For ectothermic species, temperature has long been recognized as a major environmental factor responsible for species abundance and geographical distribution (Leather et al., '93). The available literature provides information on how heat shock proteins ensure survival under conditions of environmental stress, which in the absence of a defensive reaction can lead to irreversible changes in, and permanent damage to, cells (Korsloot et al., 2004). Increased expression of certain hsp genes at high temperature recorded in laboratory conditions has been found to correlate positively with the survival of an organism (Gong and Golic, 2006; Dahlhoff and Rank, 2007; Bettencourt et al., 2008; Benot et al., 2011). Gong and Golic (2006) also showed reduced resistance to heat stress following the deletion of hsp70 in Drosophila. However, such a relationship was not observed by Jensen et al. (2010). Unfortunately, most of the studies in this area have indeed been conducted on genes from the Hsp70 family, as...
well as a few from the small heat shock protein (sHSP) family. It also appears that some hsp genes do not affect thermotolerance, for example, there was no effect of deletions in hsp27 in the resistance to heat stress manifested by Drosophila melanogaster, although the action was found to be associated with resistance to starvation (Hao et al., 2007).

Most research on the impact of hsp genes on thermotolerance in the order Diptera (flies and midges) has been carried out on Drosophila spp. and mosquitoes (reviewed by de Nadal et al., 2011; Zhao and Jones, 2012; King and MacRae, 2015). Some publications also refer to sarcophagids (Rinehart and Denlinger, 2000; Rivers et al., 2010), calliphorids (Tachibana et al., 2005; Sharma et al., 2006; Concha et al., 2012), and therphids (Kokolakis et al., 2009).

Current research is the first attempt of hsp expression profiling in the dipteran family Phoridae (scuttle flies). The most expansive genus of the Phoridae is Megaselia, with around 1,500 species described (R. H. L. Disney, personal comm.), including the cosmopolitan Megaselia scalaris (Loew, 1866). The larvae of M. scalaris have been described as detritivore, parasite, and parasitoid, consuming a wider spectrum of organic materials of both animal and plant origin than any other insect (Tumrasvin et al., ‘97; Koller et al., 2003; Disney, 2008). As an adult, M. scalaris has been reported as a polyphagous organism, generally acting as saprophagous, sarcophagous, or necrophagous (Costa et al., 2007). This species is easy to culture in laboratory conditions and is now emerging as a potential model for ecology and evolutionary biology (Disney, 2008; Rasmussen and Noor, 2009; Zhong et al., 2014; Hoehn and Noor, 2015).

Studies by Prescher et al. (2002), Durska et al. (2010), and Bonet et al. (2011) on scuttle flies in burned forests showed that Megaselia species, mostly saproxylic, dominate in the phorid communities immediately after a fire as well as during some years after the disturbance (Durska, 2013)—thus, making this phorid fly an appropriate insect model to study thermotolerance and heat shock response. Because forest fires are unpredictable acute heat stress, we anticipated that differences in temperatures leading to upregulation or induction of hsp genes and affecting viability of Megaselia are important factors with respect to thermosteresistance. Some members of Phoridae are able to burrow vertically <1 m into soil as both adults (Disney, ‘94) and larval instars (Oliva, 2004; Pastula and Merritt, 2013). Presumably, elevated thermosteresistance and ability to penetrate the soil can be important features allowing Megaselia species to survive forest fires and inhabit post-fire areas.

Because some phorid species are adapted to post-fire areas, temperatures upregulating or inducing hsp should be relatively low to provide sufficient time for synthesis of protective heat shock proteins. The objectives of our study were, thus, to estimate the hsp gene expression pattern in larvae and imagos of M. scalaris in a developmental context from the perspective of understanding adaptive processes.

**MATERIALS AND METHODS**

**Sample Collection and Heat Treatments**

We conducted our experiments on 12 M. scalaris groups. Each group derived from a single female collected at Lomna near Warsaw (52°22′57″N, 20°46′34″E) in August 2013. Flies were maintained in 70 mL bottles at 25°C and 70% relative humidity on a medium that contained yeast (2.8% w/v), cornmeal (5.1%), agar (0.8%), sugar (2.5%), and propionic acid (0.4%) (Hoffmann and Shirriffs, 2002, modified), and supplemented with raw cow liver. Progeny for experiments were collected every 24 hr, and adult flies were separated by sex under CO₂ anesthesia to obtain separate sex groups, which were held on the medium at 25°C.

