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Up-regulation of heat-shock proteins in larvae, but not adults, of the flesh fly during hot summer days

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Abstract Heat-shock proteins (HSPs) are highly expressed when organisms are exposed to thermal stresses. The HSPs are considered to play significant roles in thermal adaptation because they function as molecular chaperones facilitating proper protein synthesis. The expression of HSPs under field conditions, however, has not been evaluated much, and their importance, based on the ecological contexts in nature, is still unclear. We investigated this aspect in the larvae and adults of the flesh fly, *Sarcophaga similis*. These larvae spend their larval life in the carrion or faeces of vertebrates; therefore, they are less mobile and are occasionally exposed to high temperature. In contrast, the adults of this species can fly and, therefore, they are highly mobile. Massive transcription of Hsps was detected both in the larvae and adults in a laboratory heat shock experiment. The larvae in the field showed no or less Hsp production on thermally mild days, whereas considerable upregulation of Hsp expression was detected on days with high temperature. The adults can also be exposed to thermal stress as high as 40°C or higher in the field. However, most of the flies showed no or less Hsp expression. The observations in the experimental cage under field conditions revealed behavioural thermoregulation of adults through microhabitat selection. The present study demonstrates ontogenetic alteration of the strategy to overcome thermal stress in an insect; in the field, less mobile larvae use physiological protection against heat (HSP production), whereas highly mobile adults avoid the stress behaviourally (through microhabitat selection).

Key words Behavioural thermoregulation • Flesh fly• Heat-shock protein • Mobility • Natural thermal stress

Introduction

The expression of heat-shock proteins (HSPs) is highly induced when organisms, including insects, are exposed to environmental stresses, such as extremes of temperatures. HSPs function as molecular chaperones, facilitating proper synthesis and preventing aggregation and denaturation of other proteins. Thus, in many organisms, HSPs have been considered to play an important role in survival under thermal stress (Parsell and Lindquist 1993; Yiangou et al. 1997; Feder and Hofmann 1999; King and MacRae 2015). In insects, four major families of HSPs are recognized, namely the small heat shock proteins (sHSPs), HSP60, HSP70, and HSP90. The sHSPs, acting independently of ATP, are the first line of cell defence, preventing irreversible denaturation of substrate proteins and facilitating subsequent refolding by ATP-dependent chaperones. The remaining HSPs interact with proteins and promote protein folding, degradation, disaggregation, and localization in an ATP-dependent manner (Basha et al. 2012; Clare and Saibil 2013; King and MacRae 2015). The primary function of HSP70 is to bind to unfolded or partially unfolded proteins to prevent their aggregation and to release them for folding. HSP90 also prevents aggregation of non-native proteins, but it appears to be more selective for substrates compared to other general chaperones (Clare and Saibil 2013).

Laboratory studies have demonstrated a clear relationship between HSP production and acquisition of heat tolerance. For example, when larvae of the fruit fly *Drosophila melanogaster* are exposed to severe heat shock, the majority of animals die. If, however, a mild heat shock, which induces the production of HSPs, is applied immediately before a severe heat shock, approximately 50% of the animals survive, because they have become thermotolerant or protected (Mitchell et al. 1979). Similar results were also observed in other organisms (Patriarca and Maresca 1990; Denlinger et al. 1991; Gehring and Wehner 1995). Adaptive changes in HSP expression over seasons (Fader et al. 1994; Hofmann and Somero 1995; Minier et al. 2000;

61 Dieterich et al. 2013) also support the ecological significance of HSPs in natural populations.
62 Engineering of gene expression further verified the significance of HSPs in thermotolerance in
63 insects (Feder et al. 1996; Feder and Krebs 1998; Rinehart et al. 2007; Colinet and Hoffmann
64 2010; Koštál and Tallarová-Borovanská 2009; Lü and Wan 2011). Thus, HSPs are the
65 candidates for playing a significant role in adaptation in natural populations. However, despite
66 HSPs being extremely important for survival following heat shock under laboratory conditions,
67 their ecological relevance and adaptive importance under field conditions has only been rarely
68 investigated directly and is less clear (Sørensen 2010). Especially, some insects with high
69 mobility possibly regulate their body temperature to avoid "overheating" by microhabitat
70 selection (May 1979; Chown and Nicolson 2004). This implies that less mobile insects, which
71 are unable to escape from detrimental environments, may rely on physiological mechanisms,
72 such as HSP production to protect themselves from heat damage. On the contrary, highly
73 mobile insects, which can avoid overheating behaviourally, may not rely on such physiological
74 protection, because HSP production is costly and sometimes deleterious (Huang et al. 2007;
75 Krebs and Feder 1997; Hoekstra and Montooth 2013). However, this issue has been paid little
76 attention (Sørensen 2010, but see Feder et al. 1997, 2000).

77 Here, we investigated relationships between insects' mobility and HSP production in
78 larvae and adults of the flesh fly, *Sarcophaga similis*, in the laboratory as well as in the field.
79 Adult males of this species occur from late April to the end of November in Osaka, Japan
80 (Tachibana and Numata 2006). This species is the most dominant sarcophagid species in our
81 research field (the campus of Osaka City University; Tachibana and Numata 2006). It is
82 important to note that Osaka City is now one of the hottest cities in Japan, and August is the
83 hottest month in Osaka (Japan Meteorological Agency, 2017; see Fig. S1). The adult males of *S.*
84 *similis* establish temporal territories on sunlit places, wait for a female to pass, and frequently
85 fly to pursue females and begin copulation in flight, as observed in other sarcophagid species.

