Title: Integrated effects of thermal acclimation and challenge temperature on cellular immunity in the plusiine moth larvae *Chrysodeixis eriosoma* (Lepidoptera: Noctuidae)

Running Heads: Temperature on cellular immunity

Authors

Yumi Takano[1], Takuma Sakamoto[1], Hiroko Tabunoki[1]*, Jin Yoshimura[2,3,4,5,6], Kikuo Iwabuchi[1]

Affiliations

[1] Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509, Japan

[2] Department of Mathematical and Systems Engineering, Shizuoka University, Hamamatsu, 432-8561, Japan

[3] Department of International Health and Medical Anthropology, Institute of Tropical Medicine, Nagasaki University, Nagasaki 852-8523, Japan

[4] Department of Biological Sciences, Tokyo Metropolitan University, Hachioji, Tokyo 192-0397, Japan

[5] The University Museum, University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan

[6] Marine Biosystems Research Center, Chiba University, Kamogawa, Chiba 299-5502, Japan

*Correspondence:

Hiroko Tabunoki

Affiliation: Faculty of Agriculture, Tokyo University of Agriculture and Technology

email: h_tabuno@cc.tuat.ac.jp

postal address: Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509, Japan

Abstract. Temperature is one of the most influential factors for animals. The acclimation (rearing) and challenge temperatures are often more important than the given temperature per se. These effects on physiological responses has been known, but not well understood on immune responses. Here, we investigated the integrated effects of rearing and challenge temperatures on hemocyte populations in larvae of a plusiine moth, *Chrysodeixis eriosoma*. We hypothesize that the hemocyte concentration is decreased (increased) at higher (lower) temperatures from rearing temperatures and that the proportions of hemocyte types exhibit directional changes at higher (lower) temperatures to compensate for immune reactions. We expect that increasing (decreasing) the challenge temperature from the rearing temperature enhances (reduces) phagocytic activity. We found that higher temperatures slightly decreased the hemocyte concentration. We detected small changes in the proportions of hemocyte types among rearing temperatures, but the changes were non-directional and most of them were statistically insignificant. We also found the integrated effects only with increases in the challenge temperatures, which resulted in increased phagocytosis, whereas no apparent reactions were detected with decreases in the challenge temperatures. Our results show that the hemocyte concentration is significantly affected by the rearing temperature, which implies that hematopoiesis depends on the ambient temperature. We discuss some adaptive and non-adaptive components for the positive integrated effects of increases in the challenge temperatures. We also discussed the obtained non-responsiveness in the integrated effects with decreases in the challenge temperatures.

Keywords. Innate immunity, temperature, hemocyte, Lepidoptera, *Chrysodeixis eriosoma*

Introduction

The ambient temperature generally affects ectotherms (e.g., reptiles, fishes, amphibians, molluscs and insects) more than endotherms (e.g., humans, other mammals and avians) (Angilletta, 2009; Martin et al., 2010). In particular, small terrestrial ectotherms (e.g., worms and insects) are closely affected by the ambient temperature (Stevenson, 1985; Deutsch et al., 2008; Paaijmans et al., 2013).

In insects, the ambient temperature not only affects physiological responses and metabolic profiles but also profoundly affects the overall resistance of the hosts to pathogens (Blanford and Thomas, 1999; Inglis et al., 1996) and parasitoids (Adamo, 1998; Geden, 1997; Hance et al., 2007). The insect immune system comprises humoral and cellular defence responses (Beckage, 2008): the humoral immune response is based on the products of immune genes, such as antimicrobial peptides and the prophenoloxidase (PO)-activating system, and the cellular immune response is performed by hemocytes that exhibit phagocytosis, nodule formation and encapsulation. In lepidopteran larvae, five types of hemocytes have been discovered: prohemocytes, granulocytes, plasmatocytes, spherulocytes and oenocytoids (Wigglesworth, 1972). Oenocytoids synthesize prophenoloxidase, and granulocytes and plasmatocytes exhibit phagocytosis, nodule formation and encapsulation (Lavine & Strand, 2002; Jiang et al., 2010). The cellular immune system is affected by the ambient temperature. For example, in *Protopulvinaria pyriformis*, the encapsulation of parasitoid eggs is correlated with temperature (Blumberg, 1991), but in *Anopheles stephensi*, phagocytic activity does not simply scale with temperature (Murdock et al., 2012).

The effects of temperature on physiological events appear to be very complex, and past and current temperatures exert separate as well as integrated effects (Huey et al., 1999; Angilletta, 2009). The thermal history (i.e., acclimatization and hardening) also influences various phenotypic responses, such as the oxygen consumption rate, locomotor performance, sweat gland functions, and seasonal morph production, in endothermal and ectothermal organisms (Parsons, 2001; Prudic et al., 2011; Sato et al., 1990; Brakefield et al., 1998; Sgro et al., 2016; Demas & Carlton, 2015). However, in most experiments investigating the effects of the challenge temperature, the effects of a high or low temperature were the main objective, and the effects of increasing/decreasing the challenge temperature compared with the rearing temperatures are not considered.

