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Analysis of heat shock protein genes expression in spruce bark beetle *Ips typographus* and their importance for survival upon exposure to heat

Master Thesis

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Annotation

The aim of this work was to examine the expression of certain *Hsp* genes upon heat exposure in spruce bark beetle *Ips typographus*. We determined the level in unstressed and heat-exposed animals, and attempted to assess the importance of Hsp proteins for animals' survival upon heat treatment. We used RNA interference to knock down the expression of these genes and analyzed the influence on animals exposed to elevated temperatures.

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Kateřina Švehlová

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1. Introduction

1.1 Spruce bark beetle *Ips typographus*

Eight-spined bark beetle (*Ips typographus* (L.)) is a common European pest of spruce tree woods throughout Europe. We decided to use this insect species for our study of heat shock proteins for two reasons: firstly, it undergoes diapause. In a later part of this study, it would permit us to compare the heat shock response of nondiapausing animals with the reaction of diapausing individuals. Secondly, as a species that lives in a temperate climate, it survives a wide range of temperatures that it needs to cope with during its development and adulthood. As a non-model organism we decided to use the RNA interference technique to knock down one of the heat shock proteins - *Hsp70A* - and elucidate its function and function of other heat shock proteins in *I. typographus* during stress response.

1.2 RNA interference

RNA interference was discovered in 1998 by Fire and coworkers (Fire et al., 1998) in a nematode *Caenorhabditis elegans*, and has immediatelly attracted much attention as a novel molecular tool in biology. The method was especially appealing, because it enabled the study of genetically nontransformable species using reverse functional genomics.

Double-stranded RNA can trigger the silencing of genes of complementary sequence, a characteristic that is widely conserved among eukaryotic organisms - both plants and animals (Cogoni et al., 1996, Napoli et al., 1990, Cerutti et al., 2000). RNAi pathway is triggered by either endogenously produced or exogenously delivered RNA, and causes degradation of homologous transcripts. Inside the cell, the dsRNA is cleaved by Dicer, an RNAse III-type enzyme (Bernstein et al., 2001), producing short RNA molecules (small interfering RNAs, siRNAs). These are loaded into RNA-mediated silencing complex (RISC) which then targets and degrades mRNAs of corresponding sequence (Hammond et al., 2000). The main component of RISC complex are proteins from the Argonaute family that mediate the target recognition and cleavage (Hammond et al., 2001). The basic principle is shown in Fig. 1.

In genetically transformable species like *Drosophila melanogaster*, a transgene containing an inverted repeat of a gene fragment can be expressed in the animal which then produces a dsRNA hairpin, and initiates the RNAi pathway (Kennerdell et al., 2000). In non-model organisms, the dsRNA can be delivered in several ways such as: injecting, feeding, soaking, virus-mediated

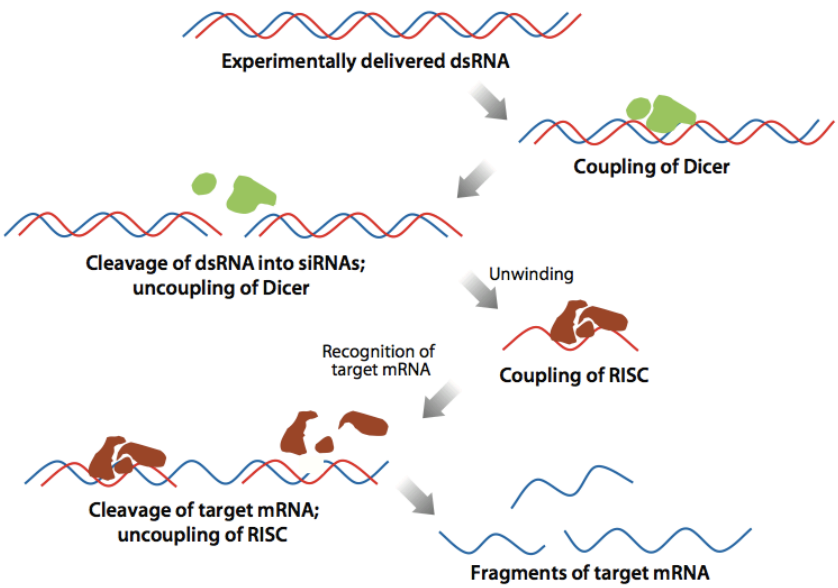


Fig. 1. Basic mechanism of RNA interference. The double-stranded RNA is cleaved by Dicer into short fragments which are then unwound and the anti-sense strand is loaded into RISC (RNA-induced silencing complex). The anti-sense RNA acts as a template for recognition of the target mRNA. Thus, the appropriate mRNA can blocked and degraded by RISC. Adapted from Bellés, 2010.

(Uhlirova et al., 2003), or transmitted from the previous generation (so called parental RNAi; Bucher et al., 2002).

1.2.1 RNAi in different species

For a species to be considered RNAi-sensitive, it is important that the interference has both transitive and systemic properties (May et al., 2005). In *C.elegans*, transitive properties derive from the production of secondary siRNAs through the action of RNA-dependent RNA polymerase (RdRP). In this process, primary siRNAs function as primers on target mRNA causing a number of secondary dsRNAs and subsequent siRNAs to be generated. By this process the RNAi signal is amplified, and is then able to silence the vast amount of target mRNA (Alder et al., 2003; see Fig 2.). Note that at this step when these otherwise unstable primary siRNAs are not able to find a homologous target, they are quickly degraded and the reaction dies out leaving undetectable siRNA levels (Plasterk, 2002).

Systemic properties refer to the spread of RNAi effect throughout the body and various tissues (Sijen et al., 2001). In *C. elegans*, *sid-1,* a gene coding for multi-transmembrane domain protein, is believed to function as a passive dsRNA channel that mediates this effect (Winston et al., 2002). Recently, it has been discovered that cellular endocytosis, and even active trans-membrane transport is likely to play an important role in dsRNA uptake as well (Saleh et al., 2006; Sundaram et al., 2006).

In certain species the RNAi effect is spread throughout the body while others do not show a robust systemic RNAi response (Miller et al., 2008). In fact the RNA interference mechanism per se can be divided into two parts: one is the core mechanism of RNA silencing inside the cell, and the

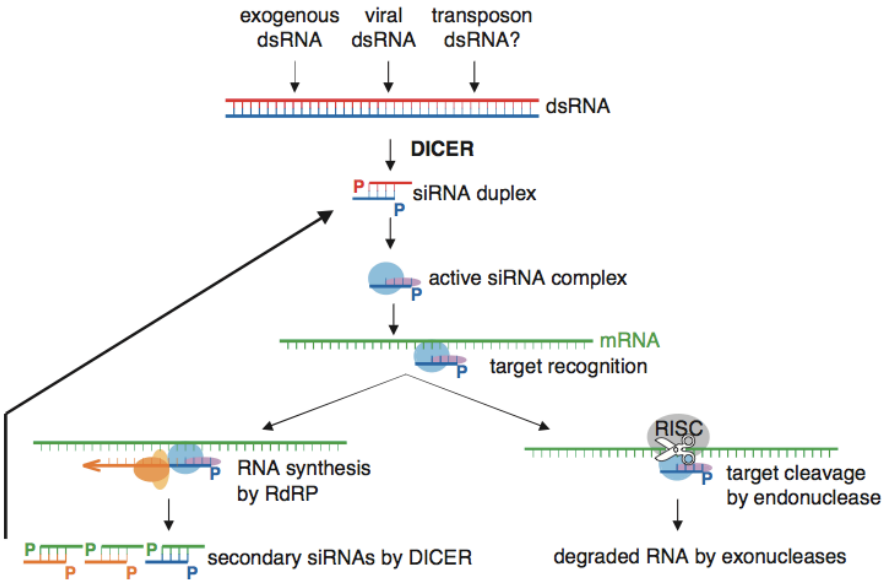


Fig. 2. A model of RNAi signal amplification. After cleavage of dsRNA by Dicer, the anti-sense fragment binds to the target mRNA and acts as a primer for synthesis of complementary strand of the mRNA. This dsRNA is then cleaved by Dicer producing a large amount of secondary siRNAs and vastly amplifying the RNAi signal. Adapted from Plasterk, 2002.

other is the ability of cells to uptake and amplify the dsRNA. While the core mechanisms, such as the protein Dicer and Argonaute, appear to be conserved between a nematode *C. elegans* and insects (Tomoyasu et al., 2008), genes involved in the uptake, spread, and amplification of dsRNA signal seem to be poorly conserved or absent in available insect genomes compared to *C. elegans*. Indeed, when Miller and coworkers overexpressed dsRNAs in the cells of *D. melanogaster*, it readily induced RNAi in tissues normally resistent to systemic RNAi (Miller et al., 2008). Their work suggests that if a species is poorly sensitive to RNAi treatment, it is rather due to a faulty or absent cell penetration and transmission of RNAi signal rather than to the core RNAi machinery itself. Several possible causes of RNAi insensivity are summarized in Table 1.

Table 1. Possible causes of RNAi insensivity in insects. Adapted from Bellés, 2010.

Species-specific	Exogenous dsRNAs are efficiently degraded
	Deficient amplification and spreading of dsRNA signal
	Low response of core RNAi machinery genes after dsRNA introduction
Tissue-specific	Tissue is badly permeable to dsRNAs
	Low response of RNAi machinery genes in the tissue
Gene-specific	Particular dsRNA is efficiently degraded

Species-specific	Exogenous dsRNAs are efficiently degraded
	Increased transcription of the gene to overcome the effect of dsRNA
	Target mRNA is protected against RNases
	Transient or insufficient expression of gene to be silenced

We have tried to establish RNA interference in bark beetle *Ips typograpus*, and attempted to exploit this method to study the function and dynamics of some of the heat shock proteins upon heat shock exposure in this species.

1.3 Heat Shock Proteins

Heat shock proteins (HSPs) are a class of proteins essential for keeping homeostasis in the cell. They execute it by means of protein folding, protein trafficking, protein aggregation into complex, or proteolysis. HSPs were first discovered in 1962 by Feruccio Ritossa who observed an enlargement of a certain part of chromosomes in flies *Drosophila melanogaster* that were exposed to heat (Ritossa, 1962), hence the name heat shock proteins. Since then, it has been shown that their expression is upregulated by a wide variety of environmental and metabolic stresses such as anoxia, starvation, exposure to toxins (heavy metal ions, ethanol, nicotine), surgical stress, and infection; a process termed (heat shock) stress response. After these findings, the name "heat shock proteins" might be a bit misleading, therefore they are often called stress proteins.