After copulation, *S. similis* females carries the egg until hatching and then deposits the newly hatched 1st instar larvae (ovoviviparous) on carrion or animal faeces. The entire feeding phase of larval development is spent within the carrion or faeces, and when feeding is completed, the larvae leave the food (wandering stage), burrow into the soil, and pupariate and pupate. In the present study, we investigated the levels of expression of *Hsp70*, *Hsp23*, and *Hsp83* in the larvae and adults in the laboratory. Thereafter, we investigated their expression in the field-collected larvae and adults. We also examined the behavioural thermoregulation of adults in the experimental cage in the field.

Materials and methods

Insect rearing

The laboratory colony of *S. similis* originally collected on the campus of Osaka City University, Osaka, Japan (34.6°N, 135.5°E), in 2013, was maintained at 25°C under long-day conditions (LD 16:8 h). The adult flies were provisioned with water and sugar, and fed on a piece of beef liver 2 days after the emergence of adults. Ten days later the females larvipoisted on the liver, and the larvae were reared on a new piece of liver under the same environmental conditions. The larvae used for the experiments were not sexed, whereas only males were used for experiments on adult flies.

Hsp23 sequence

The nucleotide sequences of *S. similis Hsp70* and *Hsp90* were found in the database (DDBJ/GenBank/EMBL accession no. LC176075 and AB196477, respectively), but that of *Hsp23* was not found. Therefore, we cloned the gene and identified its sequence. A DNA fragment of *S. similis Hsp23* was obtained by RT-PCR. An adult male was exposed to 41°C in a water bath for 15 min, and total RNA was isolated from the whole body with Trizol Reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was synthesized from the RNA with M-MLV reverse transcriptase and oligo $(dT)_{12\text{-}13}$ primers (Invitrogen). PCR was performed using GoTaq DNA Polymerase (Promega, Madison, WI, USA). The primers, which were designed from the conserved *Hsp23* nucleotide sequence among several dipteran species, were as follows; hsp23cl-F, 5′-CGT CAT CGA GGG CAA GCA YGA RGA RMG-3′ and hsp23cl-R, 5′-GGC GGG GCC GGY YTG YTG DAT YT-3′. The amplified fragment, approximately 300 bp in length, was purified with Wizard Plus SV Minipreps DNA Purification System (Promega) and sequenced on 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with BigDye Terminator v3.1 Cycle Sequence Kit (Applied Biosystems). The nucleotide sequence of *S. similis Hsp23* was deposited in the database (DDBJ/GenBank/EMBL accession no. LC150013).

Laboratory heat shock

The feeding 3rd (final) instar larvae (3 days after larviposition) and adult males (5 days after adult emergence) were exposed to various temperatures for 15 min in water baths in the laboratory.

Field observations of larvae

Approximately 100 feeding 3rd instar larvae in 100–200 g of beef liver were placed on sunlit ground on 14-, 15-, 19-, and 20-Aug-2014. The probes (φ6 mm × 3 cm) of the data logger (TR-71wf, T and D, Matsumoto, Japan) were inserted approximately 2 mm beneath the top surface and 2 mm above the bottom surface of the liver to measure the temperature where larvae reside. Since larvae stayed in the liver during the experiments, the body temperatures of larvae were considered to fall within the range of these two temperatures. The temperatures in these two positions and air temperature near the liver were recorded at intervals of 15, 30, and 60 min from around 10:30 to around 17:30 h (JST).

Two or three larvae were removed from the liver every 15, 30, and 60 min, and total RNA was isolated from single larvae to assess gene expression.

Field observation of the adults

From 16-Jul-2013 to 25-Sep-2013 and from 22-Jul-2014 to 9-Oct-2014, the body-surface temperatures (surface temperature of the thorax) of the *S. similis* adult males attracted by a piece of beef liver in the field were measured using an infrared thermographic camera (i3, FLIR,

Wilsonville, OR, USA). The air temperatures around the flies were also measured using the temperature data logger (TR-71wf). We adopted body-surface temperature in this experiment, because of the difficulty in the measurement of immediate body temperatures of the flies in the field. It was difficult to identify the species of the flying sarcophagid males in the field, but it was reported that *S. similis* was the most dominant sarcophagid species on this field area (Tachibana and Numata 2006). To confirm this, we collected 95 males after body-surface temperature measurements and observed their genitalia under the stereoscopic microscope in our laboratory. We confirmed that all of the males that we captured were *S. similis.* Thus, we measured the body-surface temperature and isolated RNAs by immediate homogenization from 28 flies without species identification. The observed data were fitted to a non-linear regression line with the nonlinear least squares curve fitting (http://www.colby.edu/chemistry/PChem/scripts/lsfitpl.html).

Body-surface temperature and body temperature of adults

To see the difference between the body-surface temperature and body temperature (inner temperature of the thorax), we simultaneously monitored these temperatures in the male flies in the laboratory. A type K thermocouple (φ0.127 mm; ST-55K-TC-1.2M, Graphtec, Yokohama, Japan) was inserted into the thorax of a fly. To simulate the direct sunlight exposure, the flies were placed under a reflector lamp (500W) at a distance of 10–15 cm and their body-surface and body temperatures were monitored simultaneously with a data logger (midi Logger GL220, Graphtec) and a thermographic camera.

