee gut bacterial population

Regarding the abundance of total gut bacteria, qPCR data revealed that 16*S r*RNA gene copy numbers in the larval guts of both honeybee subspecies were higher during the heating treatment than those under the control condition (Fig. 4), although the difference was statistically significant only for *A. mellifera sinisxinyuan* ($p \leq 0.05$). The bacterial abundance of *A. mellifera sinisxinyuan* larvae increased significantly ($p \leq 0.05$) from 1.73×10^7 copies g^{-1} fw under the control condition to 9.21×10^7 copies g^{-1} fw under the heating treatment. On the other hand, bacterial population of *A. mellifera ligustica* larval gut increased from 1.67×10^7 copies g^{-1} fw under the control to 2.89×10^7 copies g^{-1} fw under heating treatment (Fig. 4). Moreover, no significant difference ($p \geq 0.05$) was found between the gut bacterial abundance of *A. mellifera ligustica* (1.67×10^7 copies g^{-1} fw) and *A. mellifera sinisxinyuan* (1.7×10^7 copies g^{-1} fw) under the control. In contrast, under heating treatment, *A. mellifera sinisxinyuan* gut bacterial abundance (9.21×10^7 copies g^{-1} fw) was three times higher than that of *A. mellifera ligustica* (2.89×10^7 copies g^{-1} fw) ($p \geq 0.05$).

Gut bacterial abundance as quantified from imago (adult) honeybees showed that 16*S r*RNA gene copy numbers of *A. mellifera sinisxinyuan* recorded during the control and heating treatments significantly differed from

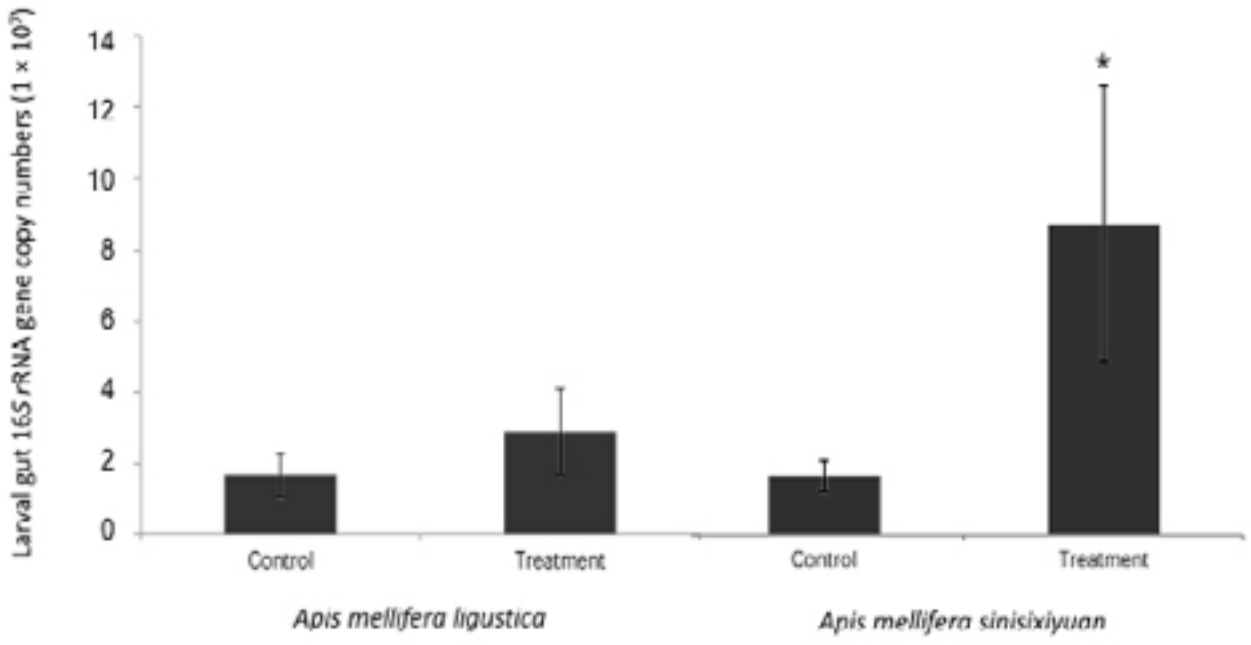


Fig. 4. Bacterial 16S ribosomal RNA gene abundance (mean±SD; n=40) in larval guts of *A. mellifera lingustica* and *A. mellifera sinisxinyuan* during control and heating treatments. * indicates significant difference between treatments for each subspecies (two-sample Student's t-test; $p \leq 0.05$).