To minimize a possible effect of genetic diversity in thermotolerance between isofemale lines, 10 individuals from each line were collected into containers (total 120 in each case). Additionally, two individuals from each line were added into the same containers for RNA isolation. The bottoms of containers were covered with wet filter paper to prevent desiccation during the treatment. Thermotolerance was measured in an incubator (Innova CO-48, New Brunswick Scientific, Enfield, CT, USA), with incubator temperature uniformity at a level of ±0.2°C.

To measure basal thermotolerance, third-instar larvae and 2–3-day-old adult males and females (N = 120 in each case) were acutely exposed to one of several test temperatures for 60 min. Temperature ranged from 37 to 44°C. Experiments were performed in three biological replications. Survivors were counted 24 hr later. After temperature treatment, 10–20 individuals were taken immediately for RNA isolation while the rest of them were transferred to 25°C.

To induce thermotolerance, Megaselia individuals at the same stage of development as in the case of the basal thermotolerance measurement were transferred to the containers covered with wet filter paper and exposed to an inductive treatment (60 min in an incubator). The inductive temperature (31, 33, 35, 38, 40, and 41°C) was chosen on the basis of hsp gene-expression experiments from the basal thermotolerance experiment. After heat treatment, all flies were transferred back to control conditions and allowed to recover for 1 hr (Carmel et al., 2011). To measure induced thermotolerance, third-instar larvae and 2–3-day-old adult males and females (N = 120 in each case) were exposed to one of several test temperatures (ranging from 37 to 46°C) for 60 min.

Three replicates were performed in each block of experiments. The data collected were the proportion of surviving flies from among the total numbers exposed. The heat tolerance of Megaselia was measured as “lethal temperature 50%” (LT₅₀), which was defined as the temperature at which 50% of the animals die and 50% survive. LT₅₀ was estimated using the probit regression model available in Statistica 64 ver. 10 (StatSoft, Inc., Tulsa, OK, USA; http://www.statsoft.com). The level of significance of each pre-treatment temperature as a whole has been analyzed.
statistically using one-way ANOVA. To determine significant differences ($P < 0.05$) between group means, Scheffe’s test was used. This test is considered to be one of the most conservative post hoc tests providing confidence intervals that are fairly wide (Statistica, ANOVA module). As no significant sex-related differences in gene expression were noted, the presented data refer to females only.

**RNA Extraction**

Total RNA was extracted with a TRI Reagent (Sigma–Aldrich, St. Louis, MO, USA), following the manufacturer’s instructions. Briefly, 10–20 individuals from each biological replication were homogenized in 1 mL of TRI Reagent. To complete dissociation of nucleoprotein complexes, homogenates were incubated for 5 min at room temperature. Subsequently, 0.2 mL of chloroform was added, and the sample was vortexed and incubated at room temperature for 15 min. The resulting mixture was centrifuged at 12,000 g for 15 min at 4°C. The upper phase was transferred to a fresh tube and RNA precipitated through the addition of 0.5 mL of isopropanol. RNA was stored at −70°C. The amount of total RNA extracted and its purification from protein and polysaccharides was determined with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA integrity was verified electrophoretically in a 1.5% agarose gel stained with ethidium bromide. Only samples that satisfied both the quality and integrity requirements were used in subsequent experiments. Three high-quality RNA samples (i.e., biological replicates) were obtained for each condition. To eliminate the probe contamination by genomic DNA, the total RNA was treated with RNase-free DNase I (Sigma–Aldrich). One microgram of RNA was treated with 1 U of DNase 1 for 15 min at room temperature. The reaction was stopped by adding stop solution, and DNase was inactivated with 1 U of RNase-free DNase I (Sigma–Aldrich). One microgram of RNA was treated with 1 U of DNase 1 for 15 min at room temperature. The reaction was stopped by adding stop solution, and DNase was inactivated with 1 U of RNase-free DNase I (Sigma–Aldrich) following the manufacturer’s instructions.