qPCR

Total RNA was isolated with Trizol Reagent. After treatment of the total RNA with deoxyribonuclease I (amplification grade; Invitrogen), cDNA was synthesized from each total RNA (0.3–2.0 μg) by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For quantitative real-time PCR analysis with a 7500 Real-Time PCR system (Applied Biosystems), 1 μL of cDNA was used in a final volume of 1X Go Taq qPCR Master Mix (Promega) and 0.2 μM of each primer, according to the supplier's instructions. Each reaction was performed in duplicate. As a control gene, *28SrRNA* was used for normalization (Rinehart et al. 2006; de Boer et al. 2009; Udaka et al. 2010). The primers used were: hsp70qrt-F (5′-TCT TGG TTG GCG GTT CTA-3′) and hsp70qrt-R (5′-CCA TAG GCA ACT GCT TCA TC-3′) for *Hsp70*, hsp83qrt-F (5′-TGA GCC CAA GAT TGA AGA TG-3′) and hsp83qrt-R (5′-CTT GTG TGT AGG CAA CCT TA-3′) for *Hsp83*, hsp23qrt-F (5′-GGT TAT ATC TCA CGG CAC TTT-3′) and hsp23qrt-R (5′-GGA ACA CTT ACG GTG AGA AC-3′) for *Hsp23* and 28Sqrt-F (5′-CCG ATG AAC CTG AAT ATC CAT T-3′) and 28qSrt-R (5′-AGG TTT TGA TAC CCA ATA ACT TGC-3′) for *28SrRNA*. The amplified fragments were approximately 100 bp in length. In all the reactions, the generation of only a single expected amplicon was confirmed by performing the melting-curve analysis. Quantification was performed by the standard curve methodology with known amounts of the DNA fragments amplified by PCR. The primers used to amplify the fragment for the standard curve analysis were hsp70rt-F (5′-CCC GTT TCG AAG AAT TGT GT-3′) and hsp70rt-R (5′-ACC GCC TGC TGT TTC AAT AC-3′) for *hsp70*, hsp83rt-F (5′-GAA CGC GAC CAA GAG GTT AG-3′) and hsp83rt-R (5′-CAC CAT ATT CGG CTT GTG TG-3′) for *hsp83*, hsp23rt-F (5′-ATG AGG AAC GCG AAG ACG-3′) and hsp23rt-R (5′-CTG GCG CTC ATT GGA TTT-3′) for *hsp23*, and 28Srt-F (5′-

CCG ATG AAC CTG AAT ATC CAT T-3′) and 28Srt-R (5′-GGT TTT GAT ACC CAA TAA CTT GC-3′) for *28SrRNA*.

Sun-shade preference of the adults

On 29-Aug-2014 and 6- and 9-Sep-2014, we investigated the sun-shade preference of *S. similis* males on the campus of Osaka City University. Five-day-old males were individually placed in a framed cage (40-cm width, 20-cm depth, 15-cm height) with all its faces covered with a nylon mesh to allow air circulation into the cage from outside. One half of the cage was shaded using aluminium foil to make a shaded part. The cage was set 1-m above the sunlit ground. The position of the male, i.e., in the sunlit or shaded part of the cage, was observed every 2 min, and simultaneously, the body-surface temperature was measured with an infrared thermographic camera. The air temperature in the cage was also recorded with the temperature data logger. Eighteen flies were used in this experiment.

Results

Larval Hsp expression in the laboratory

The larvae were exposed to a variety of high temperatures for 15 min in the laboratory and the relative abundance of *Hsp70*, *Hsp23*, and *Hsp83* transcripts were measured (Fig. 1). *Hsp70* mRNA was almost undetectable at the normal rearing temperature, but its level was

considerably increased at higher temperatures. Massive transcription started at 32°C (a 17-fold increase was observed with the increase in temperature from 25 to 32°C). The highest transcript level was observed at 40°C and the level in this case was 479-fold higher than the level prior to the heat shock (25°C). *Hsp23* also responded to the heat shock and much mRNA accumulation was observed at 36–44°C. Because of weak expression at the rearing temperature, the maximum level of expression was 15-fold higher than the level prior to the heat shock. *Hsp83* was constitutively expressed at the rearing temperature and also responded to the heat shock, and thus, only a 2.4-fold change in expression was noted.

Field observation of the larvae

We placed the larvae on a piece of beef liver on sunlit ground and measured the air temperature and the temperature inside the liver in August 2014 (Fig. 2). Although both the air temperature and temperature inside the liver gradually increased toward mid-day and decreased toward evening in all these days, the exact temperatures were greatly variable among the different days. On cloudy days of 14- and 15-Aug, the maximum air temperature never reached 40°C, and the top surface temperature of the liver was 1–5°C lower than the air temperature and was as high as 38°C. In contrast, it was bright on 19- and 20-Aug. On these days, the maximum air temperatures were much higher than that on 14- and 15-Aug. The top surface temperature of the liver was nearly identical to the air temperature on 19-Aug and reached 45°C during mid-day. The highest temperature of the bottom of the liver was as high as 40°C. Air temperature change on 20-Aug was similar to that on 19-Aug, but the top surface of the liver reached 50°C. Among each observation, we found 2–5 dead larvae at the end of the experiments on 19- and 20-Aug, but no such larvae was found on 14- and 15-Aug.

The larvae inside the liver were collected every 15, 30, and 60 min and the expression of *Hsp* was investigated (Fig. 2). On the mild days of 14- and 15- Aug, *Hsps70* expression remained at low levels and never reached the highest mean level observed in the laboratory heat shock experiment. Similarly, *Hsp23* and *Hsp83* expression remained at low levels on these days, although some individuals showed slightly higher expression levels during mid-day. In contrast, on the severe days of 19- and 20-Aug, considerable upregulation of these *Hsp* genes was noted during mid-day. The highest levels of *Hsp70*, *Hsp23*, and *Hsp83* mRNA were 4.8-, 36.0-, and 14.6-times higher than those observed in the laboratory heat shock experiment. The larvae in the field suffered extensive heat shock on severe days.