HSPs are ubiquitous, occuring in all studied organisms from bacteria to humans. They are classified on the basis of their molecular weight that ranges from 8 to 150 kDa (see Table 2). Some of the proteins are expressed continuously while expression of others is increased after stress induction.

Despite the case of their discovery, HSPs play an important role in cells under normal conditions as well. Nickamed "molecular chaperons", they bind to a newly-synthesized peptides and prevent it from unwanted association with other proteins until it is correctly folded into a functional protein (Bukau et al., 1998, Hartl, 1996). Their role in cell signaling has been deduced from their impact on the activity of various signaling molecules (Pratt et al., 2003). Hsp90 is for example known to guide kinase pp60 from cytosol into cellular membrane where the chaperone dissociates and allows pp60 to become an active kinase (Xu et al., 1993). In response to protein-denaturing stressors their transcription rate increases rapidly for the HSPs to assist in the repair of

denatured proteins, or, in case of irreversible damage, to mark them for subsequent degradation. On the other hand, expression levels of *Hsp70* that are either too high (Feder et al., 1992) or too low (Elefant et al., 1999) hinders or stops the cell growth in *Drosophila melanogaster*, indicating that correct regulation of HSPs expression is essential for cells survival.

Table 2. A selection of proteins belonging to the *Hsp* family, their molecular weight, and basic function in the cell.

HSP	Molecular Weight	Function In the Cell	Constitutive/ Inducible	Note
Hsp90	90 kDa	protein folding, protein trafficking and stabilization,regulation of client protein activity	constitutive	client proteins mostly signal transducers - kinases, steroid receptors
Hsp70	72 kDa	protein re/folding	highly inducible	down-regulates HSF-1 activity
Hsc70	73 kDa	de novo protein folding, complex formation, protein trafficking	constitutive	Hsp40 as co-factor
Hsp23	23 kDa	protein folding co-chaperone, protein stabilization against aggregation during cell stress		co-factor of Hsp90
Hsp23 pseudogene	N/A	might function as a transcription regulator for other *Hsp* genes (Rinehart et al., 2007)		obsereved also in *S. crassipalpis*
Ubiquitin	8 kDa	involved in protein degradation in proteasome		

1.3.1 Heat shock protein folding machinery

Heat shock proteins do not determine the terciary structure of folded proteins, but they rather help them find their natural tertiary structure more efficiently, and rescue them from “folding traps”. In this way they are not true cataysts because they increase the yield, not the speed, of the folding reaction. Still, the process of refolding requires energy that HSPs obtain from ATP hydrolysis

(Milarski et al., 1989). ATP cleavage results in conformational change of the chaperone that is essential for the folding procedure.

HSP monomers never work alone. They form either oligomers, or are helped by co-chaperones. The group of co-chaperones and other additional proteins that assisst the folding process are different for various client proteins, and so contribute to the substrate specificity (Bukau et al., 1998). In case of Hsp60 oligomeric folding complex, the client protein is completely isolated from the cytosol. It enters the Hsp60 complex inner cavity and then is separated from the outer environment by a Hsp10 heptameric "cap". Due to this insulation, the protein is protected from aggregation to other proteins. Subsequent ATP hydrolysis causes Hsp60 conformational change which loosens up the hydrophobic core of the substrate. During this partial unfolding process, water can enter into the core and facilitate reorganization (Csermely, 1999). ATP hydrolysis and binding of a new substrate at this time opens the Hsp10 cap and releases the client protein. For most proteins though, one round of this refolding process is not enough, and have to enter the cycle several times to eventually aquire the correct conformation.

1.3.2 Regulation of HSP genes transcription

Regulation of HSP genes transcription after stress is regulated at two levels: by heat shock transcription factor-1 (HSFs) and HSPs themselves. HSF-1 itself is partly capable to function as a stress-sensing molecule. Although the regulation of HSF-1 activity itself is complex, it is best described in a model termed "cellular thermometer". According to this concept, upon heat induction HSF-1 changes its conformation that enables this

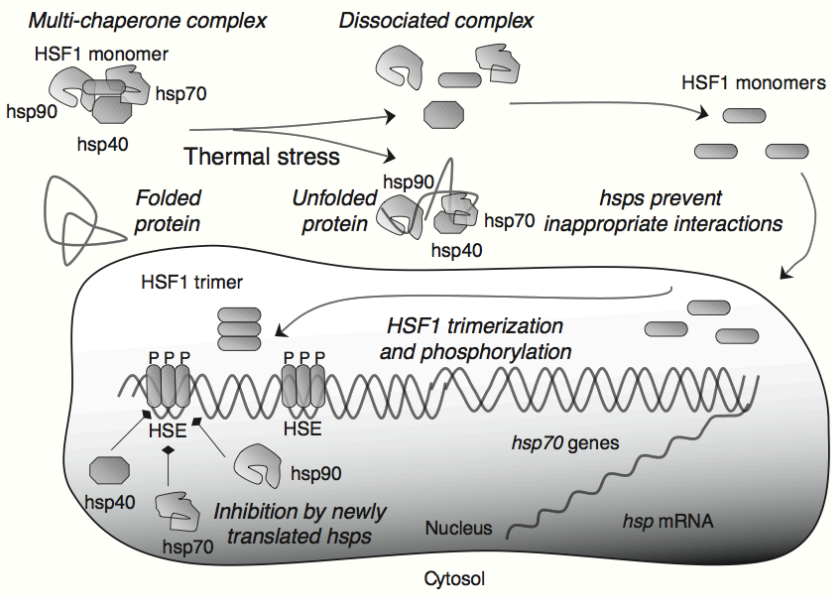


Fig. 3. A model for transcriptional regulation of *Hsp* genes expression. Under nonstresful conditions, heat shock factor 1 (HSF-1) monomers are bound to Hsp complex consisting of several heat shock proteins. Upon thermal stress, the chaperones dissociate to assisst the unfolded proteins in the cell, and free the HSF-1 monomers. These monomers can now move to the nucleus, trimerize and bind to heat shock element (HSE). Upon phophosphorylation, they become transcriptionally competent, and initiate the synthesis of heat shock proteins. Adapted from Tomanek et al., 2001.

monomeric molecule to assemble into trimers that, after their phosphorylation, induce HSPs production (Goodson et al., 1995). The second means of transcription regulation is the HSPs themselves. If they are not involved in chaperoning other proteins, they bind to HSF-1 monomer; particularly proteins from the 40 kDa, 70 kDa, and 90 kDa size classes (Baler et al., 1992). Thus, they decrease the levels of free HSF-1 monomers that otherwise trimerize and bind to heat shock elements, a regulatory sequence upstream of most HSP genes. Upon stress conditions and therefore elevated misfolded proteins, HSPs preferentially bind to these proteins, and release HSF-1 allowing it to move to te nucleus and induce production of more heat shock proteins. Thus, HSPs themselves play an autoregulatory role in determining the number of free HSF-1 monomers.

During stress, all posttranscriptional processes are generally stopped in the cell, therefore heat shock protein mRNAs had to devise various strategies how to bypass this drawback. Most HSPs for example do not have introns in their transcripts or the open reading frame lies after the intron, so that the translation can start even during the cellular proteosynthesis shutdown (Csermely et al., 1998).

2. Goals of Work

I. Identify genes encoding heat shock proteins in *Ips typographus*, namely *Hsp90*, genes from the 70 kDa family, and *sHsps*.

II. Determine lethal temperature of nondiapausing individuals of *I. typographus*.

III. Assess transcription level curves of isolated *Hsp* genes after heat exposure.

IV. Assess transcription level of isolated *Hsp* genes after RNA silencing against *Hsp70A* and determine RNA interference eficiency.

V. Determine survival rates of *I. typographus* after exposure to elevated temperatures.

3. Methods and Materials

3.1 Beetles Rearing

Spruce bark beetles *Ips typographus* were reared in logs at a constant temperature of 25 °C and a constant long-day light regime consisting of 16 hours in the light and 8 hours in darkness. The logs were sprayed with water two to three times per week. Every three to four weeks fresh uninfected logs were introduced to facilitate the newly emerged generation. The beetles collected for experiments were adult individuals.

3.2 Heat Shock Application

Adult beetles were collected from the breeding logs or low mason jars (depending on the particular-experiment setup) and placed in pairs into 0.2 mL PCR tubes. Beetles were then placed into cycler (XP Cycler, Bioer) and subjected to respective temperatures for 1 hour. After this treatment they were kept in mason jars with damp paper towels at 25 °C until further treatment:

i. In case of Lethal temperature Determination:

After 24 hours dead and living individuals were counted. Number of beetles in each group varied from 30 (mostly the case) to 59 animals.

ii. In case of Determining Expression og *Hsp* genes after heat exposure over time:

Animals were dissected and inner organs frozen in liquid nitrogen at the following times after the start of heat exposure to 44 °C: 20, and 50 minutes. After 1 hour of the heat application, remaining beetles were kept at room temperature and dissected at the following times after the start of the heat exposure: 90, 120, and 180 minutes. Another group of beetles was kept at room temperature throughout the whole experiment to determine the background level of measured *Hsp* genes.

iii. In case of Survival rate after RNAi experiment:

Dead and living animals were counted in 24-hour intervals starting 24 hours after the heat exposure until all individuals died (usually up to 168 hours).

iv. In case of Expression of Expression of *Hsp* genes after RNAi against *Hsp70A*:

120 minutes from the start of the heat exposure to 44 °C, animals were dissected and inner organs frozen in liquid nitrogen for RNA isolation.

3.3 RNA Isolation

RNA was isolated from abdomen inner organs dissected from one individual. The tissue was frozen on dry ice and stored at -80 °C. For the actual RNA isolation, the tissue was homogenized with plastic micro pestles in 1 mL of TRIzol Reagent (Invitrogen). Samples were incubated at room temperature for 5 minutes, and 0.2 mL of chloroform was added. The tubes were vortexed thoroughly for 30 seconds, incubated at room temperature for 10 minutes, and finally centrifuged for 15 minutes at 14,000 rpm and 4 °C. The aqueous phase containing the RNA was extracted and precipitated on ice with 0.5 mL of isoporpanol for 10 minutes. The tubes were centrifuged for 10 minutes at 14,000 rpm and 4 °C. The resulting pellet was washed with chilled 75% ethanol and centrifuged at 14,000 rpm for 5 minutes. Then the supernatant was discarded and the air-dried pellet was dissolved in 30 to 50 µL of DEPC-treated water. RNA concentration was measured using NanoDrop 2000 Spectrophotometer (ThermoScientific).