Following incubation for 10 min at 70°C, all remaining components were added and the reaction was run at 45°C for 50 min. cDNA samples were generated from each of three replicates derived from different individuals. The cDNA was used immediately in the PCR or stored at −20°C.

**Real-Time PCR**

We analyzed the expression of seven hsp genes, of molecular weights that were low (hsp22, hsp23, hsp26, and hsp27), medium (hsp40), or high (hsp70 and hsp83). To analyze expression of heat shock protein genes, we used the primers described by Colinet et al. (2010). Melting profiles of amplicons obtained with these primers had a clear single melting peak, suggesting that — although *Megaselia* is not closely related to *D. melanogaster* — the primers correspond to conserved *hsp* regions. Electrophoresis of Real-time PCR products in 3% agarose gel also showed that amplicons form a single band. Real-time PCRs were performed on the RotorGene 6000 system. Reactions were carried out using LuminoCt SYBR Green qPCR Master Mix (Sigma–Aldrich), while cycle threshold (Ct) estimates were obtained using the relative quantification module in the software package. PCR reactions were performed in a final volume of 20 μL containing 1 μL of cDNA sample, 2.0 μL of the primer mix (5 μM of each primer), 10 μL of the 2× LuminoCt SYBR Green qPCR Master Mix, and 7 μL of H2O. The assay included no template control and each of the test cDNAs from three biological replications. After 3 min at 95°C, the cycling conditions were as follows: 40 cycles at 95°C for 30 sec, 53–57°C for 30 sec, and 72°C for 30 sec. To validate the specificity of amplification, a post-amplification melt-curve analysis was performed. Amplicons were first denatured at 95°C for 60 sec, and then cooled to 72°C, and the temperature was then gradually raised to 95°C with increments of 0.5°C each step. Fluorescence data were recorded continuously during this period, and analyzed subsequently using the *T*ₘ calling module in the RotorGene 6000 software.

The $2^{ΔΔCt}$ method (Livak and Schmittgen, 2001) was used in calculating the relative ratio, but instead of value 2, the correct amplification efficiency was used. We used a noise-resistant iterative nonlinear regression algorithm (Real-time PCR miner; www.miner.eWindup.info) to determine the efficiency of the PCR reaction (Zhao and Fernald, 2005). mRNA levels were counted from three PCR reactions for each sample. Five genes were tested as candidate reference genes, that is, the forkhead domain 68A gene (*fd68A*; frequently known as *Mnf*), as well as the genes coding for alpha-actin (*Act*), elongation factor 1 alpha (*E1alpha*), alpha-tubulin (*Tub*), and ribosomal protein S20 (*Rps20*). The one selected was to show the highest level of stability of expression within and between thermal treatments (e.g., Ponton et al., 2011), hence, the stability of the candidate genes was evaluated using BestKeeper (Pfafli et al., 2004), NormFinder (Andersen et al., 2004), geNorm (Vandesompele et al., 2002), the comparative delta-CT method (Silver et al., 2006), and RefFinder (Xie et al., 2011). The geNorm algorithm first calculates an expression stability value for each gene and then performs a pair-wise comparison of this gene with the others. In turn, NormFinder ranks the stability of tested candidates, albeit independently of one another, whereas BestKeeper determines the standard deviation of the cycle threshold, with the user selecting the best genes by reference to these variables. On the basis of the rankings from each program, RefFinder assigns an appropriate weight to an individual gene and calculates the geometric mean of these weights for the overall final ranking.

**Amplicons Sequencing**

Excess dNTPs and unincorporated primers were removed from the PCR product using the Clean-Up Purification Kit (A & A Biotechnology, Gdynia, Poland). DNA was eluted in 40 μL of H2O.