Body temperature and body-surface temperature of adults

We measured the body temperature (temperature inside the thorax) and body-surface temperature (surface temperature of the thorax) of the adult flies simultaneously under a reflector lamp in the laboratory, to see how these temperatures were different (Fig. 3). Upon increasing the body-surface temperature, the body temperature rose linearly. The body surface temperature was found to be 2.3 ± 1.7°C higher than the body temperature in this experimental setup.

We measured the air temperature and the body-surface temperatures of free-living adult males in the field (Fig. 4). When air temperatures were higher, the body-surface temperatures were also higher, in general. However, when air temperature was higher than approximately 37°C, the increment rate of body-surface temperatures became smaller. Thus, free-living flies seem to maintain their body-surface temperature as high as 40°C even under severe thermal

conditions. The highest air temperature and the highest body-surface temperature that we observed were 48.1 and 44.5°C, respectively.

Adult Hsp expression in the laboratory

The adults were exposed to various temperatures for 15 min in the laboratory and relative amounts of *Hsp70*, *Hsp23*, and *Hsp83* transcripts were measured (Fig. 5, left panels). The expression patterns of all the three *Hsps* were mostly similar. The highest expression levels of *Hsp70*, *Hsp23*, and *Hsp90* were detected at 40, 42, and 42°C, respectively, and they were 58-, 44-, and 3.7-fold higher than the levels prior to heat shock (25 ˚C).

Field observation of the adults

We captured flies in the field just after measurement of their body-surface temperatures by an infrared thermographic camera, and investigated their *Hsp70*, *Hsp23*, and *Hsp83* expression (Fig. 5, right panels). The body-surface temperatures ranged from 29.3°C to 44.5°C and the interquartile range were 36.5–41.2°C (median = 39.9°C). Despite the higher body-surface temperatures, strong expression of *Hsp*s was not observed; only a few individuals showed the expression comparable to that in the laboratory heat shock experiment and others showed almost undetectable levels of *Hsp* mRNAs. Such low expression levels in the adults in the field were greatly different from the expression in the larvae in the field (Fig. 2).

Sun-shade preference of the adults

We released an adult male into the experimental cage, half of which was covered with aluminium foil to form a shaded part, and the cage was placed on sunlit ground. We observed the position of the fly in the cage and measured air temperature and body-surface temperature of the fly every 2 min, to clarify the relationship among these factors. The total number of flies that were in a sunlit or shaded part are shown in Figure 6A. Flies preferred to stay in the shaded part when the air temperature was higher, whereas they preferred to stay in the sunlit part when the air temperature was lower (Mann-Whitney *U* test, $P < 0.05$). It is also important to note that no significant difference was detected when the body-surface temperatures of the flies in sunlit and shaded parts were compared (Mann-Whitney *U* test, $P > 0.05$; Fig. 6B).

Discussion

Our laboratory experiments showed that *Hsp70* and *Hsp23* are heat-inducible in *S. similis* similar to their expression in other dipteran species (Goto and Kimura 1998; Yocum et al. 1998; Rinehart et al. 2000; Tachibana et al. 2005; Lopez-Martinez and Denlinger 2008; Concha et al. 2012). *Hsp90* is constitutively expressed but it also responds to heat shock. Such expression pattern is also reported in other fly species (Rinehart and Denlinger 2000; Chen et al. 2005; Tachibana et al. 2005).

Although the expression of Hsps has been examined in various organisms, most of the studies focus on the physiological mechanisms of the heat-shock response or on the comparison

of the response among species to find the significance of HSPs in their thermotolerance (Hoffmann et al. 2003; King and MacRae 2015). We still lack an ecological context on whether Hsp expression is fine-tuned to naturally-encountered heat shock (Sørensen 2010). In the present study, we found considerable upregulation of these genes in *S. similis* larvae in the field on severe days (19- and 20-Aug-2014), when air and liver temperatures exceeded 40–45°C. The environmental conditions on these days were so severe that the larvae protected themselves against thermal stress by expressing large amounts of Hsps. Feder et al. (1997) reported similar results in *D. melanogaster*. The larvae collected from the necrotic fruit that were on the sunlit ground in the field accumulated large amounts of HSP70 protein and the levels were high in comparison to the levels after standard heat shock in the laboratory. Lopez-Martinez and Denlinger (2008) also investigated heat-shock response of the apple maggot *Rhagoletis pomonella* in the field. This species lays eggs on an apple and the larvae spend their larval life inside it. The larvae are frequently exposed to summertime apple temperatures that exceed 40°C. The field temperature cycles ranging from 16 to 47°C elicit strong *Hsp70* and *Hsp83* expression. Thus, the natural heat shock is sufficiently intense to induce HSP expression in these dipteran larvae.

The expression of all the *Hsp*s was also greatly enhanced by laboratory heat shock in the *S. similis* adults, as observed in the larvae. However, drastic induction was scarcely detected in adults in the field, despite their high body-surface temperatures. These results indicate that the free-living adults in the field do not rely on HSP expression to survive in the field in contrast to larvae, and further imply that the adult flies were scarcely damaged by hyperthermia in the field. Feder et al. (2000) investigated the heat-shock response in the field-captured *D. melanogaster* adults. The levels of HSP70 in most of the flies captured on warm days were very low and comparable to the levels previously reported in unstressed flies in the laboratory. Flies showed frequent responses only when they were caged and placed in direct sunlight. These

results indicate that even on warm days most of the flies avoid thermal stress, presumably through microhabitat selection. Behavioural thermoregulation to avoid overheating of body temperature has been reported in various ectotherms (Kleckova et al. 2014; Sunday et al. 2014). Thermoregulation of *S. similis* adults was also evident in the present study. The increment in the body-surface temperature was suppressed at higher environmental temperatures in the field and flies maintained their body-surface temperatures as high as 40°C. Based on the laboratory experiments, a 15-min exposure to 40°C induced considerable upregulation of *Hsp* mRNAs, but such increased expression was not observed in the field. These results indicate that the body temperature is far lower than the temperatures that induce the *Hsp* expression. Furthermore, the present study clarified that the adult flies frequently fly from shade to sunlight or *vice versa* and this behaviour maintains their body-surface temperatures at a certain level. Although adult males of *S. similis* are also exposed to sun heat for many hours in the field, they may regulate their body temperatures so as not to be affected with the extreme thermal stress by frequent movement between sunlit and shade places, as suggested in *D. melanogaster* (Feder et al. 2000).