Isolated RNA was consequently treated with TURBO DNA-free kit (Ambion) to avoid genomic contamination. 3.5 µL of 10x TURBO DNase Buffer and 1 µL of TURBO DNase were added to 30µL of RNA sample and the reaction was incubated at 37 °C for 30 minutes. The DNase was then inactivated by 3.5 µL of DNase Inactivation Reagent. After 5 minutes of incubation at room temperature, the sample was centrifuged at 14,000 rpm for 3 minutes, and the RNA-containing supernatant was transferred into a fresh tube.

3.4 cDNA Synthesis and Amplification

Complementary DNA strand was synthesized using SuperScript III Reverse Transcription kit (Invitrogen). 1 µL of 50 µM oligo(dT) primer, 4 µL of 10mM dNTP, and 4 µL of DEPC-treated water were added to 4 µL (1.2 - 1.5 µg) of purified RNA. The mixture was incubated at 65 °C for 5 minutes, and then transfered onto ice for at least 1 minute. Subsequently, 4 µL of 5x First-Strand Buffer, 2 µL of 0.1 M DTT, and 1 µL of SuperScript III Reverse Transcriptase were added into the reaction. Content was mixed gently, and incubated at 50°C for 50 minutes. After the DNA strand was synthesized, the enzyme was inactivated at 70 °C for 15 minutes. The cDNA concentration was

determined using NanoDrop, and all samples were diluted to the final concentration of 200 ng/μL. The cDNA was then stored at -80 °C until further use.

3.5 Identification of *Ips typographus* Gene Orthologs

Orthologs of Heat Shock Protein genes 23, 70 inducible, 70 cognate and 90 were searched for in the NCBI database (http://www.ncbi.nlm.nih.gov/). Protein sequences of genes of interest in closely related insect species were obtained, and aligned in program MEGA 4 (Tamura et al., 2007) using the Clustal W system. Gene specific degenerated primers, presented in Table 3., were designed to isolate the *I. typographus* transcript orthologs.

Table 3. Sequences of degenerated primers used for isolation of *Ips typographus Hsp* gene orthologs using touchdown PCR.

Target transcript	Forward Primer (5‘ – 3‘)	Reverse primer (5‘ – 3‘)
Hsc70	CTACWGCCGGWGAYACMCAYTTGG	GGCCTCGTCYGGGTTRATGGAT
Hsp70 A *Hsp70B*	TAYGGNYTNGAYAARAAYYTNAA	YTTRTCCATYTTNGCRTC
sHspA	AGGARATYWCYGTSAARBTG	GWRATRSTCARMACYCCATC
sHsp23B	AGGARATYWCYGTSAARBTG	GWRATRSTCARMACYCCATC
Hsp90	GATGCBWTRGAYAARATCCG	GAAAYGTTCAARGGYARRTC
actin	GTBGGWCGYCCCMGDCAYCA	CCRGTGGTACGACCRGADGC

Touchdown PCR was performed on beetle cDNA, and the resulting products were analysed by gel electrophoresis. DNA of appropriate size was extracted from the gel, and cloned into pBluscript KS$^-$ vector. The vector was amplified in *Escherichia coli* strain XL1, and the insert sequenced. Obtained sequences were then blasted into NCBI database to check if they correspond to the desired gene transcript ortholog available in the database.

3.6 Touchdown PCR

The PCR reaction was prepared just as a standard PCR (described below) except for the primer molarity that was 100 μM instead of the usually-used 20 μM. The temperature profile of touchdown PCR was as follows: 2-minute denaturation at 94 °C, and 25 cycles of the following steps: 94 °C for 20 seconds, primer annealing for 30 seconds, and 72 °C for 80 seconds. The primer annealing temperature in the first cycle was 55 °C, and each step it was decreased by 0.5 °C to permit the most specific primer binding to occur early in the reaction and allow the transcript of interest to outcompete other less specifically binding sequences. This imbalance was further enforced by another set of 30 cycles of the following steps: 94 °C for 20 seconds, 45 °C for 30 seconds, 72 °C for 80 seconds; and final elongation at 72 °C for 5 minutes.

3.7 DNA Extraction From Agarose Gel

To extract and purify DNA products from agarose gel, MinElute Gel Extraction Kit (Qiagen) was used, and the instructions from the manual were followed. The method is based on solubilization of DNA-containing agarose and adsorption of nucleic acids to silica-gel particles at high-salt concentration. Bound DNA is then eluted in a low-salt solution of the Elution Buffer.

3.8 Cloning and Plasmid Transformation into *E. coli*

Purified PCR products were cloned into pBluescript KS^- vector (Stratagene) using T-A cloning system. 6 μL of the product were added to 2 μL of the vector, 1 μL of 10x T4 Ligation Buffer, and 1 μL of T4 DNA ligase (both USB). The reaction was incubated at 4 °C overnight.

6 μL of the ligation reaction was then transferred onto thawed *E. coli* XL1 competent cells, and let sit for 20 minutes on ice. Induction of the cells was achieved by heat shock at 42 °C for 45 seconds and subsequent cooling on ice for 2 minutes. The cells were then mixed with 300 μL of SOC medium, and incubated while shaking at 37 °C for 45 minutes. Approximately 100 to 150 μL of the bacterial suspension was spread onto an LB-agar plate containing carbenicillin (100 μg/mL), and X-gal (80 μg/μL). Plates were incubated at 37 °C overnight.

Colonies carrying vector were pre-selected by blue-white selection. To confirm that the vector also contains the insert of expected size, PCR was performed using portion of the colony as

template. The reaction set-up was as follows: 0.4 µL of 20 µM universal forward and reverse primer (for sequence see Table 4.), 10 µL of PPP Master Mix (Top-Bio), and 8.2 µL of MilliQ water. Finally, part of the colony was transferred into the reaction on a pipette tip.

Table 4. Sequences of universal primers used in PCR to confirm the presence of insert of the expected size. Primers are specific to sequences flanking the multiple cloning sites of KS(-) BlueScript vector.

Forward Primer (5' - 3')	Reverse Primer (5' - 3')
GTAAAACGACGGCCAGT	GCGGATAACAATTTCACACAGG

The PCR temperature profile was set: initial denaturation at 94 °C for 5 minutes; 25 cycles of the following sequence: denaturation at 94 °C for 20 seconds, primers annealing at 55 °C for 20 seconds, elongation at 72 °C for 50 seconds; and final elongation at 72 °C for 5 minutes. PCR products were analyzed by 0.75% SeaKem LE agarose gel electrophoresis (Lonza). Plasmids that contained insert of the expected size were amplified in *E. coli* XL1 strain and sent for sequencing.

3.9 Plasmid Amplification and Isolation

Colony carrying desired plasmids were inoculated into a 4 mL LB-medium tube with carbenicillin and incubated for 16 hours at 37 °C with shaking.

Plasmid DNA was isolated using High Pure Plasmid Isolation Kit (Roche) according to the manual instructions. The system relies on alkaline lysis of the cells to free the plasmid DNA. The solution is then cleared of cell debris and chromosomal DNA, and plasmids are bound to glass fiber filter present in the Filter Tube. Because this bond is unique only to nucleic acids, the tube can then be washed from remaining cellular residues. To elute the plasmids, the nucleic acid-glass bonds are disrupted by low-salt Elution Buffer.

The plasmid DNA was then stored at 4 °C or -20 °C until further use.

3.10 DNA Sequencing

Vectors containing inserts of interest were sequenced commercially by Macrogen, Inc. (http://dna.macrogen.com/).

3.11 qRT-PCR Primers Design and Testing

qRT-PCR primers were designed in MEGA 4 program to recognize specifically individual heat shock proteins and their respective isoforms. Their biochemical properties were inspected in PrimerSelect program (Lasergene package, DNASTAR). Eligible pairs were then comercially synthetized by Generi-Biotech, and tested. In case of genes with different isoforms, vectors containing corresponding parts of individual isoforms as insert were used as templates to exclude possible annealing to both transcripts.

Primers used for qRT-PCR are shown in Table 5.

Table 5. Primers used for qRT-PCR that are specific for individual isoforms of genes from the *Hsp* family and actin in *Ips typographus*.

Target Transcript	Forward Primer (5' - 3')	Reverse Primer (5' - 3')
Actin		
sHspA		
sHspB		
Hsp70B		
Hsp70A		
Hsc70		
Hsp90		

3.12 qRT-PCR

The relative amount of specific transcripts in individual cDNA samples were determined by qRT-PCR. The reaction mixture consisted of 0.25 µL of 20 µM specific forward and reverse primer, 6 µL of iQ SYBR Green Supermix (Bio-Rad), 2.5 µL of DEPC-treated water, and 3 µL of cDNA template. The temperature profile of the reaction was: 94 °C for 40 seconds; 40 cycles of the following succession: 94 °C for 30 seconds, 53 °C for 30 seconds, 72 °C for 35 seconds. After each cycle the amount of SybrGreen fluorescence was measured, and at the end of the PCR melting curve analysis (gradual temperature rise from 65 °C to 95 °C) was performed to check for the presence of multiple products.

3.13 dsRNA Design, Synthesis, and Application

Hsp70B dsRNA was designed as long as possible from the available portion of transcript sequence, but to still allow to design one of the two specific qRT-PCR primers outside the dsRNA section. This was to avoid the unlikely but theoretically possible contamination of subsequent cDNA samples with extraneously delivered template DNA while injecting animals with dsRNA.

The dsRNA portion was then amplified using primers shown in Table 6 (row "1st reaction") and cloned into pBluescript KS- vector. T7 RNA polymerase promoter was located upstream of the insert sequence, while downstream T7 RNA polymerase promotor was added during subsequent PCR. Primers that were used for the amplification are in Table 6 (row "2nd reaction"). Custom-synthetized reverse primer contains T7 RNA polymerase promotor sequence so that the resulting PCR product is flanked with T7 RNA polymerase promotors from both sides. The product was analyzed with agarose gel electrophoresis and then purified using MinElute Gel Extraction Kit (Qiagen).