In insects, the acclimation temperature is also an important factor, and a shift in temperature is

often more important than the given temperature per se. That is, the activity level at a given temperature depends on the temperature at which the organism was maintained during the previous period. This effect has well been described with respect to physiological responses such as locomotion activity and oxygen uptake (Cossins & Bowler, 1987; Wigglesworth, 1972). The immune functions of insects could also be affected by both the current temperature and the previous temperature (thermal acclimation) (Angilletta, 2009; Blumberg, 1991; Catalan et al., 2012; Triggs & Knell, 2012; Ferguson et al., 2016; Murdock et al., 2012). In butterflies, a higher rearing temperature during the larval stage (i.e., green-veined white butterfly *Pieris napi*) or heat treatment after adult emergence (i.e., mycalesine butterfly *Bicyclus anynana*) decrease the hemocyte numbers at the adult stage (Bauerfeind & Fischer, 2014; Karl et al., 2011). In the deer fly *Lipoptena cervi*, the early life temperature modifies adult immunity, and 3 days of exposure to low temperatures during pupal diapause exerts a favourable effect on the encapsulation activity of adults that emerge 3 months after treatment (Kaunisto et al., 2015). However, the combined effects of these temperatures, such as the effect of the acclimation temperature on immune responses, are poorly understood.

The previous studies imply the following immunological reaction in thermal changes (Cossins and Bowler, 1987). During acclimation, organisms should adapt to get the best metabolism at acclimation temperature by compensation of energy metabolism. The physiological performance of the higher temperature acclimated organisms should be depressed at lower temperatures more than those expected for non-acclimated ones. Conversely, the opposite should occur at higher temperatures in lower temperature acclimated organisms. Therefore, we propose the following two hypotheses: (1) increasing the challenge temperature from the previous (rearing) temperature enhances phagocytic activity; and (2) decreasing the challenge temperature from the previous temperature reduces phagocytic activity. Here, we examined the combined effects of the challenge immune temperature and thermal acclimation (rearing temperature) on the cellular immune function of lepidopteran larvae (plusiine moth, *Chrysodeixis eriosoma*). For that purpose, the hemocyte concentrations (total number of hemocytes), differential hemocyte counts and phagocytic activities at different temperatures were examined.

Materials and Methods

Insects

A laboratory stock (ca. 20-40 adults at the adult stage) of *Chrysodeixis eriosoma* (Lepidoptera,

Noctuidae) three to four generations after field collections was used for the experiments. The stock originated from adults and larvae collected on August and September 2014 in Tokyo, Japan. The adults and larvae were maintained at 25°C under a 16 L:8 D photoperiodic regime according to the method described by Nishikawa et al. (2013). The adults were fed a 10% sugar solution. The eggs were collected daily, and the larvae were reared on an artificial diet (Kawasaki et al., 1987). For each test, larvae were randomly selected from the laboratory stock. For the acclimation experiments, *C. eriosoma* eggs collected from the stock (which originated from various females) were randomly assigned to one of five temperature regimes (18, 20, 25, 30, and 32°C) on the first day after oviposition and reared individually at these temperatures.

Hemocyte concentration and differential hemocyte counts

Hemolymph was collected by piercing the second proleg of the host larvae with a microneedle on day 3 of the 6th (final) instar. The hemolymph was bled onto parafilm placed on ice and then transferred to a Thoma hemocytometer. The hemocytes were counted under a light microscope, and the hemocyte concentration is expressed as the number of cells per mm^3 of hemolymph. To obtain the differential hemocyte counts (DHCs), the hemolymph was directly bled onto a glass slide and stained with a mixed solution of brilliant cresyl blue/Sudan III (Kurihara et al., 1992). Similar to the results obtained with most other lepidopteran insects, the hemocytes of *C. eriosoma* larvae consist of a complex (combination) of five types of mesodermal cells: granulocytes, plasmatocytes, oenocytoids, spherulocytes and prohemocytes (Nishikawa, et al., 2013). From each sample, 100 hemocytes were identified under a phase-contrast microscope.

Hemocyte response to foreign objects

Polystyrene microbeads (Polybead® Polystyrene Dyed Red 10 Micron Microspheres – Polysciences Inc.) suspended in phosphate buffered saline (PBS) (9.78 M K_2HPO_4, 7.44 M KH_2PO_4, and 0.44% glucose, pH 6.8) were used as the foreign objects. First, 50 µl of PBS with microbeads (1.0×10^6 beads/ml) was injected into the proleg of 3-day-old 6th-instar larvae that were reared at various temperatures (18, 20, 25, 30, and 32°C) using a hand-pulled 10-µl Drummond microdispenser (Drummond Scientific, Broomall, PA, USA). Immediately after immune challenge by bead injection, the larvae were transferred to different temperature conditions (18, 20, 25, 30, and 32°C). Preliminary experiments showed that phagocytosis started at 30 min and stopped within 2 hours after injection. Therefore, at 2 hours post-immune challenge, hemolymph was dropped into physiological saline, and the number of completely phagocytosed and non-phagocytosed beads was counted under a phase-contrast microscope

until a total of 50 beads were counted.