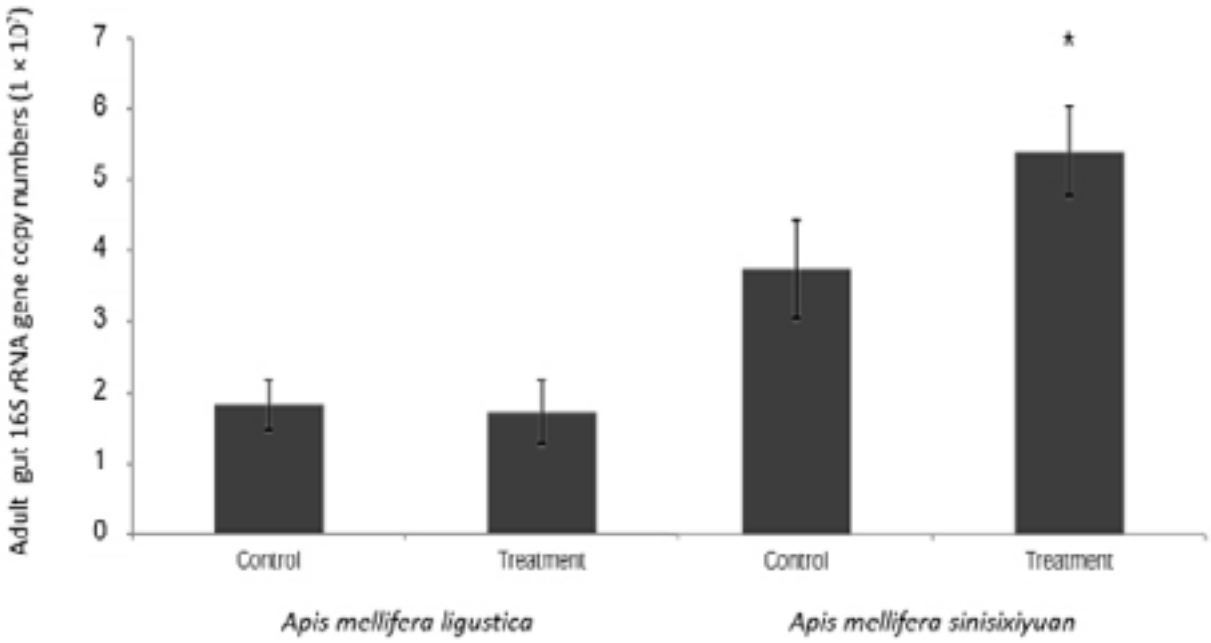


Fig. 5. Bacterial 16S ribosomal RNA gene abundance (mean±SD; n=40) in guts of newly emerged adults of *A. mellifera lingustica* and *A. mellifera sinisxinyuan* under control and heating treatments. * indicates significant difference between treatments for each subspecies (two-sample Student's t-test; p≤0.05).

each other and from those of *A. mellifera ligustica* (p≤0.05). However, the population of gut bacteria in *A. mellifera ligustica* adults did not change significantly between the two treatments (p≥005) (Fig. 5).

DISCUSSION

Climate warming has been evidenced to impact the ecology, biology and physiology of many animal species, particularly of insect ectotherms (Deutsch et al., 2008; Huey et al., 2009; Aragón et al., 2010; Huey et al., 2012; Walters et al., 2012; Paajimas et al., 2013). Most insect species, including honeybees, harbour a diverse community of symbiotic microbes in their guts, which play an essential role in mediating their body physiology (Round & Mazmanian, 2009; Sommer & Bäckhed, 2013; Hroncova et al., 2015; Raymann et al., 2017). This study assessed how ambient and simulated warmer climatic conditions impacted the brood nest microclimate, maximum thermal thresholds and gut bacterial abundance for two honeybee subspecies i.e. *A. mellifera ligustica* and *A. mellifera sinisxinyuan*.