Table 6. Sequences of two primer pairs used in two successive PCR reactions to synthesize *Hsp70A* dsRNA.

	Forward Primer (5' - 3')	Reverse Primer (5' - 3')
1st reaction	CTTTGATCTGGGAGGCGGAACT	GTAACTTTGGTGTAGAAGTCG
2nd reaction	GTAAAACGACGGCCAGT	TAATACGACTCACTATAGGGAACAAAAG

Both sense and anti-sense RNA strands were synthesized with MEGAScript RNAi Kit (Ambion) in one reaction tube. The mixture contained 8 µL of NTP, 2 µL of T7 Enzyme Mix, 2 µL of 10x T7 Reaction Buffer, and 8 µL of DNA template (or 500 ng). The reaction was incubated at 37 °C for 4 to 6 hours. Resulting complementary ssRNA products were then annealed by heating to 95 °C in a water bath and letting it cool down slowly at room temperature. dsRNA was then diluted with 5x modified Ringer solution to a final concentration of 4 µg/µL.

Adult beetles were anesthetized with flowing carbon dioxide and attached to a glass slide. Ds RNA was injected with glass capillary (type G-100, Narishige) using capillary micromanipulator (MN-151, Narishige). About 1 µL (i.e. 4 µg of dsRNA) of dsRNA solution was injected into each individual. The beetle was then placed into a mason jar filled with damp paper towels and freshly

peeled spruce bark, and kept at 25 °C for four days until further treatment. The treatment itself was of two kinds:

i. Beetles were exposed to a heat shock at 44 °C for 1 hour and mRNA levels of *Hsp* gene transcripts were measured by qRT-PCR. Twenty beetles were used for each treatment which were then divided into four groups of five individuals. The RNA was isolated one hour after the heat exposure from each group. Thus, each measured sample is a mixed cDNA from five animals.

ii. Groups of 20 to 30 beetles were exposed to temperatures ranging from 40 to 47 °C and lethality rate was obsereved after 24, 48, 72, 96, 120, 144, 168 hours from the heat exposure.

4. Results

4.1 Isolation of *Ips typographus* heat shock gene orthologs

Touchdown PCR yielded clones of partial transcripts of the gene Hsp90, and three genes from the *Hsp* 70 kDa family: *Hsc70*, and two isoforms of *Hsp70* that we designated *Hsp70A* and *Hsp70B*. Two possible isoforms from the small *Hsp* family that we isolated were matched to a putative *Hsp21* and a putative *Hsp23*. In case of *sHspA* the score for *Hsp21* and *Hsp23* was 78 % identity, 91 % positives and 69 % identity, 87 % positives respectively. In case of *sHspB* the score for *Hsp21* and *Hsp23* was 75 % identity, 94% positives and 71 % identity, 94 % positives respectively. Lengths of isolated sequences and orthologs with the highest match are summarized in Table 7. The identity of the partial transcripts was determined using blastx search in GenBank (http://www.ncbi.nlm.nih.gov/).

Table 7. Partial mRNA sequences from the *Hsp* family isolated from *Ips typographus*. Lenght and blastx closest match is displayed for each transcript.

gene isoform	sequence length	blastx closest match
Hsp90	970 bp	putative heat shock protein 90 (*Pediculus humanus*), EEB15725.1 identities 69 %, positives 84 %
Hsp70A	400 bp	heat shock protein 70 (*Anatolica polita*), ABQ39970.1 identities 97 %, positives 100 %
Hsp70B	400 bp	heat shock protein 70 (*Anatolica polita*), ABQ39970.1 identities 93 %, positives 98 %
Hsc70	603 bp	heat shock cognate 70 (*Neobathyscia mancinii*), ADZ14886.1 identities 95 %, positives 98 %
sHspA	141 bp	putative small heat shock protein (*Tribolium castaneum*), EFA09076.1 identities 78 %, positives 91 %
sHspB	156 bp	putative small heat shock protein (*Tribolium castaneum),* EFA08892.1 identities 75 %, positives 94 %
actin	307 bp	cytoplasmic actin (*Ips confusus*), ACV13203 identities 99%, positives 100%

4.2 qRT-PCR primers design

Primers used for qRT-PCR were designed and tested to be specific for individual isoforms in case of *Hsp70* and *sHsp* transcripts. PCR was performed and analysed by agarose gel analysis. Reaction with satisfactory primers yielded products only when the clone with the respective isoform was used as a template. For primers sequences see Materials and Methods.

4.3 Survival Rate After Heat Exposure

Spruce bark beetle lethal temperature had to be determined for further research. The survival rate after heat exposure to respective temperatures for 1 hour is shown in Fig. 4. The figure indicates that exposure to 47 °C is critical with survival rate of ~ 25 %, while 48 °C is lethal to all tested beetles.

In our study we decided to expose animals to the temperature of 44 °C for 1 hour; an exposure that is not lethal, but should be sufficient to induce a detectable heat stress response.

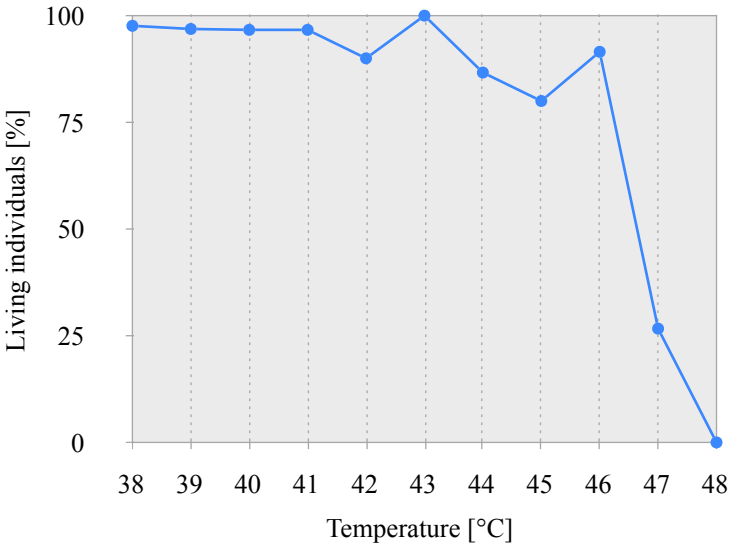


Fig. 4. Survival rate of beetles exposed to respective temperatures (from 38 to 48 °C) for one hour. Only 25 % of beetles survived the next 24 hours after exposure to temperature of 47 °C while the temperature of 48 °C was lethal to all tested beetles.

4.4 Expression of *Hsp70* family genes after heat exposure over time

To determine which heat shock protein genes are induced after exposure to heat, we measured three genes from the 70 kDa *Hsp* family. Beetles were exposed to 44 °C for 1 hour and then kept at room temperature for the remaining time. Each RNA sample was prepared from the guts of 5 individuals. Two groups, comprising of 5 animals, were dissected at each of the following times from the start of the heat exposure: 0, 20, 50, 90, 120, and 180 minutes. One group were the control individuals

that were kept at room temperature throughout the experiment. The latter were the heat-exposed animals. The relative mRNA levels of individual genes are depicted in Fig. 5.

This experiment revealed that only *Hsp70A* expression was highly elevated after exposure to 44 °C (110-fold increase after 120 minutes from the start of the exposure). At the same time, its level in non-heat exposed animals was very low. This result shows that *Hsp70A* is strongly induced after heat exposure with the mRNA level peak occuring at 120 minutes after the start of the experiment. The other two transcripts, *Hsp70B* and *Hsc70*, did not show a significant change in expression after heat exposure (See Fig 5).

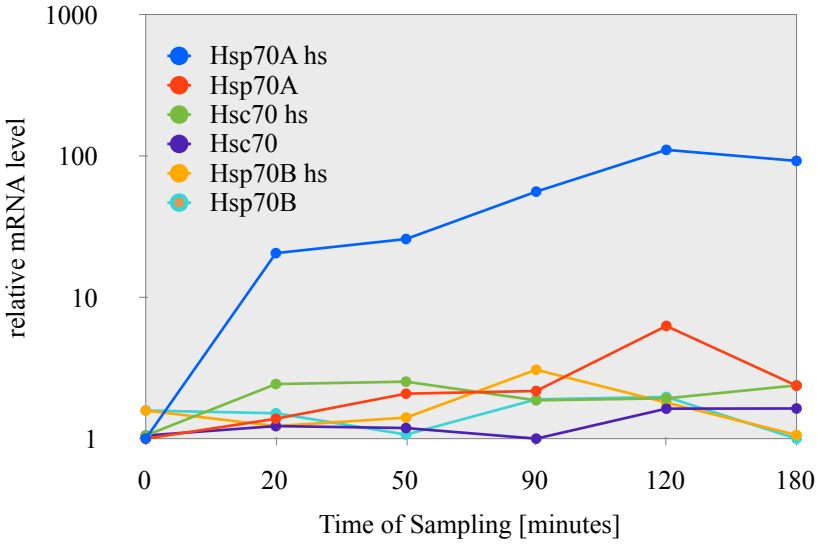
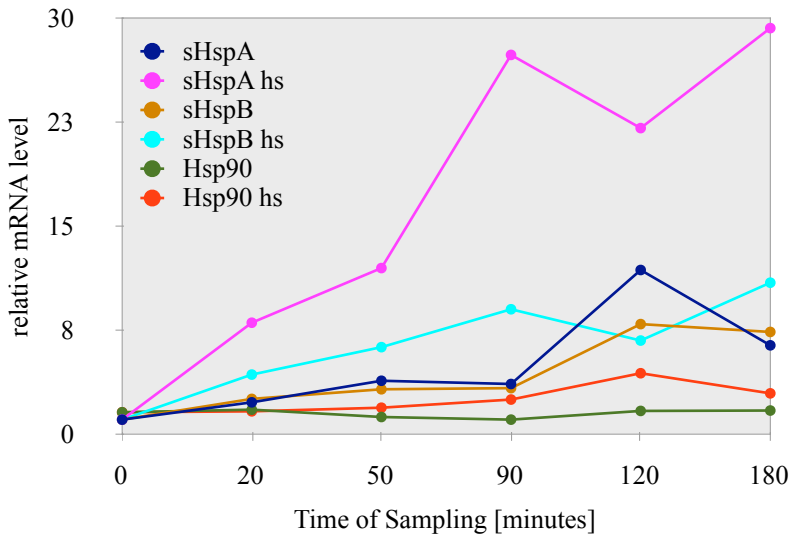


Fig. 5. Relative mRNA levels of three isoforms from the *Hsp* 70 kDa gene family. The only isoform that shows a significant increase after induction by heat is *Hsp70A*. The peak of mRNA amount is at 120 minutes after the start of the heat exposure. Negative controls (Hsp70A, Hsp70B, Hsc70) are levels of respective mRNA in animals kept at room temperature. Note the logarithmic scale.