Statistical analysis

The statistical analyses of the data were conducted using the Kruskal-Wallis test with Dunn's multiple comparison test.

Results

We first examined the effects of the rearing temperature (during embryonic and postembryonic development) on the number of hemocytes. The rearing temperature markedly affected the hemocyte concentration (number of hemocytes per ml) of the 6 th-instar *C. eriosoma* larvae (Fig. 1A, Table S1). The hemocyte concentrations obtained with rearing temperatures of 18°C, 20°C and 25°C were significantly different from those obtained with rearing temperatures of 30°C and 32°C (Table S6). The highest concentration of hemocytes was found in the larvae reared at the lower temperatures, particularly at 18°C, and the hemocyte concentration decreased with increases in the rearing temperatures until half of the highest concentration was obtained with the highest rearing temperature (32°C). The analysis of the DHCs revealed that the rearing temperature did not affect the counts of all hemocyte types except oenocytoids (Fig. 1B, Table S2), and significant but non-directional differences in the oenocytoid counts were detected (Table S6).

We then examined the integrated effects of several combinations of rearing temperatures and immune challenge temperatures on phagocytic activities. The phagocytic activity was tested by microbead injection into final-instar *C. eriosoma* larvae. We first measured the effects of the rearing temperature alone on phagocytosis by maintaining the challenge temperature identical to the rearing temperature (Fig. 2A, Tables S3, S7). The effects of the rearing temperatures were as follows. At moderate and high rearing temperatures (20 to 32°C), approximately 50% of the microbeads were phagocytosed, indicating no difference in phagocytic activity. In contrast, a rearing temperature of 18°C decreased the percentage phagocytosis to only 23% (Fig. 2A). We then set the rearing temperature to 25°C and tested the effects of the challenge temperature (Fig. 2B, Tables S4, S7). The results showed that the percentage of phagocytosed microbeads was higher at high temperatures (30 and 32°C) than at moderate and lower temperatures (18, 20 and 25°C) (Fig. 2B). Significant differences in phagocytic activity were not observed among the various temperature groups (30-32 and 18-25°C). Thus, phagocytosis increased with increases in the challenge temperatures (30 and 32°C), whereas no significant changes (increase or

decrease) were detected with a lower and unchanged challenge temperature (challenge temperature of 18-25°C and rearing temperature of 25°C). We subsequently tested the effects of thermal acclimation ('past' rearing temperature) on immune function by maintaining the immune challenge temperature at 25°C (Fig. 2C, Tables S5, S7). The percentage of phagocytosis at 18°C and 20°C was higher than 50%, whereas slightly decreased phagocytosis was obtained with a challenge temperature of 25-32°C (Fig. 2C). These results indicated that insects that were transferred from lower rearing temperatures after bead injection (18 and 20°C) exhibited higher phagocytic activity than those that were reared at moderate and higher temperatures (25 to 32°C) (Fig. 2C). However, significant differences in phagocytic activity were not found between the two temperature groups (18-20 and 25-32°C) (Table S7).

Based on these results, we found that the hemocytes of larvae that were transferred from their rearing temperature to a higher immune challenge temperature exhibited enhanced phagocytic activity. Unexpectedly, however, no enhancement in phagocytic activity was observed with the larvae transferred from their rearing temperature to the same or a lower immune challenge temperature (Fig. 3).

Discussion

Immunological activity represents a type of animal performance and is also affected by the ambient temperature (Mondal & Rai, 2001; Hung et al., 1997). In general, each animal species has an optimal temperature at which their performance level is maintained (Cossines & Bowler, 1987). The effect of temperature on cellular immune function is observed at the cellular level, and it is expected that the most effective cellular immune responses occur at the optimal temperature (Mondal & Rai, 2001). The current results indicate that the optimal temperature range for *C. eriosoma* larvae is 20-32°C because rearing difficulties are observed at temperature higher than 32°C.