_J. Exp. Zool._
Sequencing PCR reactions were performed using 1 \( \mu \)L of BigDye (ABI Cycle Sequencing Kit, Foster City, CA, USA), 2 \( \mu \)L of BigDye sequencing buffer, 1 \( \mu \)L (5 mM) of forward or 1.6 (10 mM) of reverse primer, and \( \text{H}_2\text{O} \) to 10 \( \mu \)L total volume. The thermal profile for sequencing reactions consisted of 25 cycles at 96°C for 60 sec, 96°C for 10 sec, 50°C for 5 sec, and 60°C for 105 sec. Amplicons were sequenced on an ABI 3500xl genetic analyser (Applied Biosystems, Foster City, CA, USA). Obtained sequences were compared by BLASTN with transcriptome sequences of *M. scalaris* (accession no. SRX208999) deposited in the Sequence Read Archive (SRA) and with the nucleotide database of insects available in GenBank.

**RESULTS**

Survival of Larvae and Imagoes

We measured the basal temperature sensitivity and estimated the lethal temperature (LT50) at 42.2, 41.3, and 41.4°C for third-instar larvae, adult males and females, respectively (probit regression model; see also Fig. 1). These results showed that larvae are more resistant to high-temperature treatment than imagoes. No significant sex-related differences in stress tolerance were noted.

Specificity of Gene Amplification

All amplicons were sequenced (Table S1), and the sequences obtained were found to have 97–100% identity with the corresponding transcriptome sequences of *M. scalaris* available in the sequence read archive (SRA). In line with the very low E-values obtained (ranging from 1e-06 to 8e-19), it was established that the identity referred to was not accidental. Because the SRA sequences are unannotated, the amplicons were also compared with known insect nucleotide sequences deposited in GenBank (Table S2). The sequences of all the amplicons were found to display 85–98% similarity with the corresponding insect genes.

Evaluation of Candidates for Reference Genes

We measured the cycle threshold (Ct) for the five candidate reference genes in third-instar larvae and 2–3-day-old adults (Table S3). Their expression was evaluated using five computational programs available currently. Analysis using delta-CT, NormFinder, and RefFinder indicated tubulin for use as the reference gene in the case of larvae; two other programs (BestKeeper and geNorm) suggested elongation factor 1 alpha rather than tubulin. In turn, according to RefFinder, the ranking of most to least stable expression in different tissue samples of larvae was *Tub>*Ef1a>*Rps20>*fd68A>*Act. In the case of the adults, all programs pointed to tubulin as the best reference gene (Table 1).

Expression of Hsp Genes in Larvae

The effect of temperature treatment on gene expression was measured by exposing third-instar larvae to selected temperatures for 1 hr. The analysis of *hsp* gene expression showed that from among the seven genes analyzed, only *hsp70* is induced by the thermal stress (Fig. 2A). Expression of *hsp70* was detected only after treatment of temperature 37°C or higher, expression of this gene in larvae at temperatures lower that 37°C was below qPCR detection level. All other genes are already expressed at 25°C, with expression showing an increase after treatment at high temperature. Incubation of larvae at 27, 29, and 31°C does not

### Table 1. Ranking order of the candidate reference genes as determined by RefFinder in third-instar larvae and imagoes of *M. scalaris*.

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Rank</th>
<th>Gene</th>
<th>Geometric mean of ranking values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Third-instar larvae</td>
<td>1</td>
<td><em>Tub</em></td>
<td>1.19</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td><em>Ef1a</em></td>
<td>1.41</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td><em>Rps20</em></td>
<td>3.00</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td><em>fd68A</em></td>
<td>4.00</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td><em>Act</em></td>
<td>5.00</td>
</tr>
<tr>
<td>Imagoes</td>
<td>1</td>
<td><em>Tub</em></td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td><em>Ef1a</em></td>
<td>1.68</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td><em>Rps20</em></td>
<td>3.00</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td><em>fd68A</em></td>
<td>4.00</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td><em>Act</em></td>
<td>5.00</td>
</tr>
</tbody>
</table>

