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ORIGINAL PAPER

# *Hsp70* affects memory formation and behaviorally relevant gene expression in *Drosophila melanogaster*

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## Abstract

Heat shock proteins, in particular Hsp70, play a central role in proteostasis in eukaryotic cells. Due to its chaperone properties, Hsp70 is involved in various processes after stress and under normal physiological conditions. In contrast to mammals and many Diptera species, inducible members of the Hsp70 family in *Drosophila* are constitutively synthesized at a low level and undergo dramatic induction after temperature elevation or other forms of stress. In the courtship suppression paradigm used in this study, *Drosophila* males that have been repeatedly rejected by mated females during courtship are less likely than naive males to court other females. Although numerous genes with known function were identified to play important roles in long-term memory, there is, to the best of our knowledge, no direct evidence implicating Hsp70 in this process. To elucidate a possible role of Hsp70 in memory formation, we used *D. melanogaster* strains containing different *hsp70* copy numbers, including strains carrying a deletion of all six *hsp70* genes. Our investigations exploring the memory of courtship rejection paradigm demonstrated that a low constitutive level of Hsp70 is apparently required for learning and the formation of short and long-term memories in males. The performed transcriptomic studies demonstrate that males with different *hsp70* copy numbers differ significantly in the expression of a few definite groups of genes involved in mating, reproduction, and immunity in response to rejection. Specifically, our analysis reveals several major pathways that depend on the presence of *hsp70* in the genome and participate in memory formation and consolidation, including the cAMP signaling cascade.

**Keywords** Hsp70 · Courtship suppression paradigm · *Drosophila* · Learning and memory · Transcriptome analysis

## Introduction

*Drosophila melanogaster* has been employed as a model system to understand the genetic and molecular basis of learning and memory in eukaryotic organisms (Tully et al. 1990; Akalal et al. 2010; Mery et al. 2007; Zhuravlev et al. 2015). The use of different paradigms including a memory of courtship rejection approach in males (Kamyshev et al. 1999; Winbush et al. 2012; Gerstner and Yin 2010; Keleman et al. 2007) has provided insights into molecular mechanisms underlying the formation of long- and short-term memories (Tully et al. 1994; Beck et al. 2000; Davis 2005; Dubnau and Tully 1998; Griffith and Ejima 2009; Jones et al. 2018). Fruit flies have a memory system that permits sophisticated forms of learning and information processing necessary for survival and reproduction (Mery et al. 2007; Ishimoto et al. 2009). However, the numerous genes and signaling pathways discovered in flies to date (Akalal et al. 2011; Bozler et al. 2017; Kahsai and Zars 2011) apparently represent only a small number of the factors participating in learning and memory formation. In particular, various findings and observations suggest some interactions between memory formation and storage and very ancient stress–response systems including heat shock genes, innate immunity genes, and genes involved

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in the production of gaseous signaling molecules such as $H_2S$ (Bozler et al. 2017; Barajas-Azpeleta et al. 2018; Kuntz et al. 2017; Nagpure and Bian 2015; Snijder et al. 2016).

Heat shock proteins (Hsps) are at the heart of proteostasis in eukaryotic cells, given their essential roles in protein folding and degradation pathways (Hartl et al. 2011). Hsps participate in damaged protein degradation and clearing either by the UPS or autophagy (Carman et al. 2013; Ciechanover and Kwon 2017; Witt 2013; Wyatt et al. 2013). Under normal physiological conditions, Hsp70 binding to client proteins in the early stages of protein folding controls the formation of proper protein folding and transport of mature proteins, while inhibiting aggregate formation. Due to these chaperone properties, Hsp70 is involved in various processes including adaptation to stress (Evgen'ev et al. 2014), development and apoptosis (Kennedy et al. 2014; Kumar and Tiwari 2018), and probably learning and memory formation (Thekkuveettil and Lakhotia 1996; Gyurko et al. 2014). Heat shock treatment (HS) can restore memory in *D. melanogaster* mutant flies ("agnostic") and mollusks, as well as several other eukaryotic organisms (Hooper et al. 2016; Porto et al. 2018; Savvateeva-Popova et al. 2017). There is abundant information concerning the involvement of individual Hsps induced by HS or administration of recombinant Hsps in memory formation and storage in various organisms (Kandel 2001; Foster et al. 2015; Sunada et al. 2016; Lukowiak et al. 2010). Furthermore, in our experiments, administration of recombinant Hsp70 by different means including intranasal injections has been found to ameliorate the cognitive impairments in mouse models of AD (Bobkova et al. 2014; Bobkova et al. 2015). Similarly, it has been shown that the expression of Hsp70 significantly improves memory in transgenic flies carrying Aβ42 oligomers (Martín-Peña et al. 2018). To this end, the heat shock transcription factor (HSF1), which induces the expression of *hsp70* and other heat shock genes, plays the central role in synaptic fidelity and memory consolidation (Hooper et al. 2016; Murshid et al. 2010).