The rearing temperature affects the hemocyte concentration of lepidopteran larvae (plusiine moth, *Chrysodeixis eriosoma*). As might be expected, the hemocyte concentration obtained at higher temperatures (30 and 32°C) was lower than that obtained with lower temperatures (18, 20 and 25°C) (Fig. 1A). This result is consistent with previous studies (Bauerfeind & Fischer, 2014; Karl et al., 2011), and increased hemocyte concentrations at low temperatures might be commonly found in lepidopteran insects. In contrast, unexpectedly, the rearing temperature had no effect on the DHCs (proportions of hemocyte types) (Fig. 1B). Thus, the hematopoiesis activity of *C. eriosoma* might be regulated by temperature, probably due to long-term thermal

247 acclimation during embryonic and postembryonic development, without changes in the
248 proportion of hemocyte types. In humans, hyperthermia increases the number of hematopoietic
249 stem cells (Capitano et al., 2012), and the mechanism of increased hematopoiesis activity at
250 lower temperatures has not been studied in insects. The current results (increased hemocyte
251 concentration at lower temperatures) might imply that hematopoiesis is increased at lower
252 temperatures in this insect species (Fig. 1A). The present study also showed that the phagocytic
253 activity of *C. eriosoma* was retained under various temperatures higher than 20°C but was
254 greatly decreased at the lower temperature of 18°C (Fig. 2A). The cellular immune response
255 was markedly affected by past-experienced temperatures. The phagocytic activity of larvae
256 reared at 25°C was greatly increased by transferring the larvae to higher temperatures (30 and
257 32°C) after immune challenge (Fig. 2B). Enhancements in function in response to a
258 temperature increase should be beneficial for maintaining resistance against parasitoids.
259 Successful parasitization is lower at higher temperatures because the eggs and larvae of
260 parasitoids are more often killed by encapsulation (Thomas & Blanford, 2003; Fellowes et al.,
261 1999; Hance et al., 2007). Moreover, the encapsulation ability of hemocytes might compensate
262 for the disadvantage of the lower concentration of hemocytes in larvae that develop at higher
263 temperatures (Fig. 1). In addition, the increased hemocyte concentration observed with the low
264 temperature of 18°C might represent adaptive compensation to the decreased immune activity
265 observed at lower temperatures. Increased immunity induced by exposure to cold temperatures
266 is thought to be an adaptive response to cold-associated pathogen stress (Sinclair et al., 2013).
267 Notably, no obvious change in phagocytic activity was observed in the larvae transferred to
268 lower temperatures (18 and 20°C). Thus, the phagocytic activity might be stable at lower
269 temperatures and at a decreased temperature. In contrast, the analysis of larvae reared at
270 different temperatures and transferred to 25°C after immune challenge revealed that the
271 phagocytic activity was markedly increased in the larvae reared at 18 and 20°C (Fig. 2C). In this
272 case, no noticeable change was observed in the larvae transferred from higher temperatures (30
273 and 32°C). Additionally, in this experiment, stable phagocytic activity was found at challenge
274 temperatures lower than the rearing temperatures. Thus, our hypothesis that a challenge
275 temperature higher than the previous (rearing) temperature enhances phagocytic activity was
276 thus confirmed. However, unexpectedly, a challenge temperature (25°C) lower than the previous
277 (rearing) temperature (30 and 32°C) does not decrease the phagocytic activity.
278
279 This study implies that thermal acclimation might increase the basal level of immune activity at
280 temperatures that are higher than the previous temperatures. In general, the behavioral activity
281 of insects is increased by increasing temperature, in which the increased behavioral activity is
282 mediated by the elevation of neuronal activity and is dependent on the rate of increase of

temperature (Morrissey and Edwards, 1979; Abrams and Pearson, 1982; Inan et al., 2011). The increase in hemocyte function observed in response to increasing temperatures is thought to be an adaptive response to natural enemies such as parasitoids whose performance is supposed to be increased with increasing temperatures. However, the effect of increase in temperature on parasitoid performance is not known (Thomas & Blanford, 2003; Hance et al., 2007). In addition, acute elevation of temperature increases the resistance of insects to pathogens, probably due to immune-neural connection, although whether the increase in temperature, but not high temperature, is indeed effective or not remains unknown (Moore, 2002). Therefore, whether the acclimation responses of the phagocytic activity of lepidopteran larvae actually enhance the fitness or are merely unavoidable consequences of temperature changes remains to be determined.

We identified the effect of increasing immune challenge temperatures on the immune responses (Fig. 3). This finding supported our proposed hypothesis regarding increasing temperatures. However, decreases in temperatures exerted no noticeable changes in the immune responses. This finding rejects the second proposed hypothesis regarding decreases in temperature. The lack of effect observed with decreasing temperature is a very interesting question from the physiological (functional, mechanistic) and adaptive perspectives. Although we currently have no related information, we suspect that it might be more prevalent in endotherms in which thermal homeostasis is important (Cossins and Bowler, 1987). As might be expected, an immune challenge temperature higher than the acclimation temperature induces an increase in phagocytic activity. Thus, the phagocytic activity of *C. eriosoma* larvae is enhanced by an increase in temperature but not by a high temperature per se (Figs. 2B, 2C and 3). At the cellular level, temperature increase would positively affect the metabolism rate (Schulte, 2015). However, further studies are needed to elucidate the molecular mechanism and the ecological significance of the enhancement in immune activity caused by an increase in temperature. Furthermore, whether this phenomenon is commonly involved in the immune system of ectotherms and endotherms and whether the effect is observed in both the innate and acquired immune systems remain to be resolved. In general, functionally transient receptor potential (TRP) channels are crucial for sensing the rate of temperature increase (Kwon et al., 2008; Luo et al., 2017). TRP channels in vertebrates and invertebrates are distributed in neurons as well as immunocytes (Dhaka et al., 2006; Saito & Tominaga, 2015; Wei et al., 2015; Kashio et al., 2012). TRP channels might explain the mechanisms underlying these integrated effects of temperatures on cellular immunity.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Hemocyte concentration of the last instar larva reared at various temperatures. (related to Fig. 1A)