Here, we clarified that, in the field, the flesh fly larvae with low ability to escape from their habitat protect themselves from thermal damage by expressing *Hsp*s. On the other hand, adults with high ability to escape from heat stress by flying can regulate their body temperatures and, therefore, they do not have the necessity of the expression of *Hsp*s. The flesh flies appear change their strategy, during ontogeny, from physiological protection against heat damage to behavioural protection against heat exposure.

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478

479

480 **Figure legends**

481

482 **Fig. 1** Relative amounts of *Hsp70* (top), *Hsp23* (middle), and *Hsp83* (bottom) mRNAs in
483 *Sarcophaga similis* larvae that were exposed to various temperatures for 15 min in the
484 laboratory. *n*= 3–4 for each temperature. The highest mean levels of *Hsp70*, *Hsp23*, and *Hsp83*
485 mRNA at 40, 38, and 44°C, respectively, were set at 1.0. Lines were drawn through the means
486 of biological replicates

487

488 **Fig. 2** Air temperature, temperatures of a piece of beef liver in which *Sarcophaga similis* larvae
489 reside, and relative amounts of *Hsp70*, *Hsp23*, and *Hsp83* mRNA in the larvae, on 14-, 15-, 19-,
490 and 20-Aug-2014. A piece of beef liver in which the larvae reside were placed on sunlit ground
491 on each day and air temperature (*closed squares*) and temperatures of top (*open triangles*) and
492 bottom (*closed triangles*) surfaces of the beef liver were recorded (top panels). The larvae were
493 taken out from the liver and the relative amounts of *Hsp70*, *Hsp23*, and *Hsp83* mRNAs were
494 measured. The highest mean expression levels of each gene in the laboratory heat shock
495 experiment (40, 38, and 44°C for *Hsp70*, *Hsp23*, and *Hsp83*, respectively; Fig. 1) were set at 1.0
496 and are shown in the horizontal grey bars. The expression levels in 21–37 larvae were plotted

497

498 **Fig. 3** Body-surface and body temperatures of *Sarcophaga similis* adults under a reflector lamp
499 in the laboratory. A linear regression line is also shown. The data of 248 temperature
500 measurements from 6 flies were plotted

501

Fig. 4 Body-surface temperatures of *Sarcophaga similis* adults in the field and air temperatures at which the body-surface temperatures were measured. A non-linear regression line (*solid line*) is also shown. Circles and diamonds are the data from flies captured in 2013 and 2014, respectively, and open and closed marks are the data of flies, the species of which were identified and unidentified, respectively. Data from 151 flies were plotted

Fig. 5 Relative amounts of *Hsp70*, *Hsp23*, and *Hsp83* mRNAs of *Sarcophaga similis* adult flies that were exposed to various temperatures in the laboratory (left panels) and were captured in the field (right panels). The body-surface temperatures of the adults were also recorded. The highest mean levels of *Hsp70*, *Hsp23*, and *Hsp83* mRNAs at 40, 42, and 42°C in the laboratory heat shock experiment were set at 1.0. n = 3–4 for each temperature in the laboratory heat shock experiment. n = 27–28 for the expression of *Hsp*s in adults in the field

Fig. 6 Sun-shade preference of *Sarcophaga similis* adults. An adult fly was released into the experimental cage, half of which was covered with aluminium foil to form a shaded part. The cage was placed on sunlit ground. The air temperature, body-surface temperature, and the position of the fly (sunlit or shaded part) were recorded continuously. (A) air temperature and the total number of flies that were in a sunlit or shaded part. (B) body-surface temperatures of the flies in a sunlit or shaded part. Median, interquartile, maximum, and minimum values are shown. The data from 275 observations in 18 flies are shown