*Tub*, alpha-tubulin; *Ef1a*, elongation factor 1 alpha; *Rps20*, ribosomal protein S20; *fd68A*, forkhead domain 68A; *Act*, alpha-actin.
change hsp gene expression. After treatment at 33°C, the temperature expression of three low-molecular-weight heat shock protein genes (hsp22, hsp23, and hsp26) was found to increase 6.9–11.8-fold relative to the 25°C treatment. Expression of the hsp23 gene increased proportionally to temperature treatment up to 39°C, reaching the maximum of upregulation (equal to 27.6-fold). An increase of treatment temperature from 39 to 42°C was not shown to generate further changes in gene expression. Expression of the hsp26 gene after treatment involving temperatures in the range 35–42°C was slightly higher than at 33°C (Fig. 2A). In the case of the hsp22 gene, the expression profile after treatment involving temperatures in the range 25–35°C was similar to those noted for the hsp23 and hsp26 genes, reaching the highest level of upregulation (18.7-fold) at 35°C, while expression levels decreased from the maximum at higher temperatures (Fig. 2A).

Expression in the hsp40 gene increased 12.5-fold following treatment at 37°C, as well as 13.9–16.9-fold where temperature levels were still higher. Lower temperatures did not increase expression in this gene. Expression of the hsp70 gene was induced at 37°C, under that temperature, the expression of hsp70 was below the detection limit. The expression of hsp70 increased with treatment temperature up to 40°C, at which point expression was 20.3-fold higher than at 37°C. The expression of the hsp27 and hsp83 genes only increased following treatment at 41°C. The results obtained show that the expression of investigated hsp genes increases after treatment at temperatures that did affect the survival of larvae.

Figure 2. Changes in hsp gene expression in (A) third-instar larvae and (B) females of M. scalaris (mean ± SEM) following temperature treatment.
Effect of Temperature Pre-treatment on Thermoresistance

Upregulation or induction of hsp genes at temperatures lower than those leading to loss of viability suggested that pre-treatment should increase thermotolerance. In fact, it was the case in our study as temperature pre-treatment as a whole had statistically significant effect on induced thermotolerance in larvae (one-way ANOVA, $F_{5, 12} = 657.47, P < 0.001$) and adult males ($F_{5, 12} = 196.32, P < 0.001$) and females ($F_{5, 12} = 241.74, P < 0.001$). Pre-treatment at a 31°C temperature which did not change hsp expression had no effect on larvae and adult thermotolerance (Table 2). Pre-treatment of larvae at 33°C, which stimulated upregulation of hsp22, hsp23, and hsp26, leads to a small but statistically significant increase in thermotolerance. A small (0.3°C) but significant increase of thermotolerance at 33°C pre-treatment was also observed in females (but only hsp26 upregulated; Fig. 2B), while pre-treatment of adult males had no effect (Table 2). An increase in pre-treatment temperature to 35°C was found to upregulate expression, not only of hsp26, but also of hsp40. Upregulated expression of the two genes did increase thermotolerance in adult insects. A further increase in pre-treatment temperature (to 38°C) induced expression of hsp70, while 40°C for adult and 41°C for larvae upregulated expression also of hsp83. Induction/upregulation of these genes correlated positively with thermotolerance in both larval and adult Megaselia.

DISCUSSION

In nature, insects may face regular exposure to such stresses as heat, cold, desiccation, and chemical toxins, making it likely that they will have evolved mechanisms to cope with the associated effects. Thermal stress, for example, gives rise to several changes in the insect transcriptome and proteome. Phoridae have not hitherto been studied from the point of view of thermotolerance and regulation of hsp gene expression by elevated temperature. Recently, there is available only one dataset of unassembled and unannotated transcriptome of M. scalaris (SRA: SRX208999) and Megaselia abdita (SRA: ERX168854).

Table 2. Influence of pre-treatment temperature on induced thermotolerance in third-instar larvae and imagoes (males and females) of M. scalaris (mean for three replicates).