Table S2. Differential hemocyte counts of the last instar larva reared at various temperatures. (related to Fig. 1B)

Table S3. Effect of temperature through the entire period on phagocytic activity. (related Fig. 2A)

Table S4. Effect of temperature at immune challenge on phagocytic activity. (related Fig. 2B)

Table S5. Effect of rearing temperature on phagocytic activity. (related Fig. 2C)

Table S6. Statistical Inferences for Figure 1. Statistical test using Kruskal-Wallis test with Dunn's multiple comparison test for hemocyte number or ratio.

Table S7. Statistical Inferences for Figure 2. Statistical test using Kruskal-Wallis test with Dunn's multiple comparison test for phagocytic rate.

Acknowledgements

We thank Shin Goto, the handling editor, for his invaluable comments that almost deserve coauthorship if not the editor. This work was supported by JSPS KAKENHI (Grant Numbers: 20K20571 to HT, 26257405 and 15H04420 to J. Y. and 15H02483 to K.I.).

Authors' contributions:

Y. T. and K. I. designed and performed the experiments, T. S. and K. I. performed the statistical analyses, J. Y. and H. T. provided conceptual insights, and Y. T., J. Y., H. T. and K. I. wrote the manuscript. All the authors agree to be held accountable for the content in the manuscript and have approved the final version of the manuscript.

Competing interests:

We have no competing interests.

Data Availability Statement

The data that supports the findings of this study are available in the supplementary material of this article.

355

356 **References**

357 Abrams, T.W. & Pearson, K.G. (1982) Effects of temperature on identified central neurons that
358 control jumping in the grasshopper. *The Journal of Neuroscience* **2**, 1538-1553.
359 Adamo, S.A. (1998) The specificity of behavioral fever in the cricket Acheta domesticus.
360 *Journal of Parasitology* **84**, 529-533.
361 Angilletta, M.J.Jr. (2009) Thermal adaptation: a theoretical and empirical synthesis. Oxford
362 University Press, New York.
363 Bauerfeind, S.S. & Fischer, K. (2014) Integrating temperature and nutrition – Environmental
364 impacts on an insect immune system. *Journal of Insect Physiology* **64**, 14-20.
365 Beckage, N.E. (2008) Insect Immunology. Elsevier
366 Blanford, S. & Thomas, M.B. (1999) Host thermal biology: the key to understanding insect-
367 pathogen interactions and microbial pest control? *Agricultural and Forest Entomology* **1**,
368 195-202.
369 Blumberg, D. (1991) Seasonal variations in the encapsulation of eggs of the encyrtid parasitoid
370 *Metaphycus stanleyi* by the pyriform scale, *Protopulvinaria pyriformis*. *Entomologia*
371 *Experimentalis et Applicata* **58**, 231-237.
372 Brakefield, P.M., Kesbeke, F. & Koch, P.B. (1998) The regulation of phenotypic plasticity of
373 eyespots in the butterfly Bicyclus anynana. *The American Naturalist* **152**, 853-860.
374 Capitano, M.L., Nemeth, M.J., Mace, T.A., Salisbury-Ruf, C., Segal, B.H., McCarthy, P.L. &
375 Repasky, E.A. (2012) Elevating body temperature enhances hematopoiesis and neutrophil
376 recovery following total body irradiation in an IL-1, IL-17, and G-CSF-dependent manner.
377 *Blood* **120**, 2600-2609.
378 Catalan, T.P., Wozniak, A., Niemeyer, H.M., Kalergis, A.M. & Bozinovic, F. (2012) Interplay
379 between thermal and immune ecology: Effect of environmental temperature on insect
380 immune response and energetic costs after an immune challenge. *Journal of Insect*
381 *Physiology* **58**, 310-317.
382 Cossins, A.R. & Bowler, K. (1987) Temperature Biology of Animals, Chapmann and Hall
383 Demas, G.E. & Carlton, E.D. (2015) Ecoimmunology for psychoneuroimmunologists:
384 Considering context in neuroendocrine-immune-behavior interactions. *Brain, Behavior, and*
385 *Immunity* **44**, 9-16.
386 Deutsch, C.A., Tewksbury, J.J., Huey, R.B., Sheldon, K.S., Ghalambor, C.K., Haak, D.C. &
387 Martin, P.R. (2008) Impacts of climate warming on terrestrial ectotherms across latitude.
388 *Proceedings of the National Academy of Sciences USA* **105**, 6668-6672.
389 Dhaka, A., Viswanath, V. & Patapoutian, A. (2006) Trp ion channels and temperature sensation.
390 *Ann Rev Neurosci* **29**, 135-161.
391 Fellowes, M. D. E., Kraaijeveld, A. R. & Godfray, H. C. J. (1999) Cross-resistance following