Results revealed that simulated climate warming significantly impacted the brood nest microclimate (temperature and relative humidity) and the gut bacterial population of both larval and adult bees. Simulated climate warming increased the mean brood nest temperature by about 1.38±1.17 and 0.39±0.14°C of *A. mellifera ligustica* and *A. mellifera sinisxinyuan*, respectively, and reduced the relative humidity inside the brood nest by about 1.62±3.09 and 5.57±1.14%, respectively. Despite the augmentation of ambient temperature under heating treatment, honeybee workers maintained their brood nest temperature in the optimum range of 33-36°C (Silva et al., 2009; Kaftanoglu et al., 2011), most probably due to their fanning and water collection activities (Human et al., 2006). However, the relative humidity decreased from the optimum range suggesting that climate warming directly affected the brood nest microclimatee by decreasing the relative humidity inside the brood nest. Honeybee workers might not be able to regulate the relative humidity inside their nest during climate warming. Several studies have shown that low humidity inside the honeybee nest impacts negatively egg-hatching and brood-survival rates through desiccation of the broods (Ellis et al., 2008; Al-Ghamdi et

al., 2016) and increases the rate of infestation of honeybees by *Varroa* mites (Kraus & Velthuis, 1997). Among high temperature and low humidity occurring during the summer in temperate climate, low relative humidity may be one of the key factors leading to the collapse of honeybee colonies (Cox-Foster et al., 2007; Switanek et al., 2017). Our results showed that the adaptation of a species to environmental change might be linked to the thermal plasticity of its gut microbiota population (Hylander & Repasky, 2019; Raza et al., 2020; Sepulveda & Moeller, 2020).

Simulated climate warming exerted a strong impact on the gut bacterial population of honeybees. Gut bacterial population as assessed through the quantification of 16S *r*RNA gene copy numbers increased under warmer climate conditions (heating treatment) for both larvae and adult honeybee workers. The gut bacterial population of insects is known to be shaped by its host environment (Brune & Friedrich, 2000; Hongoh et al., 2006; Robinson et al., 2010; Colman et al., 2012; Sullam et al., 2012), and these could have considerable implications in improving their host's physical condition (Dillon & Dillon, 2004; Round & Mazmanian, 2009; Lee & Mazmanian, 2010; Hroncova et al., 2015).

A relatively higher number of gut bacteria (16*S r*RNA gene copy numbers) in the indigenous honeybee subspecies (*A. mellifera sinisxinyuan*) than in the cosmopolitan (*A. mellifera ligustica*) one could mean that indigenous honeybee subspecies from temperate climate are more sensitive to climate warming than cosmopolitan ones (Chen et al., 2016). Therefore, this subspecies harbours more bacteria to sustain the environmental stress. Many studies have documented that an increased number of gut bacteria usually helps their host insects to better tolerate the abiotic stress (Rosenberg & Zilber-Rosenberg, 2011; Crotti et al., 2012; Engel & Moran, 2013). Our results corroborate the findings of previous studies demonstrating that elevated temperatures usually increase the gut bacterial community of ectotherms (Lokmer & Wegner, 2015; Kohl & Yahn, 2016) but are not in line with the effect of climate warming on vertebrate ectotherms reported by Bestion et al. (2017).

This preliminary study cannot answer completely how climate warming impacts honeybees. Although there is increasing evidence that there may be connection between the gut microbiota balance and the tolerance of honeybees to high temperature and low humidity stresses, the underlying mechanisms are still not clear. The employment and exploitation of microorganisms in a defined environment could solve such practical problems as colony collapse disorders (CCDs) and abiotic stress under climate warming (Crotti et al., 2012). Thus, symbiotic microorganisms can exert their beneficial contribution to the host to sustain its health and to tolerate biotic and abiotic stress in different ways, i.e. by activation or stimulation of the host innate immune system. However, further *in-vitro* and *in-vivo* research is needed to clarify the molecular mechanisms of insect gut symbiosis and to better understand how the honeybee gut microbial community would impact the learning, physiological and behavioural mechanisms of the host bees under temperature extremes as manifested in Sepulveda & Moeller (2020).

ACKNOWLEDGMENTS

The authors acknowledge the Institute of Apicultural Research (Beijing, China) and the Climate Change Biology Research Group, State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences (Beijing, China) for their cooperation and assistance in ecological experimentation and molecular characterization of honeybee gut microbes, respectively. We are thankful to Prof. Dr. Ma Chun-Sen and Dr. Wei Shi for their valuable remarks and technical comments on the initial draft of the manuscript.

Funding

This study was financially supported by the China Scholarship Council (CSC), by the Agricultural Science and Technology Innovation Program (CAAS-ASTIP-2018-IAR) and by the

Taif University Researchers Supporting Project number (TURSP-2020/92), Taif University, Taif, Saudi Arabia.