<table>
<thead>
<tr>
<th>Stage and sex</th>
<th>Temperature for basal thermotolerance</th>
<th>31</th>
<th>33</th>
<th>35</th>
<th>38</th>
<th>40</th>
<th>41</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larvae</td>
<td></td>
<td>42.2$^a$</td>
<td>42.4$^a$</td>
<td>43.0$^b$</td>
<td>43.1$^b$</td>
<td>44.2$^c$</td>
<td>nd</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td>41.3$^a$</td>
<td>41.5$^a$</td>
<td>41.5$^a$</td>
<td>42.5$^b$</td>
<td>43.1$^c$</td>
<td>43.1$^c$</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td>41.3$^a$</td>
<td>41.6$^a$</td>
<td>41.9$^b$</td>
<td>42.6$^b$</td>
<td>43.1$^d$</td>
<td>43.4$^d$</td>
</tr>
</tbody>
</table>

SEM (not shown) were always < 0.1. Thermotolerance is defined as a temperature at which 50% of animals die ($LT_{50}$) as suggested by the probit regression model. Different letters in superscripts show values differing significantly from one another (Scheffe’s post hoc test, $P < 0.05$).

J. Exp. Zool.
The proper investigation of *hsp* gene expression is predicated on the correct selection of the reference gene(s). Tubulin and Efi were proposed as reference genes in the case of gene-expression profiling under temperature stress in the case of *D. melanogaster* (Ponton et al., 2011). Tubulin has also been top-ranked as a reference gene in expression studies relating to the booklouse *Liposcelis bostrychophila* (Jiang et al., 2010). Tubulin, 18S rRNA, and Rps20 were likewise reported to be the best reference genes in relation to fifth instars of the pink stem-borer *Sesamia inferens* exposed to different temperatures (Sun et al., 2015). The results of our analyses are, thus, consistent with most of these studies, given that tubulin (followed by Efi) emerged as the best reference gene (Table 1) in the case of both the third-instar larvae and the imagoes of *M. scalaris*.

We found that expression of *hsp* genes in larvae and imagoes of *M. scalaris* is upregulated at different temperatures, with only one (*hsp70*) of the seven analyzed in fact being induced by high temperature. It is not surprising as *hsp70* appears to be developmentally regulated, with lower basal levels occurring in stages in which alternative physiological strategies exist to protect the organism from stress or when rapid growth is required (Karouna-Renier and Rao, 2009; see also Fig. 2). The results reported herein are consistent with those obtained, for example, for *D. melanogaster* (Feder et al., ’97), the Mediterranean fruit fly *Ceratitis capitata* (Kalouska et al., 2009), the nonbiting midge *Chironomus dilutus* (Karouna-Renier and Rao, 2009), and the corn earworm *Helicoverpa zea* (Zhang and Denlinger, 2010). *Hsp70* is associated with *Hsp83*, the *Drosophila* homolog of the mammalian Hsp90 family of molecular chaperones. Increased expression of *hsp83* was observed in both larvae and imagoes of *M. scalaris* though the maximum level of upregulation in the former was more than threefold higher than in the latter. This correlates well with greater thermo resistance among *M. scalaris* larvae, and is consistent with the results obtained by Duncan (2005) in relation to *D. melanogaster* Schneider S2 cells, whereby increased levels of Hsp70 did not ensure thermotolerance when the activity of Hsp90 was repressed.

A widely accepted model for chaperone action suggests that denatured proteins are recognized by an Hsp40 protein that delivers the protein to Hsp70. Once in a complex with Hsp70, the J-domain of Hsp40 stimulates ATP hydrolysis on Hsp70, which promotes a conformational change in the Hsp70 substrate binding domain and increases its binding affinity for the misfolded proteins. Hsp40 is liberated from the Hsp70:protein complex following ATP hydrolysis. Protein remains bound to Hsp70 until ADP is exchanged for ATP via a diverse class of nucleotide exchange factors. Specific nucleotide exchange factors act on Hsp70 to release the bound client proteins and allow them to reature to their native state (Schlecht et al., 2011). In our experiments, upregulation of *hsp40* in *M. scalaris* began following treatment of larvae at 37°C and adults at 35°C.