392 artificial selection for increased defense against parasitoids in *Drosophila melanogaster*.
393 *Evolution* **53**, 966-972.
394 Ferguson, L.V., Heinrichs, D.E. & Sinclair, B.J. (2016) Paradoxical acclimation responses in the
395 thermal performance of insect immunity. *Oecologia* **181**, 77-85.
396 Geden, C.J. (1997) Developmental models for the filth fly parasitoids Spalangia gemina, S.
397 cameroni, and Muscidifurax vaptor (Hymenoptera: Pteromalidae) under constant and variable
398 temperatures. *Biological Control* **9**, 185-192.
399 Hance, T., van Baaren, J., Vernon, P. & Boivin, G. (2007) Impact of extreme temperatures on
400 parasitoids in a climate change perspective. *Annual Review of Entomology* **52**, 107-126.
401 Huey, R.B., Berrigan, D., Gilchrist, G.W. & Herron, J.C. (1999) Testing the adaptive
402 significance of acclimation: a strong inference approach. *American Zoologist* **39**, 323-336.
403 Hung, H.W., Lo, C.F., Tseng, C.C. & Kou, G.H. (1997) Antibody production in Japanese eel,
404 Anguilla japonica Temminck & Schlegel. *Journal of Fish Diseases* **20**, 195-200.
405 Inan, O.T., Marcu, O., Sanchez, M.E., Bhattacharya, S. & Kovacs, G.T.A. (2011) A portable
406 system for monitoring the behavioral activity of *Drosophila*. *Journal of Neuroscience*
407 *Methods* **202**, 45-52.
408 Inglis, D.G., Johnson, D.L. & Goettel, M.S. (1996) Effects of temperature and thermoregulation
409 on mycosis by Beauveria bassiana in grasshoppers. *Biological Control* **7**, 131-139.
410 Jiang, H., Vilcinskas, A. & Kanost, R. (2010) Immunity in lepidopteran insects. In: Söderhäll,
411 K. ed., Invertebrate Immunity. Springer, p.181-204.
412 Karl, I., Stoks, R., De Block, M., Janowitz, S.A. & Fischer, K. (2011) Temperature extremes and
413 butterfly fitness: conflicting evidence from life history and immune function. *Global Change*
414 *Biology* **17**, 676-687.
415 Kashio, M., Sokabe, T., Shintaku, K., Uematsu, T., Fukuta, N., Kobayashi, N., Mori, Y. &
416 Tominaga, M. (2012) Redox signal-mediated sensitization of transient receptor potential
417 melastatin 2 (TRPM2) to temperature affects macrophage functions. *Proceedings of the*
418 *National Academy of Sciences of the United States of America* **109**, 6745-6750.
419 Kaunist, S., Harkonen, L., Rantala, M.J. & Kortet, R. (2015) Early-life temperature modifies
420 adult encapsulation response in an invasive ectoparasite. *Parasitology* **142**, 1290-1296.
421 Kawasaki, K., Ikeuchi, M. & Hidaka, T. (1987) Laboratory rearing method for Acanthoplusia agnata
422 (Lepidoptera: Noctuidae) without change of artificial diet. *Japanese Journal of Applied*
423 *Entomology and Zoology* **31**, 78–80.
424 Kurihara, Y., Simazu, T. & Wago, H. (1992) Classification of hemocytes in the common
425 cutworm, *Spodoptera litura* (Lepidoptera: Noctuidae) II. Possible roles of granular
426 plasmatocytes and oenocytoids in the cellular defense reactions. *Applied Entomology and*
427 *Zoology* **27,** 237-242.

Kwon, Y., Shim, H-S., Wang, X. & Montell, C. (2008) Control of thermotactic behavior via coupling of a TRP channel to a phospholipase C signaling cascade. *Nature Neuroscience* **11**, 871-873.

Lavine M.D. & Strand M.R. (2002) Insect hemocytes and their role in immunity. *Insect Biochemistry and Molecular Biology* **32**, 1295-1309.

Luo, J., Shen, W.L. & Montell, C. (2017) TRPA1 mediates sensation of the rate of temperature change in *Drosophila* larvae. *Nature Neuroscience* **20**, 34-41.

Martin, L.B., Hopkins, W.A., Mydlarz, L.D. & Rohr, J.R. (2010) The effects of anthropogenic global changes on immune functions and disease resistance. *Annals of the New York Academy of Sciences* **1195**, 129-148.