REFERENCES

Abou-Shaara, H.F., Al-Ghamdi, A.A., Mohamed, A.A. (2012). Tolerance of two honey bee races to various temperature and relative humidity gradients. *Environmental and Experimental Biology*, *10*(4), 133-138.

Al-Ghamdi, A.A., Alsharhi, M.M., Abou-Shaara, H.F. (2016). Current status of beekeeping in the Arabian countries and urgent needs for its development inferred from a socio-economic analysis. *Asian Journal of Agricultural Research*, *10*, 87-98. https://doi.org/10.3923/ajar.2016.87.98

Aragón, P., Rodríguez, M.A., Olalla-Tárraga, M.A., Lobo, J.M. (2010). Predicted impact of climate change on threatened terrestrial vertebrates in central Spain highlights differences between endotherms and ectotherms. *Animal Conservation*, *13*(4), 363-373. https://doi.org/10.1111/j.1469-1795.2009.00343.x

Becher, M.A., Scharpenberg, H., Moritz, R.F.A. (2009). Pupal developmental temperature and behavioral specialization of honeybee workers (*Apis mellifera* L.). *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology*, *195*(7), 673-679. https://doi.org/10.1007/s00359-009-0442-7

Bestion, E., Jacob, S., Zinger, L., Di Gesu, L., Richard, M., White, J., Cote, J. (2017). Climate warming reduces gut microbiota diversity in a vertebrate ectotherm. *Nature Ecology & Evolution*, *1*(6), pp. 0161. https://doi:10.1038/s41559-017-0161

Bestion, E., Teyssier, A., Richard, M., Clobert, J., Cote, J. (2015). Live fast, die young: experimental evidence of population extinction risk due to climate change. *PLoS Biology*, *13*(10), e1002281. https://doi.org/10.1371/journal.pbio.1002281

Brune, A., & Friedrich, M. (2000). Microecology of the termite gut: structure and function on a microscale. *Current Opinion in Microbiology*, *3*(3), 263-269. https://doi.org/10.1016/s1369-5274(00)00087-4

Chen, C., Liu, Z., Pan, Q., Chen, X., Wang, H., Guo, H., ... Shi, W. (2016). Genomic analyses reveal demographic history and temperate adaptation of the newly discovered honey bee subspecies *Apis mellifera sinisxinyuan* n. ssp. *Molecular Biology and Evolution*, *33*(5), 1337-1348. https://doi.org/10.1093/molbev/msw017

Chevalier, C., Stojanović, O., Colin, D.J., Suarez-Zamorano, N., Tarallo, V., Veyrat-Durebex, C., Montet, X. (2015). Gut microbiota orchestrates energy homeostasis during cold. *Cell*, *163*(6), 1360-1374. https://doi.org/10.1016/j.cell.2015.11.004

Colman, D.R., Toolson, E.C., Takacs-Vesbach, C.D. (2012). Do diet and taxonomy influence insect gut bacterial communities?. *Molecular Ecology*, *21*(20), 5124-5137. https://doi.org/10.1111/j.1365-294x.2012.05752.x

Corn, P.S. (2005). Climate change and amphibians. *Animal Biodiversity and Conservation*, *28*(1), 59-67.

Cox-Foster, D.L., Conlan, S., Holmes, E.C., Palacios, G., Evans, J.D., Moran, N.A., Martinson, V. (2007). A metagenomic survey of microbes in honey bee colony collapse disorder. *Science*, *318*(5848), 283-287. https://doi.org/10.1126/science.1146498

Crotti E., Balloi A., Hamdi C., Sansonno L., Marzorati M., Gonella E. (2012). Microbial symbionts: a resource for the management of insect-related problems. *Microbial Biotechnology*, *5*(3), 307-317. https://doi.org/10.1111/j.1751-7915.2011.00312.x

Deutsch, C.A., Tewksbury, J.J., Huey, R.B., Sheldon, K.S., Ghalambor, C.K., Haak, D.C., Martin, P.R. (2008). Impacts of climate warming on terrestrial ectotherms across latitude. *Proceedings of the National Academy of Sciences*, *105*(18), 6668-6672. https://doi.org/10.1073/pnas.0709472105

Dillon, R.J., & Dillon, V.M. (2004). The gut bacteria of insects: nonpathogenic interactions. *Annual Review of Entomology*, *49*(1), 71-92. https://doi.org/10.1146/

annurev.ento.49.061802.123416

Ellis, M.B., Nicolson, S.W., Crewe, R.M., Dietemann, V. (2008). Hygropreference and brood care in the honeybee (*Apis mellifera*). *Journal of Insect Physiology*, *54*(12), 1516-1521. https://doi.org/10.1016/j.jinsphys.2008.08.011