4.5 Expression of two *sHsp* isoforms and *Hsp90* gene after heat exposure

Next we investigated how *sHsp* isoforms and *Hsp90* gene expression levels change after heat exposure to 44 °C. One of the *sHsp* isoforms, *sHspA,* displayed a high upregulation after the treatment compared to the level of mRNA in control animals (almost 30-fold increase 180 minutes after the start of the exposure). On the other hand, expression of the second isoform, *sHspB*, increased slighly in both heat-exposed and control animals (7- and 10-times respectively after 180 minutes from the start of the exposure). The transcript level of *Hsp90* rose mildly in heat-exposed animals compared to the untreated ones (4-times after 120 minutes from the start of the exposure). For details see Fig. 6.

Fig. 6. Relative mRNA levels of two *sHsp* isoforms and *Hsp90* gene. *sHspA* shows a significant increase in expression (sHspA hs) compared to heat-unexposed controls (sHspA). *sHspB* displays a slight increase in expression in both control and heat-exposed animals (sHspB, sHspB hs respectively). *Hsp90* mRNA level in heat-exposed animals rises mildly compared to control animals (Hsp90 hs, Hsp90 respectively) with highest level occuring 120 minutes after the heat exposure.



4.6 Expression of *Hsp* genes after RNA interference against *Hsp70A*

To test if Hsp70A protein is essential for bark beetle's survival after heat stress, we wanted to use RNA interference to down-regulate *Hsp70A* expression. To test if we can sufficiently lower the protein production, we tested the method using four groups of beetles that were treated as follows:

i. 40 animals injected with dsRNA against *Hsp70A* mRNA,
ii. 40 animals injected with dsRNA against *eGFP,*
iii. 40 animals injected with modified Ringer solution,
iv. 40 animals uninjected.

Half of each group was used to determine the background level of each gene transcript. The remaining half was exposed to temperature of 44 °C for one hour, and sampled after 120 minutes from the start of the heat exposure. Each sample denoted in Figures 7 to 9 contains a mixed cDNA from 5 individuals. Fig. 7. shows mRNA levels of *Hsp70A* in differently treated groups of beetles. As would be expected, heat-exposed uninjected animals (NC hs - negative control, heat shock) display a highly increased level of *Hsp70A* expression. On the other hand, heat-exposed beetles that were injected with *Hsp70A* dsRNA show a significantly decreased level of *Hsp70A* mRNA compared to heat-exposed uninjected animals. This might indicate that the RNA silencing was successful. Curiously, animals injected with modified Ringer solution and *eGFP* dsRNA display the same, or even greater decrease in *Hsp70A* mRNA levels.

Hsp70B, *Hsc70*, and *Hsp90* mRNA levels show a large variability among individual samples in certain groups, but overall they do not show significant differences among differently treated groups (Fig. 8).

sHsp23A and *sHspB* expression levels follow a similar pattern. Interestingly, both isoforms are highly elevated in the heat-exposed *Hsp70A* dsRNA-injected group. A somewhat higher expression was also detected in all remaining heat-exposed groups of animals (in the case of *Hsp23A* mRNA), and groups injected with modified Ringer solution and *eGFP* dsRNA (in the case of *Hsp23B* transcript; Fig. 9).

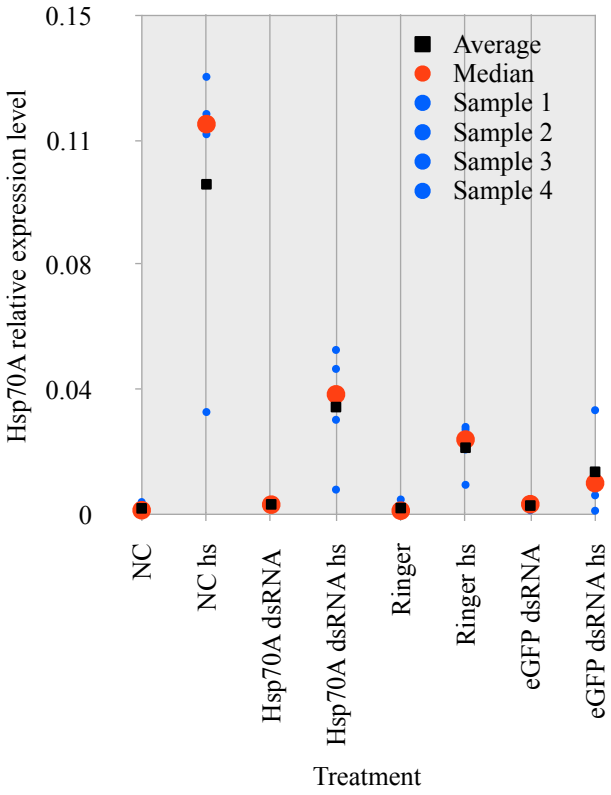
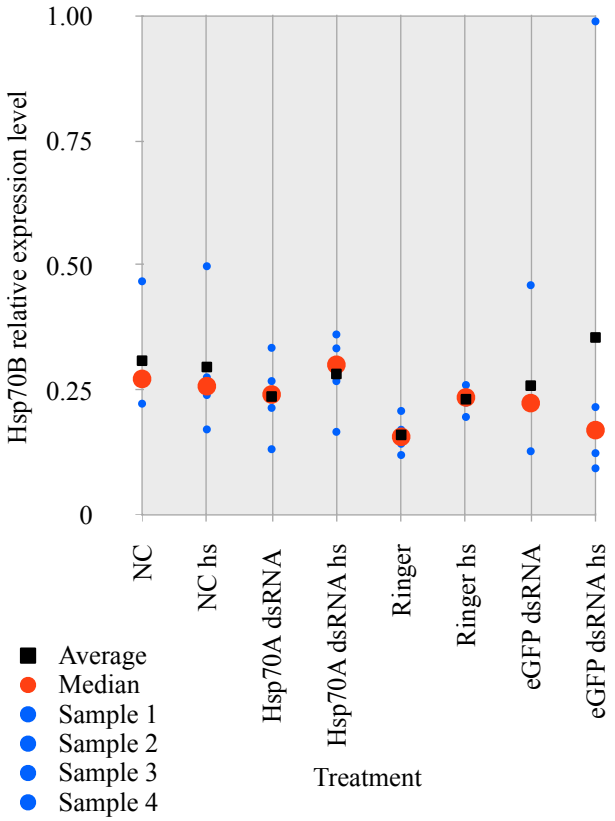
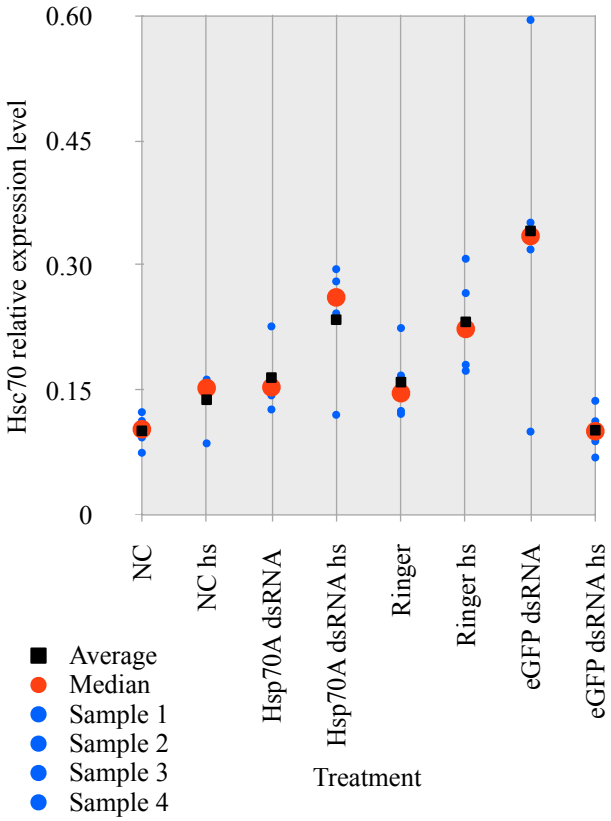


Fig. 7. Difference in transcript levels among beetle groups subjected to different treatment and subsequent exposure to 44 °C for 1 hour. *Hsp70A* expression was highly elevated only in control individuals after heat exposure (NC hs). All injected heat-exposed groups show a somewhat increased level of the expression (*Hsp70A* dsRNA hs, Ringer hs, *eGFP* dsRNA hs - *Hsp70A* dsRNA-injected, Ringer solution-injected, and *eGFP* dsRNA-injected respectively), while all non-exposed animals demonstrate a very low amount of *Hsp70A* mRNA (NC, *Hsp70A* dsRNA, Ringer, *eGFP* dsRNA - negative control, *Hsp70A* dsRNA-injected, Ringer solution-injected, and *eGFP* dsRNA-injected respectively)





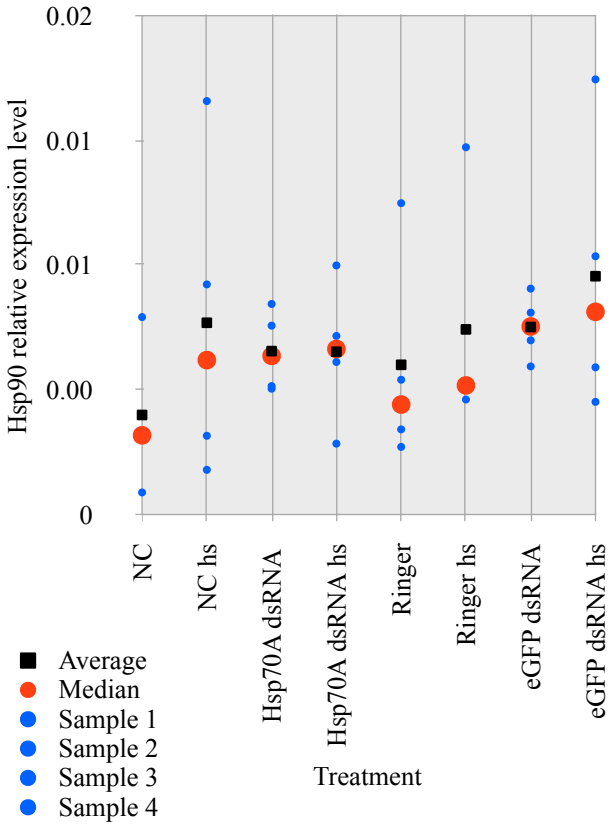


Fig. 8. mRNA level of *Hsp70B* (A), *Hsc70* (B), and *Hsp90* (C) in differently treated groups. None of these genes show a significant change in expression upon heat exposure. Group names designates as in Fig 7.