Mondal, S. & Rai, U. (2001) In vitro effect of temperature on phagocytic and cytotoxic activities of splenic phagocytes of the wall lizard, Hemidactylus flavividis. *Comparative Biochemistry and Physiology A* **129**, 391-398.

Moore, J. (2002) Parasites and behavior of animals. Oxford University Press, New York.

Morrissey, R. & Edwards, J.S. (1979) Neural function in an alpine grylloblattid: A comparison with the house cricket, *Acheta domesticus*. *Physiological Entomology* **4**, 241-250.

Murdock, C.C., Paaijmans, K.P., Bell, A.S., King, J.G., Hillyer, J.F., Read, A.F. & Thomas, M.B. (2012) Complex effects of temperature on mosquito immune function. *Proceedings of Royal Society B* **279**, 3357-3366.

Nishikawa, H., Yoshimura, J. & Iwabuchi, K. (2013) Sex differences in the protection of host immune systems by a polyembryonic parasitoid. *Biology Letters* **9,** 20130839.

Paaijmans, K.P., Heinig, R.L., Seliga, A., Blanford, J.I., Blanford, S., Murdock, C. & Thomas, M.B. (2013) Temperature variation makes ectotherms more sensitive to climate change. *Global Change Biology* **19**, 2373-2380.

Parsons, K. (2001) Human Thermal Environments: The Effects of Hot, Moderate, and Cold Environments on Human Health, Comfort, and Performance, Second Edition, CRC Press

Prudic, K.L., Jeon, C., Cao, H. & Monteiro, A. (2011) Developmental plasticity in sexual roles of butterfly species drives mutual sexual ornamentation. *Science* **331**, 73-75.

Saito, S. & Tominaga, M. (2015) Functional diversity and evolutionary dynamics of thermosTRP channels. *Cell Calcium* **57**, 214-221.

Sato, F., Owen, M., Matters, R., Sato, K. & Gisolfi, C.V. (1990) Functional and morphological changes in the eccrine sweat gland with heat acclimation. *Journal of Applied Physiology* **69**, 232-236.

Schulte, P.M. (2015) The effects of temperature on aerobic metabolism: towards a mechanistic understanding of the responses of ectotherms to a changing environment. *The Journal of Experimental Biology* **218**, 1856-1866.

464 Sgro, C.M., Terblanche, J.S. & Hoffmann, A.A. (2016) What can plasticity contribute to insect
465 responses to climate change? *Annual Review of Entomology* **61**, 433-451.
466 Sinclair, B.J., Ferguson, L.V., Salehipour-shirazi, G. & MacMillan, H.A. (2013) Cross-tolerance
467 and cross-talk in the cold: relating low temperatures to desiccation and immune stress in
468 insects. *Integrative and Comparative Comp Biology* **53**, 545–556.
469 Stevenson, R.D. (1985) Body size and limits to the daily range of body temperature on
470 terrestrial ectotherms. *American Naturalist* **125**, 102-117.
471 Thomas, M. B. & Blanford, S. (2003) Thermal biology in insect-parasite interactions.
472 *Trends in Ecology and Evolution* **18**, 344-350.
473 Triggs, A. & Knell, R.J. (2012) Interactions between environmental variables determine
474 immunity in the Indian meal moth *Plodia interpunctella*. *Journal of Animal Ecology* **81**, 386–
475 394.
476 Wei, J.J., Fu, T., Yang, T., Liu, Y. & Wang, G.R. (2015) A TRPA1 channel that senses thermal
477 stimulus and irritating chemicals in *Helicoverpa armigera*. *Insect Molecular Biology* **24**,
478 412–421.
479 Wigglesworth, F.R.S. (1972) The principles of insect physiology. 7th edition, Chapman and
480 Hall.
481
482
483