It is possible to speculate that during evolution, mechanisms underlying memory formation and the stress response were formed in parallel, and the observed simultaneous activation of Hsps synthesis and major neurotrophic factor (BDNF) production corroborates this speculation (Hooper et al. 2016; Zhao et al. 2015; Scaccianoce et al. 2003).

The cytosolic chaperone Hsp70 is evolutionarily conserved and represents one of the most abundant chaperones in mammalian cells. In contrast to mammals and many studied Diptera species, the major stress proteins in fruit flies belonging to the Hsp70 family under normal conditions are constitutively synthesized at very low levels and undergo dramatic induction immediately after heat shock and other stressful stimuli (Evgen'ev et al. 2014; Dahlgaard et al. 1998; Shilova et al. 2018). Using a "rejection" paradigm, we have previously demonstrated a severe memory deficit in the *D. melanogaster* strain (ts403) with abnormal function of the heat shock genes system (Evgen'ev et al. 1979). However, in this ts-mutant, the synthesis of all Hsps including Hsp70 was inhibited and we were not able to pin-point the observed effects to specific *hsp* genes.

Given these findings and observations, herein, we used *D. melanogaster* strains containing different *hsp70* copy numbers, including strains with deletion of all six *hsp70* genes. Our investigations exploring the mating rejection courtship paradigm demonstrated that a low constitutive level of Hsp70 present in the brain is required for the formation of short and long-term memories in male fruit flies. The transcriptomic studies revealed characteristic differences in the expression of a few definite groups of genes in response to rejection in the heads of males with different *hsp70* copy numbers.

## Materials and methods

### Fly strains

All flies were reared on standard sugar–yeast–agar medium at 25°C, 60% humidity, and the light–dark cycle 12:12 h. The following strains were obtained from Bloomington *Drosophila* Stock Center: the original strain $w^{1118}$ (Bloomington #6326) containing all six copies of *hsp70*; the derived strain $w^{1118}$ (df(3R) Hsp70A, df(3R)Hsp70B), referred to as "*hsp70-*", in which all the *hsp70* genes were deleted by genetic manipulation (Bloomington #8841) (Gong and Golic 2004). We also used a derived $w^{1118}$ strain which comprised four *hsp70* copies located in the 87B region named "*hsp70-4c*" (Bloomington # 8842). We introduced into the "*hsp70-"* strain one *hsp70* copy originating from the 87A locus via *P*-mediated transformation (Shilova et al. 2018) and designated this strain "*hsp70-1c*". Since *hsp70-* flies were phenotypically *white* and it is known that the *white* mutation influences memory characteristics (Sitaraman et al. 2008; Anaka et al. 2008), the (*w+/hsp70-*) pCaSpeR5 vector plasmid carrying the mini-*white* gene was injected into preblastoderm embryos of 8841 strain to obtain an *hsp70-* strain with red eyes, as described previously (Astakhova et al. 2015). Therefore, we developed a strain lacking all *hsp70* genes with normally pigmented eyes and used these red-eyed males in our studies as an additional control. The obtained strain was designated *w+/hsp70-*. We used the *Canton-S* strain, which carries all *hsp70* genes, in the courtship experiments. This strain is routinely used as a control in all behavior tests in our laboratory.

Strain Elav GFP/FM7; Hsp70-/Hsp70- was constructed by several consequent crosses of # 108077 strain (Genotype P{GawB}elavC155, P{UAS-mCD8::GFP. L}Ptp4ELL4, P{hsFLP}1, w*/FM7c (short name Elav GFP/FM7)), and

#8841 strain (genotype w[1118]; Df(3R)Hsp70A, Df(3R)Hsp70B (short name *hsp70-*)). In the resulted strain with deleted copies of *hsp70*, the GFP gene coding region is driven by the Elav gene promoter, expressed in neurons. Confocal microscopy was used to study the fluorescence of GFP in the brains isolated from third instar larvae and adult flies of this strain.