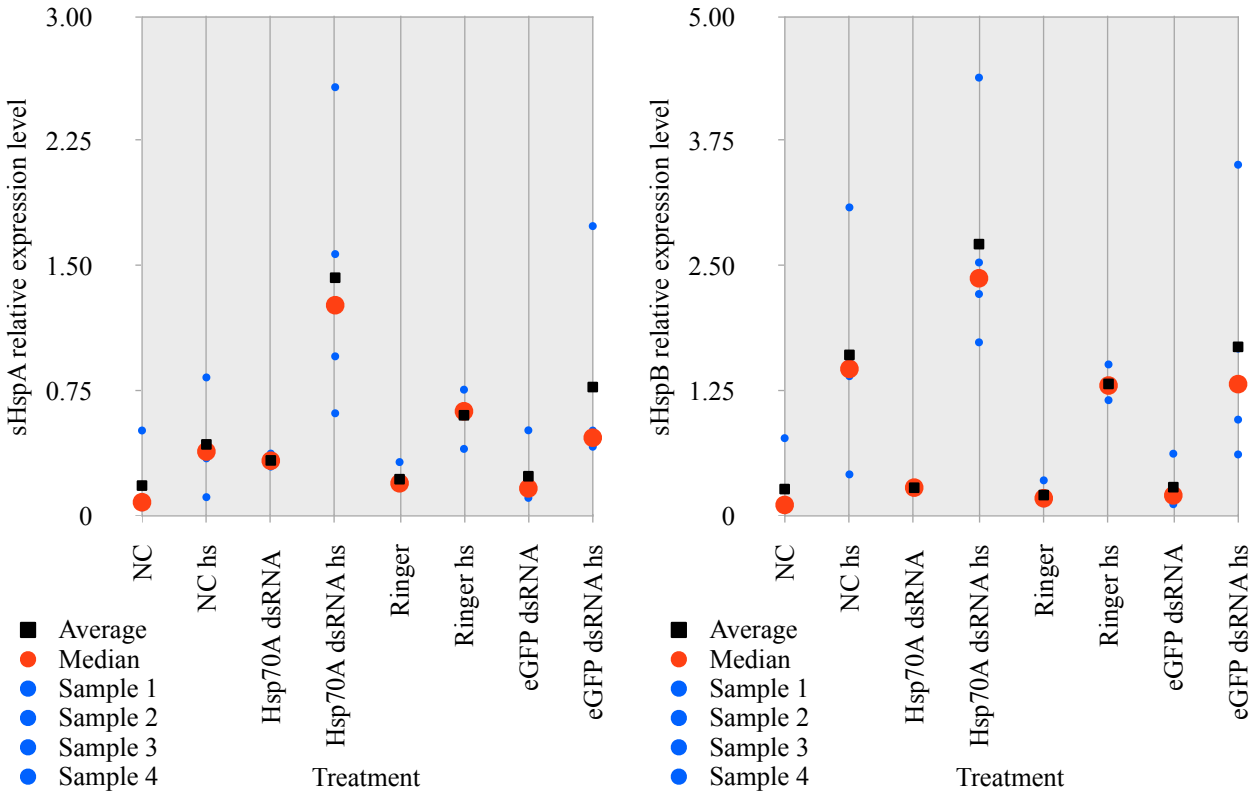


Fig. 9. Expression levels of two isoforms of small heat shock proteins in differently treated groups. Both isoforms show a significant increase in *Hsp70A* dsRNA-injected group compared to the remaining heat-exposed animals. Group designation as in Fig.

4.7 Heat exposure lethality after RNA interference against *Hsp70A*

To see how the *Hsp70A* dsRNA application affects lethality after heat exposure, we performed a test where we exposed the following groups to elevated temperatures and observed the survival rates over time:

i. animals injected with dsRNA against *Hsp70A* mRNA,
ii. animals injected with modified Ringer solution,
iii. animals uninjected.

Animals were exposed to temperatures of 40, 42, 44, 45, 46, and 47 °C, and numbers of living and dead individuals were counted in 24-hour intervals. The results and respective statistical analyses are shown in Fig. 10 to 13. For temperatures of 40, 42, and 44 °C, we observed a significantly earlier mortality of *Hsp70A* dsRNA-injected animals compared to either Ringer solution-injected or uninjected animals while the two latter groups did not display a significant difference from each other.

Hsp70A dsRNA-injected animals exposed to the temperature of 45 °C differed from both Ringer solution-injected and uninjected animals (Fig. 10, and 11).

These results indicate that prior treatment with *Hsp70A* dsRNA does affect the beetles' ability to survive elevated temperatures.

Exposure to temperatures of 46 and 47 °C severely affected all differently-treated groups suggesting that even beetles that are able to produce Hsp70A protein cannot cope with the tissue damage caused by such heat stress (Fig. 12 and 13).

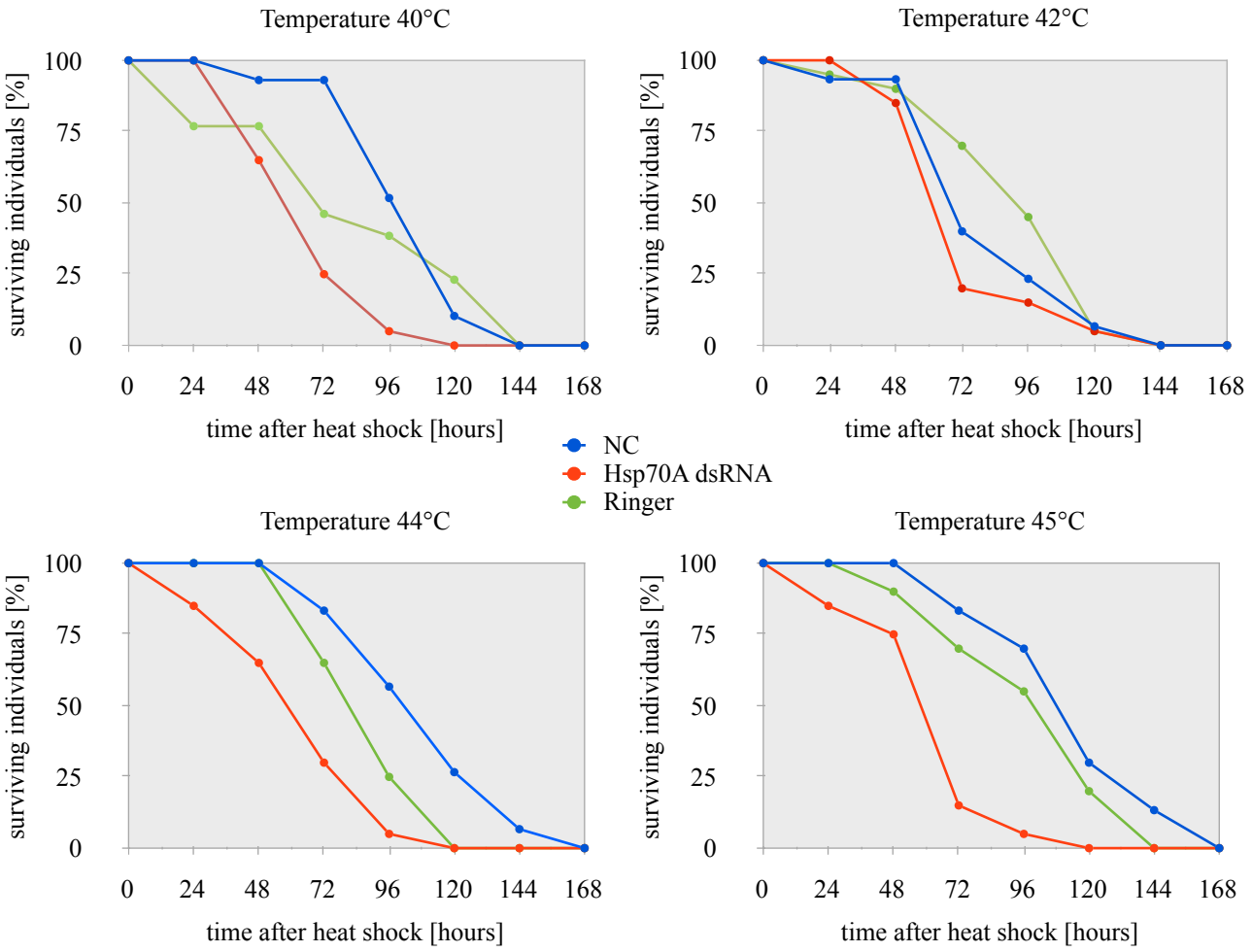


Fig. 10. Percentage of surviving animals depending on prior treatment. *Hsp70A* dsRNA injected animals (red curve) die significantly faster than animals uninjected (blue curve, temperature 40 and 44 °C), modified Ringer-injected (green curve, temperature 42 °C), or both (temperature 45 °C).