Figure Legends

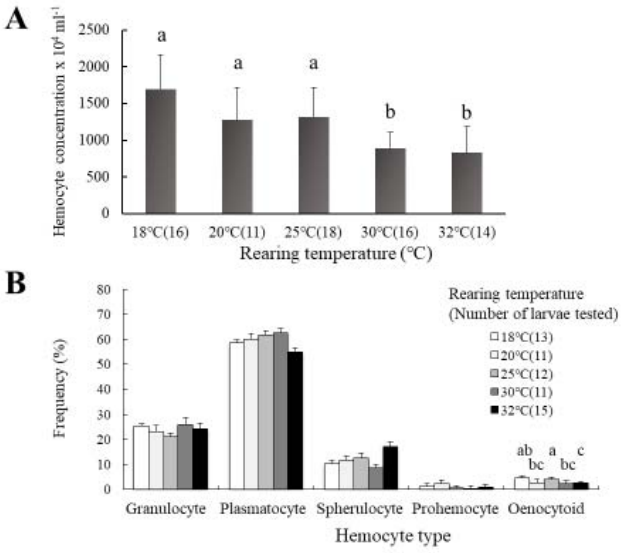

Fig. 1. Effects of the rearing temperature on hemocytes of last-instar larvae. Hemocyte concentration (**A**) and differential hemocyte count (**B**). The columns represent the means ± SDs (A) and ± SEs (B) of individual counts. Means with different letters indicate significant differences between treatments ($p < 0.05$, Kruskal-Wallis test with Dunn's multiple comparison test). The numbers in parentheses are the sample sizes. Note that no significant differences in the frequencies of granulocytes, plasmatocytes, spherulocytes and prohemocytes were found, and significant non-directional differences in the frequency of oenocytoids were detected. See Supplementary Tables S1, S2 and S6 for the data and their statistics.

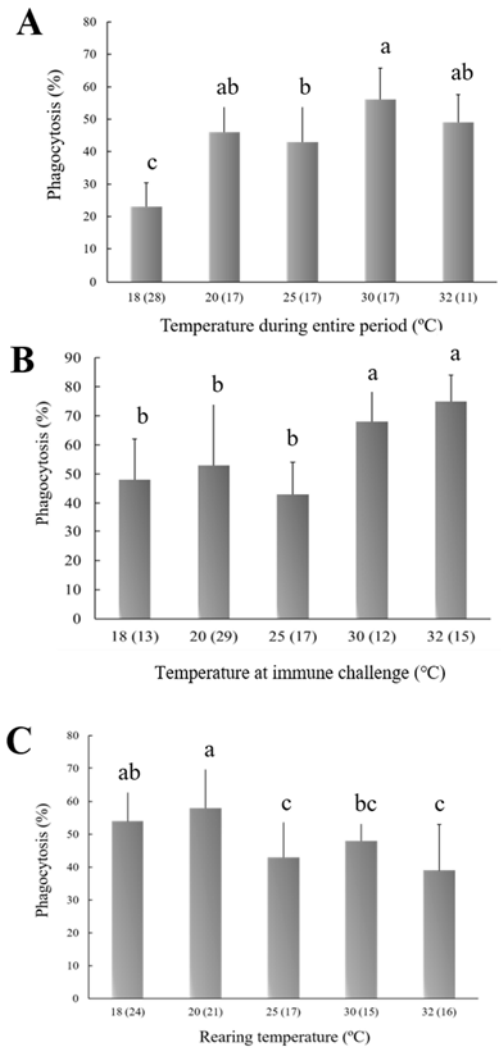

Fig. 2. Phagocytic activity to the injected microbeads in final-instar larvae exposed to various combinations of rearing and immune challenge temperatures. (**A**) The immune challenge temperature was identical to the rearing temperature. (**B**) The rearing temperature was maintained at 25℃, but the immune challenge temperatures varied from 18 to 32℃. (**C**) The rearing temperatures varied from 18 to 32℃, and the immune challenge temperature was maintained at 25℃. The test procedure was as follows: The microbeads were injected into the larvae immediately after removal from the incubator with the rearing temperature, and the injected larva were instantly placed into the incubator with the challenge temperature. Two hours later, the degree of phagocytosis was measured (number of beads with/without phagocytosis). Means with different letters indicate significant differences between treatments ($p < 0.05$, Kruskal-Wallis test with Dunn's multiple comparison test). The numbers in parentheses are the sample sizes. See Supplementary Tables S3, S4, S5 and S7 for the data and their statistics.

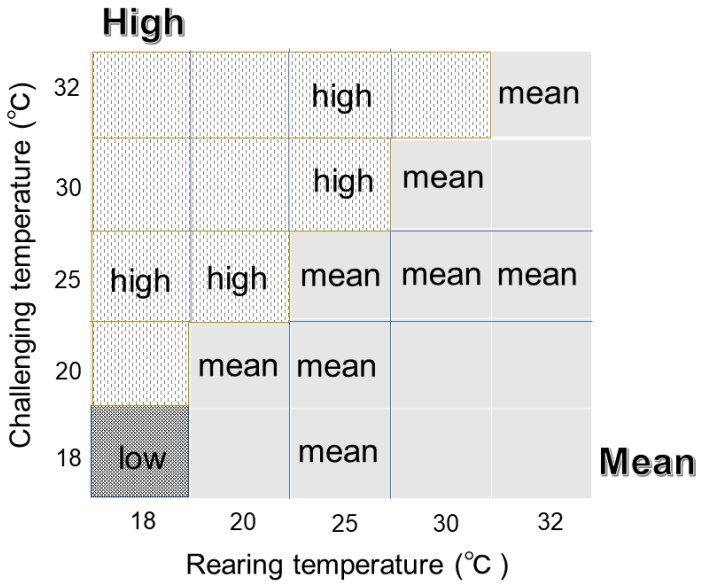

Fig. 3. Expected integrated effects of the rearing and challenge temperatures on phagocytosis.