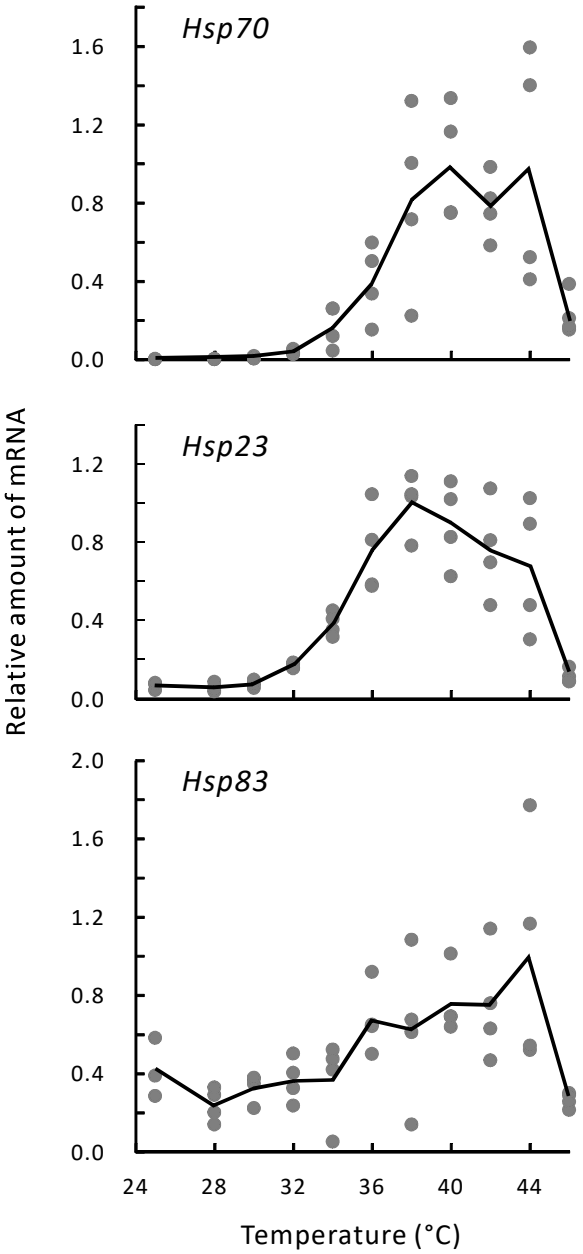
Hsp70
Hsp23
Hsp83
Relative amount of mRNA
1.6
1.2
0.8
0.4
0.0
2.0
24
28
32
36
40
44
Temperature (°C)

Fig. 1

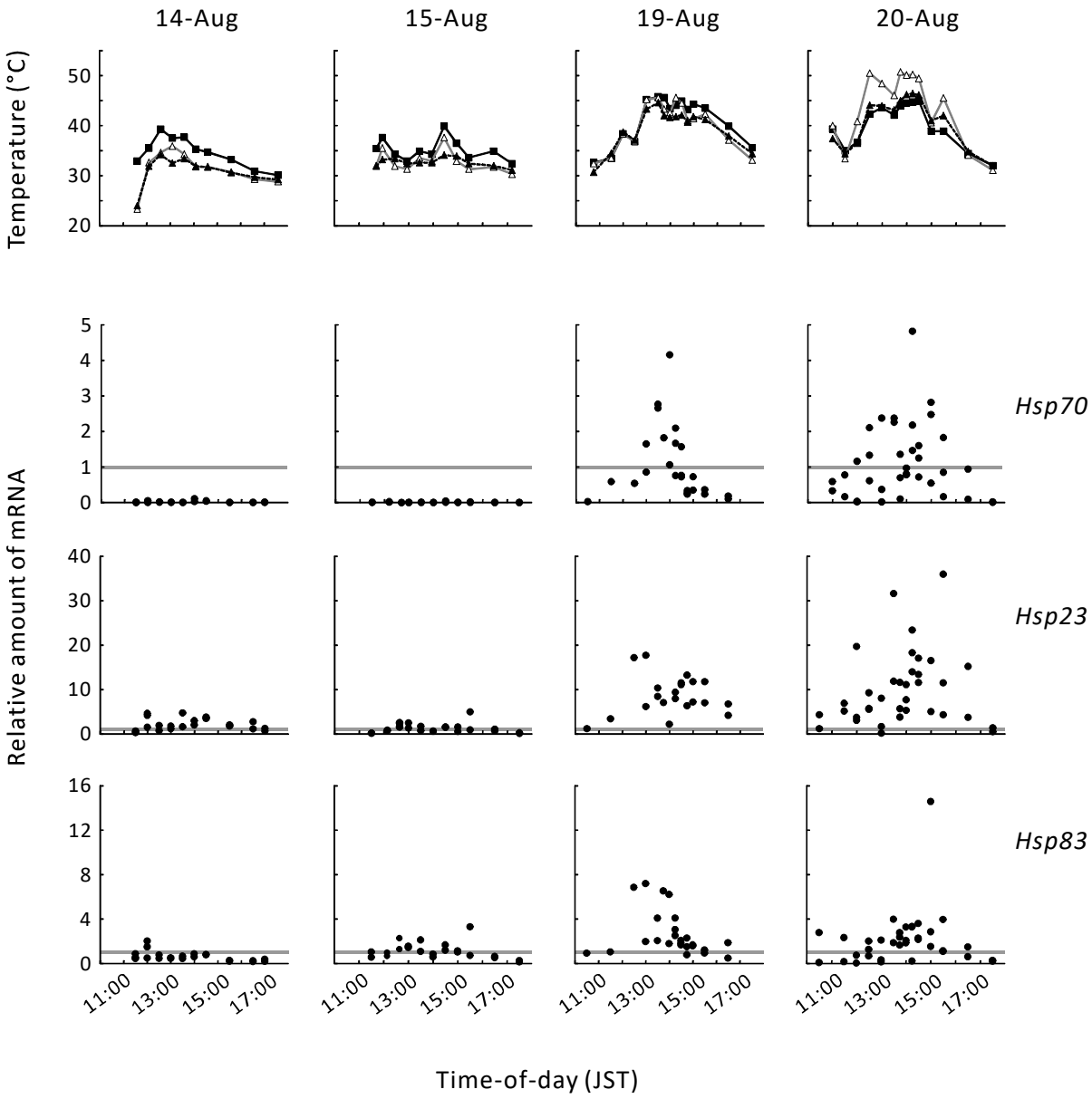
14-Aug
15-Aug
19-Aug
20-Aug
Temperature (°C)
50
40
30
20
Relative amount of mRNA
5
4
3
2
1
0
Hsp70
40
30
20
10
0
Hsp23
16
12
8
4
0
Hsp83
11:00
13:00
15:00
17:00
Time-of-day (JST)