There are three to four temperatures which activate *hsp* gene transcription. *Hsp22*, *hsp23*, and *hsp26* are genes that increase expression beyond 33°C treatment in larvae. The expression profile for thermal treatment of *hsp26* is similar in larvae and adults, but expression of *hsp22* activates the treatment at a temperature that is higher by 5°C. Expression of *hsp23* in the adult is low. Different expression patterns in response to thermal stress are consistent with previous reports, for example, in adult western flower thrips *Frankliniella occidentalis* hsp70 was induced at 31°C, while *hsp40* at 37°C (Wang et al., 2014). A similar situation was observed in the beet armyworm *Spodoptera exigua* where two different genes, *hsp70* and *hsp90*, were induced at different temperatures (Jiang et al., 2012). Jiang et al. (2012) also showed that *hsp* induction depends on the developmental stage of the insect. In the second instar larvae and elder larvae of *S. exigua*, *hsp70* was induced at 41°C and at 43°C, respectively.

Activation of *hsp* gene-expression at temperatures lower than those impairing *Megaselia* viability implies that larvae and imagoes alike have some time available to synthesize Hsp proteins, with a view to their achieving protection from acute thermal stress. Our results, therefore, suggest that it is not only the level of expression, but also the temperature interval between the activation of *hsp* genes and the viability measured as LT50, that are important in the mechanism of thermal adaptation. For correct measurement of gene expression at high temperatures, certain problems nevertheless arise, given that larvae and imagoes are comatose, while some may even be dead. Although the presence of dead insects at the end of the treatment cannot be precluded, their impact on the measurement of gene expression should be not critical given that most larvae and imagoes showed signs of life 4–5 hr after the treatment, and they were dying later.

Expression of *hsp* is known to be modulated during development—a period in which they have been found to serve different functions in the protein synthesis and turnover (Tower, 2011). *Hsp23* mRNA has been reported as undetectable in 4-day-old females of *D. melanogaster* (Mason et al., ’84). Analysis of changes in the transcriptome during *D. melanogaster* development has also shown elevated *hsp23* expression in larvae and pupae, as against limited expression in adults (Graveley et al., 2003). Increased expression of *hsp26* or *hsp27* has been found to increase stress resistance in *D. melanogaster* (Wang et al., 2004), *Apostichopus japonicus* (Zhao et al., 2011), *Chironomus riparius* (Martinez-Paz et al., 2014), and may play an important role in response to acute temperature and cadmium stress in *Larimichthys crocea* (Yang et al., 2012).
There are certain situations in which one stressor can enhance tolerance to others. In the natural habitat, insects encounter a combination of multiple acute and long-term stress factors that can affect expression of hsp genes. Mild temperature hardening is one of the most frequent stresses confronting insects in nature, and improved heat resistance following heat hardening has often been associated with the induction of heat shock proteins. Flies pre-heated at more moderate temperatures survive acute temperature stress better than untreated flies (Table 2), a finding consistent with the data obtained for Drosophila (Hoffmann et al., 2003; Morrow et al., 2006; Carmel et al., 2011). Enhanced thermotolerance as a result of increased hsp expression was inter alia reported for the flightless midge Belgica antarctica (Rinehart et al., 2006), pea leafminer Liriomyza huidobrensis (Huang et al., 2007), guava fruit fly Bactrocera correcta, and oriental fruit fly Bactrocera dorsalis (Hu et al., 2014).

According to DeBano and Klopatek ('87), depending on fire severity, temperature in the soil at 5 cm during light, moderate and high fire was below 50, at 50 and 75°C, respectively. In a Eucalyptus forest after 2 hr of fire when temperature at the surface exceeded 250°C, the soil temperature at a depth of ca. 15 cm was only 35°C (Beadle, '40). Considering that larvae of M. scalaris can penetrate the soil at more than 50 cm, they have a chance to survive forest fire by tunneling into deeper soil layers. As fire may cross an area rapidly, the greater thermotolerance in M. scalaris larvae may be another important factor in their survival.

ACKNOWLEDGMENT

We thank Justin Lack (University of Wisconsin) for his comments on the manuscript.

LITERATURE CITED


J. Exp. Zool.


Rasmussen DA, Noor MA. 2009. What can you do with 0.1x genome coverage? A case study based on a genome survey of the scuttle fly Megalothaenius scalaris (Phoridae). BMC Genomics 10:382.