Transgenic strains containing various constructs were maintained under the same conditions as the abovementioned laboratory strains.

## Heat shock treatment

Heat shock (HS) treatment was used to modulate the HS stress response. Behavioral patterns of flies were assessed after HS treatment was applied to 5-day-old adult flies 1 h before the test as described (Nikitina et al. 2003). The flies were subjected to heat shock in empty culture flasks immersed in a water bath for 30 min at 37°C.

## Test for learning and memory of flies in a conditioned courtship suppression paradigm

To evaluate memory formation in *Drosophila* males, we used a conditioned courtship suppression paradigm (CCSP) (Kamyshev et al. 1999).

Males were collected and kept individually, and virgin wild-type *Canton-S* females were maintained in small groups (10 per vial) until 5 days of age. Before the day of the experiment, the females were allowed to mate overnight. The collection of naive flies was done at the same circadian time as the trained flies. The studies were performed on adult flies at the first half of the day by the temperature +25 ± 0.5°C.

For training, a naive male (with no previous courtship experience) was placed together with a 5-day-old fertilized *Canton-S* female for 30 min in the experimental chamber (test for learning and short-term memory) or 5 h in a beaker containing medium (volume of free space of approximately 3 $cm^3$) (test for learning and long-term memory) according to an established protocol (McBride et al. 1999). In the retraining test, a trained male was placed in a fresh chamber together with another fertilized female.

To observe courtship, a male of a strain understudy and a fertilized 5-day-old wild-type female were introduced by shaking through a funnel into different halves of a Plexiglas experimental chamber (15 mm in diameter, 5 mm high) separated by a sliding opaque partition. After 45 s of adaptation, the partition was withdrawn, and the flies were left together. During 300 s of observation, the temporal sequence of behavioral elements was recorded using a specially designed program (Kamyshev et al. 1999). The elements related to courtship include orientation and pursuit, wing vibration, licking, and attempted copulation. The non-courtship elements recorded were activity (running), preening, and rest.

In the test for learning and short-term memory for each strain, three independent samples of experimental males were examined: naive males (with no previous courtship experience) and trained males (having previous experience of courtship), tested either immediately (for learning acquisition) or 3 h after training (for memory retention).

In the test for learning and long-term memory, four independent samples of experimental males were examined: naive males (with no previous courtship experience) and trained males (with previous courtship experience), tested either immediately (for learning acquisition) or 2 or 8 days after training (for memory retention). For each group (naive males, immediately following training and at given time intervals after training), 20 flies were tested.

The resulting courtship index [CI], the percentage of time spent in courtship over a 300-s period, was calculated for each male (Fig. S1). The CI was used to calculate the learning index (LI) as follows:

LI = ([CIna − Citr]/CIna) × 100 = (1 − CItr/CIna) × 100 (McBride et al. 1999), where CIna and CItr are the mean courtship indices for independent samples of naive and trained males, respectively.

Statistical comparisons of behavioral data were performed using a two-sided randomization test (Rohlf and Sokal 1981) by directly computing the probability of rejection of the null-hypothesis αR. The sampled randomization test with 10,000 permutations was used. The null hypothesis was rejected at $\alpha R<0.05$. Besides, we compared all experimental groups with each other.

## Incorporation of [$^{35}$S]_methionine into *D. melanogaster* brain proteins

The brains were dissected from 5-day old naïve males kept at 25°C (control), from males subjected to HS for 30 min at 37°C (immediately and 4 h after HS), and from males after 5-h training with fertilized females. For each point, 15 brains were dissected and incubated for 1 h at 25°C in 20 μL of PBS supplemented with 1 μL (1 μCu) of [$^{35}$S]_methionine (Amersham Biosciences, USA). Labeled brains were lysed in 20 μL Laemmli buffer. Protein extracts were separated by electrophoresis in 8% SDS_polyacrylamide gel. The incorporation of the radioactive label was evaluated by radioautography exposure.