Temperature 40°C

Multiple Comparisons p values (2-tailed); 40°C
Kruskal-Wallis test: H (2, N= 62) =17,56 p =,0002

	NC	Hsp70A dsRNA	Ringer
NC		0.000136	0.228714
Hsp70A dsRNA	0.000136		0.286840
Ringer	0.228714	0.286840	

Temperature 42°C

Multiple Comparisons p values (2-tailed); 42°C
Kruskal-Wallis test: H (2, N= 70) =6,74 p =,0343

	NC	Hsp70A dsRNA	Ringer
NC		0.710417	0.415909
Hsp70A dsRNA	0.710417		0.045073
Ringer	0.415909	0.045073	

Temperature 44°C

Multiple Comparisons p values (2-tailed); 44°C
Kruskal-Wallis test: H (2, N= 70) =23,46 p =,0000

	NC	Hsp70A dsRNA	Ringer
NC		0.000008	0.102941
Hsp70A dsRNA	0.000008		0.055703
Ringer	0.102941	0.055703	

Temperature 45°C

Multiple Comparisons p values (2-tailed); 45°C
Kruskal-Wallis test: H (2, N= 70) =25,46 p =,0000

	NC	Hsp70A dsRNA	Ringer
NC		0.000004	0.562748
Hsp70A dsRNA	0.000004		0.003687
Ringer	0.562748	0.003687	

Fig. 11. Statistical output for survival rates after exposure to 40, 42, 44, and 44 °C. P-values for pairs of groups that are lower than 0.05 (i.e. the progress in animals' survival is significantly different) are denoted in red. Survival rates of groups injected with *Hsp70A* dsRNA (HSP) are always significantly lower from groups that were uninjected (NK), Ringer solution-injected (Ringer), or both.

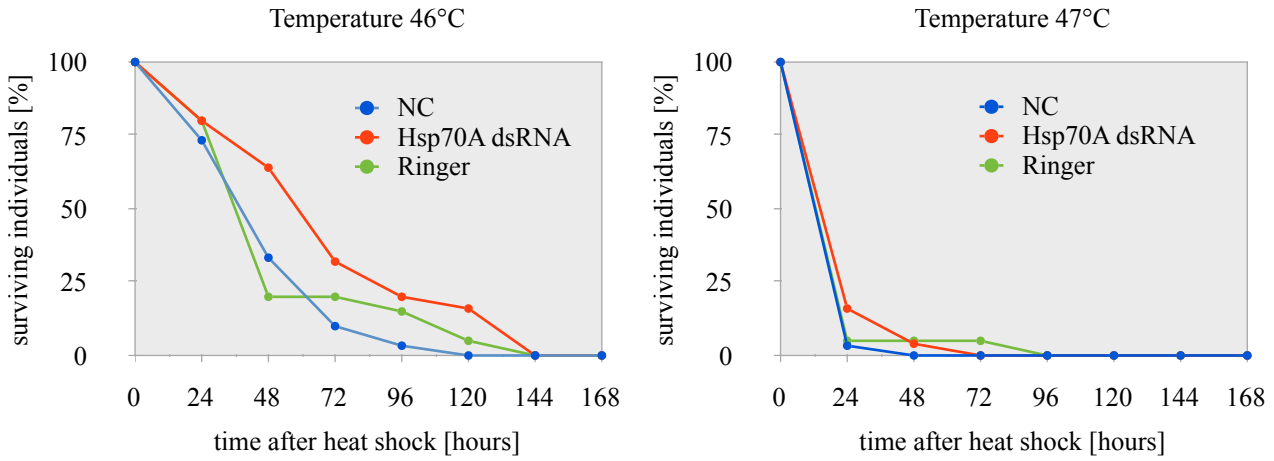


Fig. 12. Percentage of surviving animals depending on prior treatment. No significant difference is obsereved among groups of animals injected with *Hsp70A* dsRNA, Ringer solution-injected, or uninjected when exposed to temperatures of 46 and 47 °C.

Temperature 46°C

Multiple Comparisons p values (2-tailed); 46°C
Kruskal-Wallis test: H (2, N= 75) =5,23 p =,0729

	NC	Hsp70A dsRNA	Ringer
NC		0.128333	1.000000
Hsp70A dsRNA	0.128333		0.238548
Ringer	1.000000	0.238548	

Temperature 47°C

Multiple Comparisons p values (2-tailed); 47°C
Kruskal-Wallis test: H (2, N= 75) =3,15 p =,2065

	NC	Hsp70A dsRNA	Ringer
NC		1.000000	1.000000
Hsp70A dsRNA	1.000000		1.000000
Ringer	1.000000	1.000000	

Fig. 13. Kruskal-Wallis test output on survival rate of animal groups with different prior treatment. Individual groups do not show a significant difference ($p >> 0.05$) in survival rate upon exposure to temperatures of 46 and 47 °C.

5. Conclusions

I. We identified six partial transcripts from the *Hsp* family in *Ips typographus*: *Hsp90*, three isoforms of the 70 kDa family - *Hsp70A*, *Hsp70B*, and the constitutive isoform, *Hsc70*, and two genes from the small heat shock protein family that we designated *sHspA* and *sHspB*. Additionally, we isolated partial cDNA of *actin* gene that was used as a reference in qRT-PCR technique.

II. Lethal temperature of nondiapausing *Ips typographus* was determined to be between 47 and 48 °C for beetles that were kept under stable conditions at 25 °C, and in long-day light regime consisting of 16 hours in the light and 8 hours in darkness.

III. Transcription rate changes of isolated *Hsp* genes after exposure to temperature of 44 °C were examined, and a significant increase in *Hsp70A* and *sHspA* transcript level was observed. The peak of *Hsp70A* mRNA level seems to be two hours from the start of the heat exposure.

IV. After RNAi treatment with *Hsp70A* dsRNA and subsequent heat exposure, a significant decrease in *Hsp70A* transcript level was observed in all injected groups - injected with *Hsp70A* dsRNA, *eGFP* dsRNA, and Ringer solution - compared to uninjected control individuals. The reason of this phenomenon is still to be elucidated. However, we also observed a significant rise in expression of the two forms of small heat shock proteins, *sHspA* and *sHspB*, in the group treated with *HspA* dsRNA.

V. Survival tests after heat exposure to a range of temperatures showed that beetles treated with *Hsp70A* dsRNA died significantly faster than beetles injected with either Ringer solution or uninjected. However, the results are not very distinct and it would be desirable to support the outcome with additional tests on more individuals.

6. Discussion

Our study determined that the lethal temperature for adult *I. typographus* beetles is 48 °C when none of the tested beetles survived the next 24 hours after the exposure. However, the survival rate curve is quite steep with over 90 % surviving animals after exposure to 46 °C, the critical point being at 47 °C when only 25 % of beetles survive, and finally, after application of 48 °C none of the animals survived. The temperature values correspond nicely to previous studies performed by Wermelinger and coworkers. In their work they mathematically determined the upper temperature threshold for the development of *I. typographus* being 42 °C for larval stage, and 40 °C for pupal stage and eggs (Wermelinger et al., 1998), and 34 °C the maximum temperature for oviposition (Wermelinger et al., 1999).

We cloned partial cDNAs of three isoforms of *Hsp* genes from the 70 kDa family, two genes from the small *Hsp* family, and a *Hsp90* partial transcript. Our further experiments confirmed that one of the *Hsp70* genes, isoform that we designated *Hsp70A*, is strongly inducible in *Ips typographus* upon heat exposure which corresponds with previous studies of its ortholog in other species (Li et al., 2011; Rinehart et al., 2000; Yocum, 2001). Similarly, one of the small heat shock protein genes, isoform that we named *sHspA*, is significantly upregulated upon heat exposure. Because we only isolated a small fraction of the cDNA, we cannot determine which of the small *Hsp* it is exactly to compare our observed expression with previous work.

Hsp90 mRNA transcription rate increases slightly. If we take into account the fact that in unstressed cells Hsp90 is already one of the most abundant proteins in cytosol (Whitesell et al., 2005), a 4-fold increase in expression can mean a big rise in absolute numbers of *Hsp90* mRNA transcripts. At the same time, Hsp90 protein has been observed to change its activity to bind unfolded proteins more readily after stress induction than in an unstressed cell. Indeed, Yonehara and colleagues showed that porcine Hsp90 self-oligomerizes and aquires a new chaperone function after Hsp90 incubation in elevated temperature (Yonehara et al., 1996). Thus, after heat exposure, *Hsp90* gene not only slightly increases its transcription rate, but Hsp90 protein also adjusts its enzymatic activity to help protect cells from thermal damage.

Other genes and isoforms, *Hsp70B, Hsc70*, *sHspB* do not show a prominent expression change upon heat exposure. This result is not surprising in the case of *Hsc70* since no change in its expression level has been observed by other research groups either (Rinehart et al., 2000).

In our experiments that we used to test the efficiency of RNA interference, we observed several discrepencies. While RNA interference by *Hsp70A* dsRNA seems to affect the viability of

bark beetles when exposed to elevated temperatures compared to Ringer solution-injected animals, the *Hsp70A* mRNA levels seem to be very similar in these two groups. The hypothesis to explain this phenomenon is as follows:

While uninjected beetles respond to the heat exposure by highly increasing the *Hsp70A* transcription rate, all groups injected with *Hsp70A* dsRNA, *eGFP* dsRNA, and Ringer solution express *Hsp70A* at significantly lower levels. Although the levels of the *Hsp70A* transcript are similar in the injected groups, we suggest that the reason of the decrease is twofold:

In control injected groups, the injection itself triggers the stress response which leads to the synthesis of various heat shock proteins (Brown et al., 1989), Hsp70A being one of them. After four days when the heat shock is applied, the amount of Hsp70A protein is such that it effectively binds most of the heat shock factor 1 monomers (Tomanek et al.,), and negatively regulates further synthesis of Hsp70A after heat exposure.

On the other hand, in *Hsp70A* dsRNA injected group, most of the *Hsp70A* mRNA is effectively degraded by RNAi machinery soon after the dsRNA application. Thus, the animals cannot react by Hsp70A protein synthesis to either the tissue damage caused by the injection process and, or the subsequent heat exposure. Therefore, the reason of the lowered *Hsp70A* expression level is not that they already have enough of the effective agent - Hsp70A protein, but rather the silencing effect of RNAi.

This hypothesis would nicely correlate with the expression level of *sHspA* isoform that is only significantly elevated in *Hsp70A* dsRNA-injected group. Small heat shock proteins act as stabilizing agents in a stressed cell that protect misfolded proteins from aggregation until they can be refolded by a heat shock protein-containg refolding machinery (Ryan et al., 1996). Thus, we might interpret the elevated level of *sHsps* in RNA silenced animals as a biomarker of insufficiently addressed cellular stress. This speculation would have to be confirmed by further experiments, for example using Western blot to determine the level of Hsp70A protein before the start of the heat exposure in *Hsp70A* dsRNA-injected, Ringer injected, and *eGFP* dsRNA-injected animals.