Engel, P., & Moran, N.A. (2013). The gut microbiota of insects-diversity in structure and function. *FEMS Microbiology Reviews*, *37*(5), 699-735. https://doi.org/10.1111/1574-6976.12025

Groh, C., Tautz, J., Rossler, W. (2004) Synaptic organization in the adult honey-bee brain is influenced by brood-temperature control during pupal development. *Proceedings of the National Academy of Sciences*, *101*(12), 4268-4273. https://doi.org/10.1073/pnas.0400773101

Hongoh, Y., Ekpornprasit, L., Inoue, T., Moriya, S., Trakulnaleamsai, S., Ohkuma, M., Noparatnaraporn, N., Kudo, T. (2006). Intracolony variation of bacterial gut microbiota among castes and ages in the fungus-growing termite *Macrotermes gilvus*. *Molecular Ecology*, *15*(2), 505-516. https://doi.org/10.1111/j.1365-294x.2005.02795.x

Hroncova, Z., Havlik, J., Killer, J., Doskocil, I., Tyl, J., Kamler, M. (2015). Variation in honey bee gut microbial diversity affected by ontogenetic stage, age and geographic location. *PLoS One*, *10*(3), e0118707. https://doi.org/10.1371/journal.pone.0118707

Huey, R.B., Deutsch, C.A., Tewksbury, J.J., Vitt, L.J., Hertz, P.E., Álvarez Pérez, H.J., Garland Jr.,T. (2009). Why tropical forest lizards are vulnerable to climate warming. *Proceedings of the Royal Society B: Biological Sciences*, *276*(1664), 1939-1948. https://doi.org/10.1098/rspb.2008.1957

Huey, R.B., Kearney, M.R., Krockenberger, A., Holtum, J.A., Jess, M., Williams, S.E. (2012). Predicting organismal vulnerability to climate warming: roles of behaviour, physiology and adaptation. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *367*(1596), 1665-1679. https://doi.org/10.1098/rstb.2012.0005

Human, H., Nicolson, S.W., Dietemann, V. (2006). Do honeybees, *Apis mellifera scutellata*, regulate humidity in their nest? *Naturwissenschaften*, *93*(8), 397-401. https://doi.org/10.1007/s00114-006-0117-y

Hylander, B.L., & Repasky, E.A. (2019). Temperature as a modulator of the gut microbiome: what are the implications and opportunities for thermal medicine? *International Journal of Hyperthermia*, *36*(1), 83-89. https://doi.org/10.1080/02656736.2019.1647356

IPCC. Climate Change 2014 (2014) Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, Pachauri, R. K. and Meyer, L. A. (eds)]. IPCC, Geneva, Switzerland, 151 pp. https://doi.org/10.1017/cbo9781107415416

Johnson, R. (2010) Honey bee Colony Collapse Disorder. CRS report for congress. Congressional Research Service, 1-17.

Jones, J.C., & Oldroyd, B.P. (2006). Nest thermoregulation in social insects. *Advances in Insect Physiology*, *33*, 153-191. https://doi.org/10.1016/s0065-2806(06)33003-2

Jones, J.C., Helliwell, P., Beekman, M., Maleszka, R., Oldroyd, B.P. (2005) The effects of rearing temperature on developmental stability and learning and memory in the honey bee, *Apis mellifera*. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology*, *191*(12), 1121-1129. https://doi.org/10.1007/s00359-005-0035-z

Jones, J.C., Myerscough, M.R., Graham, S., Oldroyd, B.P. (2004). Honey bee nest thermoregulation: diversity promotes stability. *Science*, *305*(5682), 402-404. https://doi.org/10.1126/science.1096340

Kaftanoglu, O., Linksvayer, T.A., Page, R.E. (2011). Rearing honey bees, *Apis mellifera*, in vitro I: effects of sugar concentrations on survival and development. *Journal of Insect Science*, *11*(1), 96. https://doi.org/10.1673/031.011.9601

Ken, T., Bock, F., Fuchs, S., Streit, S., Brockmann, A., Tautz, J. (2005) Effects of brood temperature on honey bee *Apis mellifera* wing morphology. *Acta Zoologica Sinica*, *51*(4), 768-771.