Fig. 2

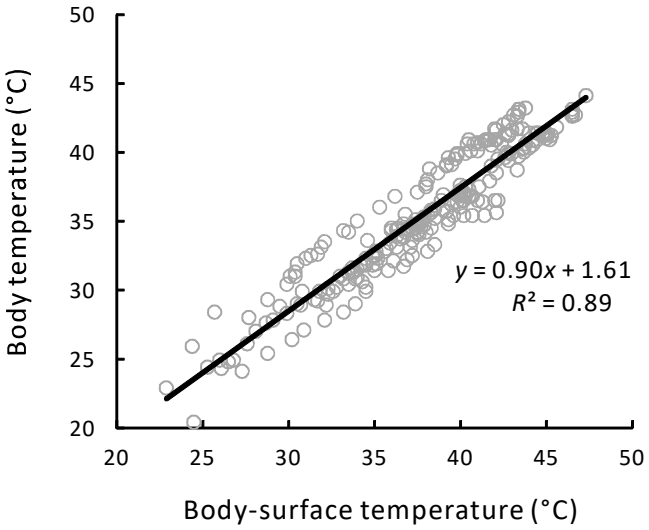
Body temperature (°C)
50
45
40
35
30
25
20
20
25
30
35
40
45
50
Body-surface temperature (°C)
y = 0.90x + 1.61
R² = 0.89

Fig. 3

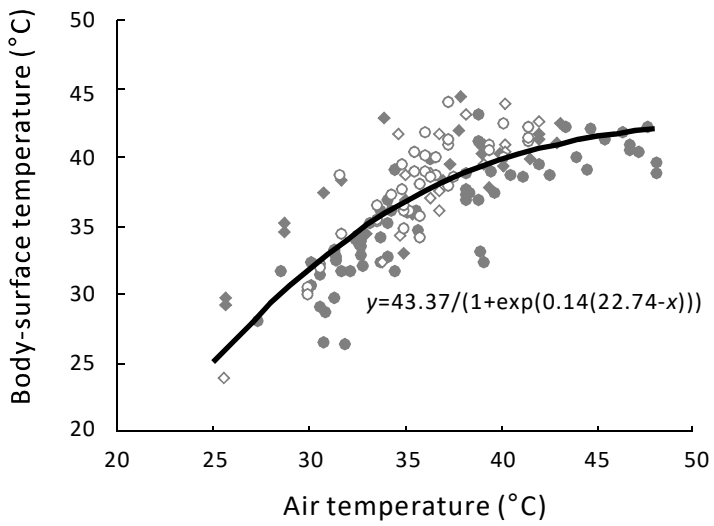
Body-surface temperature (°C)
50
45
40
35
30
25
20
20
25
30
35
40
45
50
Air temperature (°C)
y=43.37/(1+exp(0.14(22.74-x)))

Fig. 4

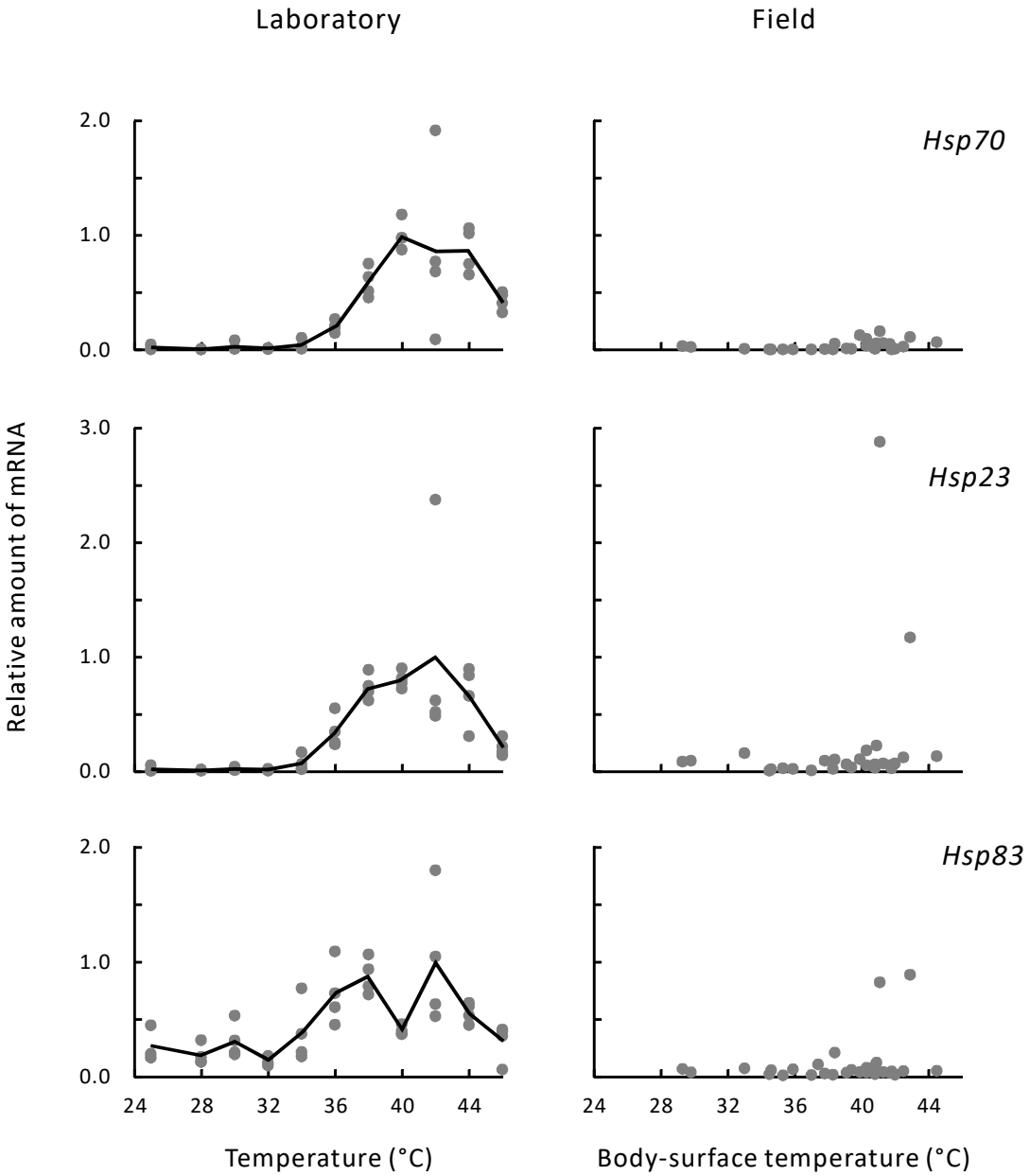
Laboratory
Field
Hsp70
Hsp23
Hsp83
Relative amount of mRNA
2.0
1.0
0.0
3.0
24
28
32
36
40
44
Temperature (°C)
Body-surface temperature (°C)