The lethality tests show a statistically significant difference in survival rate between *HspA* mRNA silenced animals and those injected with Ringer solution or uninjected. Still, the significance is not very obvious for two reasons:

i. After the *HspA* dsRNA application, we are not able to determine if the dsRNA delivery was succesful in animals that are subsequently used for the test of survival after heat exposure. Due to this fact, some of the animals that we observe in the *Hsp70A* dsRNA group might not express an *Hsp70A* RNA silenced phenotype and thus obscure the surviving rate results.

ii. Beetles that are exposed to elevated temperature are then kept in unnatural conditions (see Methods and Materials), and therefore we observe a distinctive decrease in survival of the control animals as well. Ideally, we would return the tested groups to their natural conditions in the spruce tree logs, but consequent detemining of living and dead animals would be very difficult. The comparism of individual surviving rates at 24 and 72 hours after heat exposure in different temperatues is shown in Fig.14. From the figure we deduce that the greatest difference between *Hsp70A* dsRNA-applied and Ringer-injecter or uninjected groups is between at 44 and 45 °C; 40 and 42 °C exposure being too low to induce a significant stress, and 46 and 47 °C being too high for all the tested groups to cope with the tissue damage.

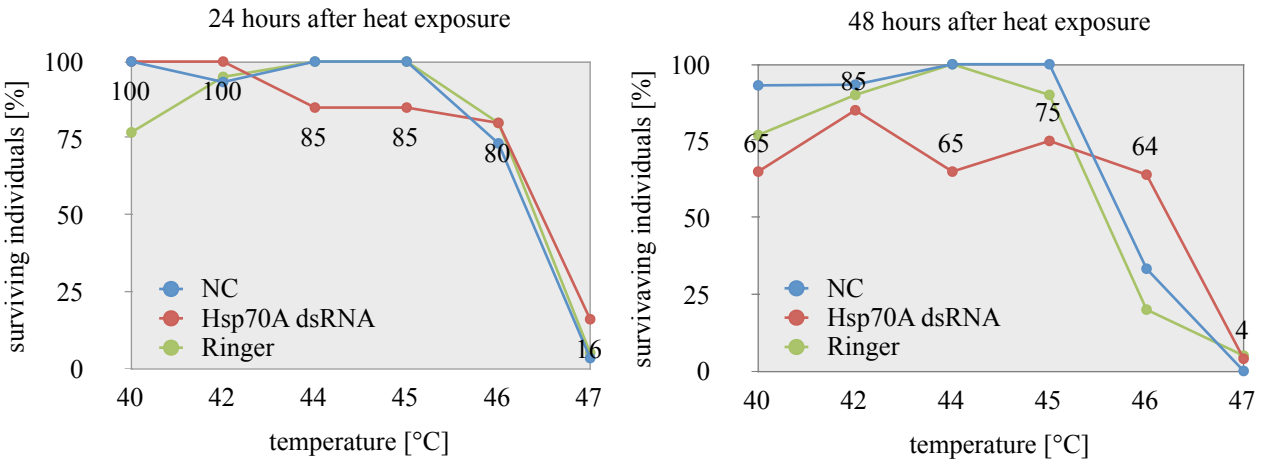


Fig. 14. Display of surviving rate of differently treated groups of animals exposed to different temperatures after 24 and 48 hours from the heat exposure. The *Hsp70A* dsRNA-injected groups (red curve) shows the greatest difference after exposure to temperatures of 44 and 45 °C. Numbers denoted at the *Hsp70A* dsRNA curve are percentage of surviving animals at respective temperatures.

7. References

Alder et al. Gene silencing in Caenorhabditis elegans by transitive RNA interference. RNA (2003) vol. 9 (1) pp. 25-32

Baler et al. Heat shock gene regulation by nascent polypeptides and denatured proteins: hsp70 as a potential autoregulatory factor. The Journal of cell biology (1992) vol. 117 (6) pp. 1151-9

Bellés. Beyond Drosophila: RNAi in vivo and functional genomics in insects. Annual review of entomology (2010) vol. 55 pp. 111-28

Bernstein et al. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature (2001) vol. 409 (6818) pp. 363-6

Brown et al. Induction of a heat shock gene at the site of tissue injury in the rat brain. Neuron (1989) vol. 2 (6) pp. 1559-64

Bucher et al. Parental RNAi in Tribolium (Coleoptera). Current biology : CB (2002) vol. 12 (3) pp. R85-6

Bukau and Horwich. The Hsp70 and Hsp60 chaperone machines. Cell (1998) vol. 92 (3) pp. 351-66

Cerutti, et al. Transgene And Transposon Silencing In Chlamydomonas Reinhardtii By A DEAH-Box RNA Helicase. Science (2000) vol. 290 (5494) pp. 1159

Cogoni et al. Transgene silencing of the al-1 gene in vegetative cells of Neurospora is mediated by a cytoplasmic effector and does not depend on DNA-DNA interactions or DNA methylation. The EMBO journal (1996) vol. 15 (12) pp. 3153-63

Csermely et al. The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review. Pharmacology & therapeutics (1998) vol. 79 (2) pp. 129-68

Csermely. Chaperone-percolator model: a possible molecular mechanism of Anfinsen-cage-type chaperones. BioEssays: news and reviews in molecular, cellular and developmental biology (1999) vol. 21 (11) pp. 959-65

Fire et al. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature (1998) vol. 391 (6669) pp. 806-11

Goodson, et al. Heat-inducible DNA binding of purified heat shock transcription factor 1. Journal of Biological Chemistry (1995) vol. 270 (6) pp. 2447-50

Hammond et al. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature (2000) vol. 404 (6775) pp. 293-6

Hammond et al. Argonaute2, a link between genetic and biochemical analyses of RNAi. Science (2001) vol. 293 (5532) pp. 1146-50

Hartl. Molecular chaperones in cellular protein folding. Nature (1996) vol. 381 (6583) pp. 571-80

Kennerdell et al. Heritable gene silencing in Drosophila using double-stranded RNA. Nature Biotechnology (2000) vol. 18 (8) pp. 896-8

Li et al. Comparative analysis on the expression of inducible HSPs in the silkworm, Bombyx mori. Molecular Biology Reports (2011) published online

May et al. RNA Interference Spreading in C. elegans. Methods in Enzymology (2005) vol. 392, pp. 308-15

Meng et al. Cloning of Chicken hsp90β: The Only Vertebrate hsp90 Insensitive to Heat Shock. Biochemical and Biophysical Research Communications (1993) vol. 190 (2) pp. 630-6

Milarski and Morimoto. Mutational analysis of the human HSP70 protein: distinct domains for nucleolar localization and adenosine triphosphate binding. The Journal of cell biology (1989) vol. 109 (5) pp. 1947-62

Miller et al. Larval RNAi in Drosophila?. Development Genes and Evolution (2008) vol. 218 (9) pp. 505-10

Napoli et al. Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. The Plant cell (1990) vol. 2 (4) pp. 279-289

Plasterk. RNA silencing: the genome's immune system. Science (2002) vol. 296 (5571) pp. 1263-5

Pratt and Toft. Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. Experimental biology and medicine (Maywood, NJ) (2003) vol. 228 (2) pp. 111-33

Rinehart et al. Developmental upregulation of inducible hsp70 transcripts, but not the cognate form, during pupal diapause in the flesh fly, Ssarcophaga crassipalpis. Insect biochemistry and molecular biology (2000) vol. 30 (6) pp. 515-21

Rinehart et al. Up-regulation of heat shock proteins is essential for cold survival during insect diapause. Proceedings of the National Academy of Sciences of the United States of America (2007) vol. 104 (27) pp. 11130-7

Ritossa. A new puffing pattern induced by temperature shock and DNP in Drosophila. Experientia (1962) vol. 18, pp. 571-3

Ryan and Hightower. Stress proteins as molecular biomarkers for environmental toxicology. EXS (1996) vol. 77, pp. 411-24.

Saleh et al. The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. Nature Cell Biology (2006) vol. 8 (8) pp. 793-802

Sijen et al. On the role of RNA amplification in dsRNA-triggered gene silencing. Cell (2001) vol. 107 (4) pp. 465-76

Sundaram et al. ATP-binding cassette transporters are required for efficient RNA interference in Caenorhabditis elegans. Molecular biology of the cell (2006) vol. 17 (8) pp. 3678-88

Tamura et al. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution (2007) vol. 24 (8) pp. 1596-9

Tomoyasu et al. Exploring systemic RNA interference in insects: a genome-wide survey for RNAi genes in Tribolium. Genome biology (2008) vol. 9 (1) pp. R10

Uhlirova et al. Use of Sindbis virus-mediated RNA interference to demonstrate a conserved role of Broad-Complex in insect metamorphosis. Proceedings of the National Academy of Sciences of the United States of America (2003) vol. 100 (26) pp. 15607-12

Wermelinger and Seifert. Analysis of temperature dependent development of the spruce bark beetle Ips typographus. Journal of Applied Entomology (1998) vol. 122 (4) pp. 185-91

Wermelinger and Seifert. Temperature-dependent reproduction of the spruce bark beetle Ips typographus. Ecological Entomology (1999) vol. 24 (1) pp. 103-10

Whitesell and Lindquist. HSP90 and the chaperoning of cancer. Nature Reviews Cancer (2005) vol. 5 (10) pp. 761-72

Winston et al. Systemic RNAi in C. elegans Requires the Putative Transmembrane Protein SID-1. Science (New York, NY) (2002) vol. 295 (5564) pp. 2456-2459

Xu and Lindquist. Heat-shock protein hsp90 governs the activity of pp60v-src kinase. Proceedings of the National Academy of Sciences of the United States of America (1993) vol. 90 (15) pp. 7074-8

Yocum. Differential expression of two HSP70 transcripts in response to cold shock, thermoperiod, and adult diapause in the Colorado potato beetle. Journal of Insect Physiology (2001) vol. 47 (10) pp. 1139-45

Yonehara et al. Heat-induced chaperone activity of HSP90. The Journal of biological chemistry (1996) vol. 271 (5) pp. 2641-5