Kohl, K.D., & Yahn, J. (2016). Effects of environmental temperature on the gut microbial communities of tadpoles. *Environmental Microbiology*, *18*(5), 1561-1565. https://doi.org/10.1111/1462-2920.13255

Kraus, B., & Velthuis, H.H.W. (1997). High humidity in the honey bee (*Apis mellifera* L.) brood nest limits reproduction of the parasitic mite *Varroa jacobsoni* Oud. *Naturwissenschaften*, *84*(5), 217-218. https://doi.org/10.1007/s001140050382

Lee, Y.K., & Mazmanian, S.K. (2010). Has the microbiota played a critical role in the evolution of the adaptive immune system? *Science*, *330*(6012), 1768-1773. https://doi.org/10.1126/science.1195568

Li, J., Rui, J., Li, Y., Tang, N., Zhan, S., Jiang, J., Li, X. (2020). Ambient temperature alters body size and gut microbiota of *Xenopus tropicalis*. *Science China Life Sciences*, *63*(6), 915-925. https://doi.org/10.1007/s11427-019-9540-y

Lokmer, A., & Wegner, K.M. (2015). Hemolymph micro-biome of Pacific oysters in response to temperature, temperature stress and infection. *The ISME Journal*, *9*(3), 670-682. https://doi.org/10.1038/ismej.2014.160

Mardan, M., & Kevan, P.G. (2002). Critical temperatures for survival of brood and adult workers of the giant honeybee, *Apis dorsata* (Hymenoptera: Apidae). *Apidologie*, *33*(3), 295-302. https://doi.org/10.1051/apido:2002017

McFall-Ngai, M., Heath-Heckman, E.A.C., Gillette, A.A., Peyer, S.M., Harvie, E.A. (2012). The secret languages of coevolved symbiosies: insights from the *Euprymna scolopes-Vibrio fisheri* symbiosis. *Seminars in Immunology*, *24*(1), 3-8. https://doi.org/10.1016/j.smim.2011.11.006

Nazzi, F., Brown, S.P., Annoscia, D., Del Piccolo, F., Di Prisco, G., Varricchio, P. (2012). Synergistic parasite-pathogen interactions mediated by host immunity can drive the collapse of honeybee colonies. *PLoS Pathogens*, *8*, e1002735. https://doi.org/10.1371/journal.ppat.1002735

Neven, L.G. (2000). Physiological responses of insects to heat. *Postharvest Biology and Technology*, *21*(1), 103-111. https://doi.org/10.1016/s0925-5214(00)00169-1

Paaijmans, K.P., Heinig, R.L., Seliga, R.A., Blanford, J.I., Blanford, S., Murdock, C.C., Thomas, M.B. (2013). Temperature variation makes ectotherms more sensitive to climate change. *Global Change Biology*, *19*(8), 2373-2380. https://doi.org/10.1111/gcb.12240

Raymann, K., Shaffer, Z., Moran, N.A. (2017). Antibiotic exposure perturbs the gut microbiota and elevates mortality in honeybees. *PLoS Biology*, *15*(3), e2001861. https://doi.org/10.1371/journal.pbio.2001861

Raza, M.F., Wang, Y., Cai, Z., Bai, S., Yao, Z., Awan, U.A., Zhang, H. (2020). Gut microbiota promotes host resistance to low-temperature stress by stimulating its arginine and proline metabolism pathway in adult *Bactrocera dorsalis*. *PLoS Pathogens*, *16*(4), e1008441. https://doi.org/10.1371/journal.ppat.1008441

Robinson, C.J., Schloss, P., Ramos, Y., Raffa, K., Handelsman, J. (2010). Robustness of the bacterial community in the cabbage white butterfly larval midgut. *Microbial Ecology*, *59*(2), 199-211. https://doi.org/10.1007/s00248-009-9595-8

Rosenberg, E., & Zilber-Rosenberg, I. (2011) Symbiosis and development the hologenome concept. *Birth Defects Research Part C: Embryo Today: Reviews*, 93(1), 56-66. https://doi.org/10.1002/bdrc.20196

Round, J.L., & Mazmanian, S.K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. *Nature Reviews Immunology*, *9*(5), 313-323. https://doi.org/10.1038/nri2515

Ryu, J.H., Kim S.H., Lee H.Y., Bai J.Y., Nam Y.D., Bae

J.W.,... Li, W.J. (2008). Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in *Drosophila*. *Science*, *319*(5864), 777-82. https://doi.org/10.1126/science.1149357

Seeley, T.D. (2014). Honeybee ecology: a study of adaptation in social life. (pp. 71-74). Princeton: Princeton University Press.