Fig. 5

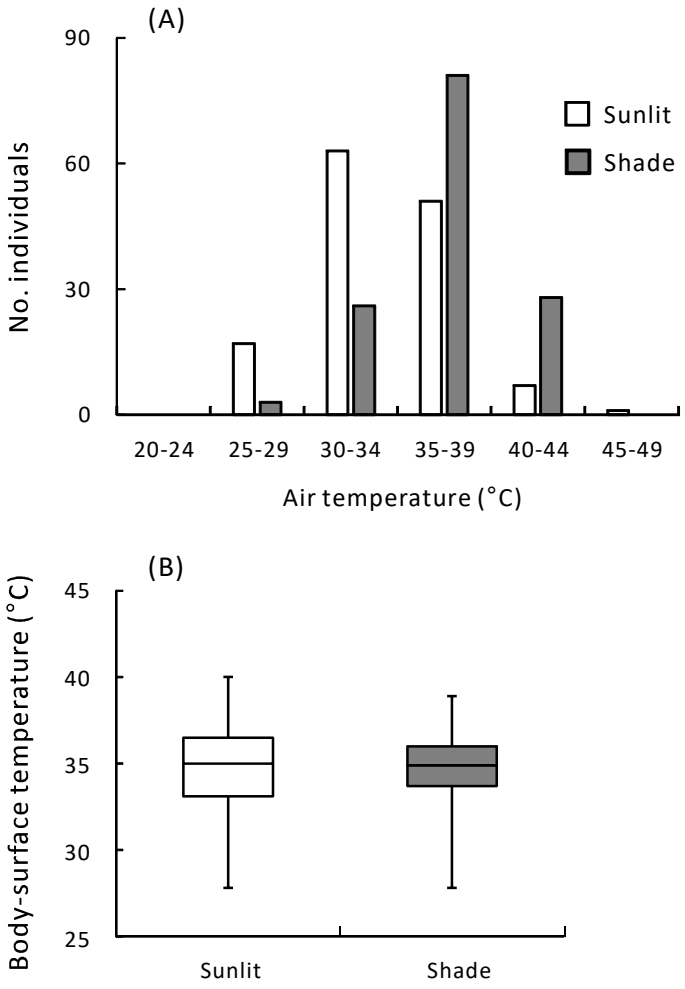
(A)
90
60
30
0
No. individuals
Sunlit
Shade
20-24
25-29
30-34
35-39
40-44
45-49
Air temperature (°C)
(B)
45
40
35
30
25
Body-surface temperature (°C)
Sunlit
Shade

Fig. 6

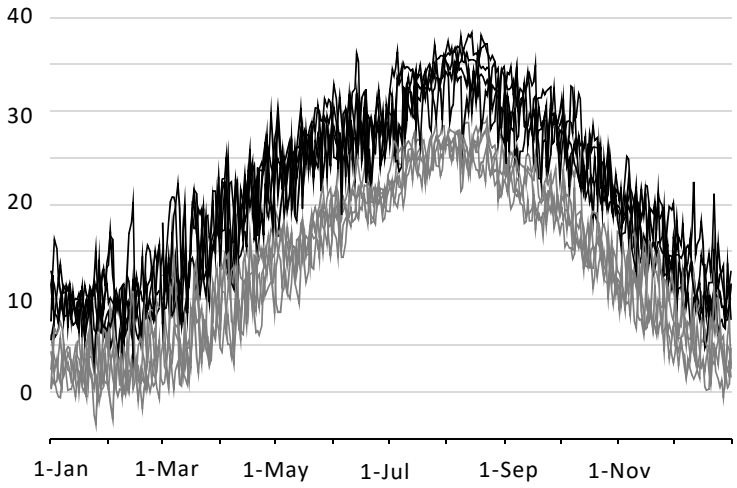

Fig. S1. The daily maximum (black lines) and minimum (gray lines) air temperatures in Osaka, Japan. Data on 2012-2016 were superimposed (Japan Meteorological Agency, http://www.jma.go.jp/jma/indexe.html, Accessed on May 18, 2017). During the period of 2012-2016, the maximum air temperature was 38.4°C on mid-August in 2013. The numbers of days on which the daily maximum temperature exceeds 35.0°C were 6-23, and the numbers of days on which the daily average temperature exceeds 30°C were 9-21, most of which were detected on August. Thus, Osaka City is now one of the hottest cities in Japan.

Fig. S1