Sepulveda, J., & Moeller, A.H. (2020). The effects of temperature on animal gut microbiomes. *Frontiers in Microbiology*, *11*, 384.

Silva, I.C., Message, D., Cruz, C.D., Campos, L.A.O., Sousa-Majer, M.J. (2009). Rearing Africanized honey bee (*Apis mellifera* L.) brood under laboratory conditions. *Genetics and Molecular Research*, *8*(2), 623-629. https://doi.org/10.4238/vol8-2kerr018

Sommer, F., & Bäckhed, F. (2013). The gut microbiota-masters of host development and physiology. *Nature Reviews Microbiology*, *11*(4), 227-238. https://doi.org/10.1038/nrmicro2974

Sullam, K.E., Essinger, S.D., Lozupone, C.A., O'Connor, M.P., Rosen, G.L., Knight, R.O.B., Russell, J.A. (2012). Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. *Molecular Ecology*, *21*(13), 3363-3378. https://doi.org/10.1111/j.1365-294x.2012.05552.x

Switanek, M., Crailsheim, K., Truhetz, H., Brodschneider, R. (2017). Modelling seasonal effects of temperature and precipitation on honey bee winter mortality in a temperate climate. *Science of the Total Environment*, *579*, 1581-1587. https://doi.org/10.1016/j.scitotenv.2016.11.178

Tautz, J., Maier, S., Groh, C., Roessler, W., Brockmann, A. (2003). Behavioral performance in adult honey bees is influenced by the temperature experienced during their pupal development. *Proceedings of the National Academy of Sciences*, *100*(12), 7343-7347. https://doi.org/10.1073/pnas.1232346100

Walters, R.J., Blanckenhorn, W.U., Berger, D. (2012). Forecasting extinction risk of ectotherms under climate warming: an evolutionary perspective. *Functional Ecology*, *26*(6), 1324-1338. https://doi.org/10.1111/j.1365-2435.2012.02045.x

Supplementary Figures:

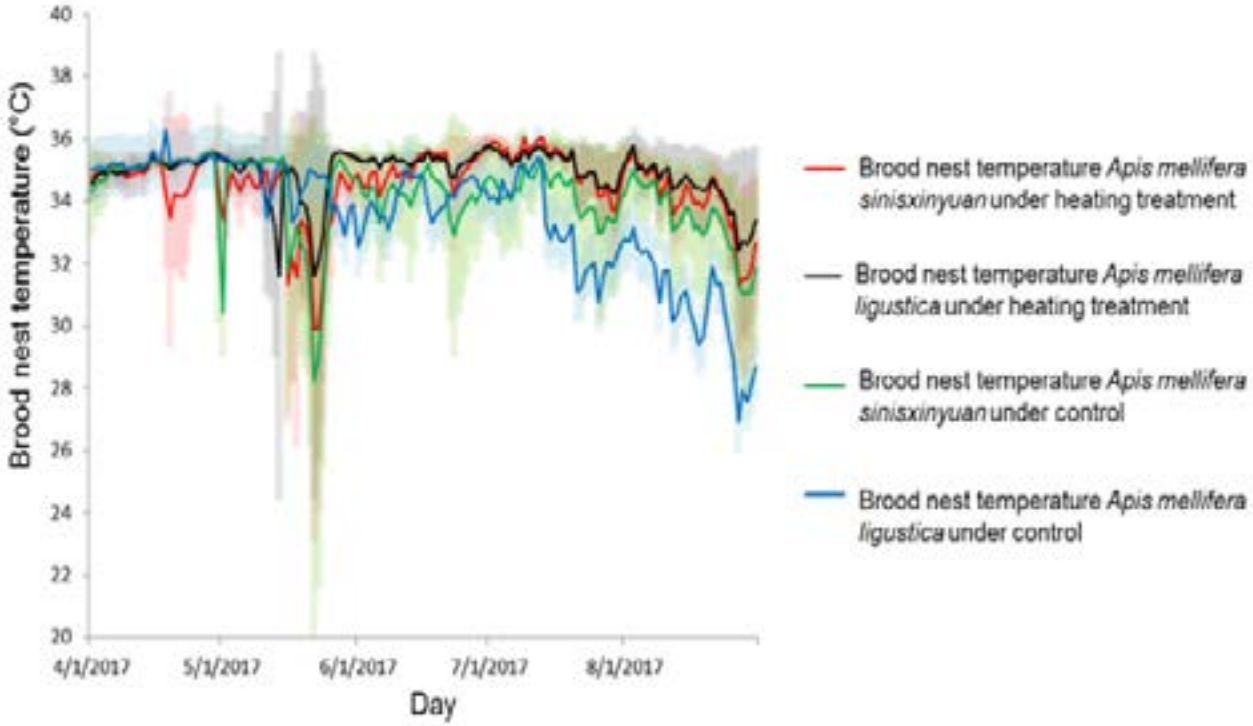


Fig. S1: Daily brood nest temperature of *A. mellifera ligustica* and *A. mellifera sinisxinyuan* during ambient (control treatment) and warmer ambient (heating treatment) climatic conditions from April to August, 2017 (cumulative data for 8 bee hives for each treatment). The shaded area indicates the standard deviation above and below the mean represented by the line of the same colour.

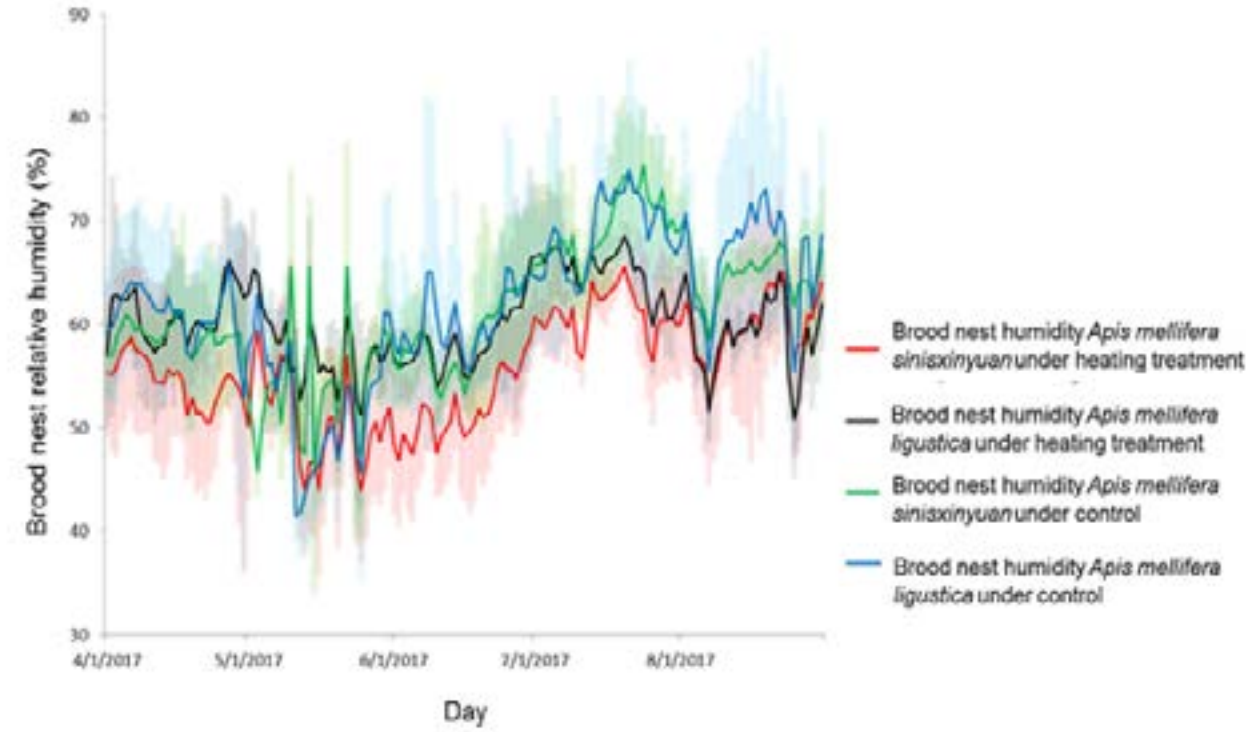


Fig. S2: Daily brood nest relative humidity of *A. mellifera ligustica* and *A. mellifera sinisxinyuan* recorded during ambient (control treatment) and warmer ambient (heating treatment) climatic conditions from April to August, 2017 (cumulative data for 8 bee hives for each treatment). The shaded area indicates the standard deviation above and below the mean represented by the line of the same colour.