### Western blotting

Protein extracts were obtained from the heads of 5-day naïve males kept at 25°C, males subjected to HS 30 min 37°C (all tested strains) and males after 5-h training with fertilized females (*Canton-S* and strain *hsp70-4c*). After electrophoresis in 8% SDS PAGE, proteins were transferred to nitrocellulose

membrane (Amersham, USA). Hsp70 was detected using monoclonal antibodies 7FB, specific for the inducible Drosophila Hsp70 (Kind gift of Dr. Suzanne Lindquist). Actin was detected with the anti-Actin antibody, clone 4C (Sigma-Aldrich). After incubation with secondary antibodies conjugated with horseradish peroxidase, immune complexes were detected on a ChemiDoc MP system (Bio-Rad, USA) using a reagent for chemiluminescent detection (Thermo Scientific SuperSignal West Pico Plus Chemiluminescent substrate) of Hsp70 after heat shock and actin as an internal control. For detection of Hsp70 under control conditions, we used the Thermo Scientific Super Signal West Femto Maximum Sensitivity substrate. The results were processed using Image J. The measured levels of Hsp70 in each sample were normalized to the amount of actin.

### Collection of fly heads and RNA extraction

To obtain the libraries, naïve males were placed with fertilized females at 10 a.m. for 5 h and, hence, the isolation of RNA from the heads of the males was carried out at 3 p.m. For each behavioral experiment, four biological replicates of approximately 100 flies were collected and frozen. Naive or trained males snap-frozen in liquid nitrogen at the appropriate time point after training were manually decapitated. The heads were collected on a dry ice-cooled surface. Total RNA extraction was performed with RNAzol reagent (Molecular Research Center, USA) according to the manufacturer's protocol. The concentration of RNA was measured with a Qubit Fluorometer (Invitrogen). The quality of RNA was determined with an Agilent BioAnalyzer 2100 using an RNA 6000 nano kit. The RNA Integrity Number (RIN) of all RNA samples taken for mRNA library preparation was not less than 8.

### cDNA library preparation and data analysis

Libraries for RNA-seq were prepared using the NEB Next Ultra II Directional RNA Library Prep Kit for Illumina (NEB, USA) according to the manufacturer's guidelines. The 75-bp single-end sequencing was conducted on an Illumina NextSeq 500 platform.

Deep sequencing provided ~15–20 million reads for each library. Processing of the raw sequence data was performed using PPLine script (Krasnov et al. 2015), which included mapping of the reads to the *D. melanogaster* genome (Dm6) with STAR (Dobin et al. 2013) following adapter, length, and quality trimming by Trimmomatic (Bolger et al. 2014). Differential gene expression analysis was performed with the edgeR package (Robinson et al. 2010). Gene Ontology and KEGG enrichment analyses were performed using the top GO (v.2.36.0) and cluster Profiler Bioconductor packages (Yu et al. 2012). Visualization of the gene set enrichment analysis (GSEA) was performed using custom scripts written in Python and R. Sequence data were deposited in the NCBI GEO database under the number - GSE152647.

### Quantitative PCR

One microgram of total RNA was used for cDNA synthesis with an MMLV RT kit (Evrogen, Moscow, Russia). All qRT-PCR reactions were conducted using the SYBR Green fluorescent dye (Evrogen, Russia) in an ABI PRISM VR 7500 device (Applied Biosystems). The relative expression of the studied genes was calculated based on the ΔΔCt method (Schefe et al. 2006). Quantifications were normalized to the housekeeping gene *rp49* (Ponton et al. 2011). Experiments were performed with three replicates and three experimental replicates. The primer sequences are listed in Table S1.

## Results

Classical studies involving various learning paradigms and courtship rejection have identified major genes and learning and memory circuits in the *Drosophila* brain (Griffith and Ejima 2009; Kahsai and Zars 2011). However, to the best of our knowledge, there are no direct data concerning the role of the constitutive expression of the major *Drosophila* stress gene *hsp70* in LTM and STM formation and consolidation. The data presented below help to define the role of *hsp70* transcription in LTM and STM and reveal various gene systems that interact with Hsp70 in the processes of learning and memory formation and consolidation in male flies.

### Learning and short-term memory

The conditioned courtship suppression paradigm (CCSP) is based on naturally occurring *Drosophila* mating behavior stimuli. In male courtship of a fertilized female, two unconditioned stimuli are combined: attracting (courtship-stimulating hormone, aphrodisiac) and aversive (courtship-repressing hormone, antiaphrodisiac). The antiaphrodisiac is characteristic only of fertilized females, which release it in response to male courtship. As a result of the combination, the attracting stimulus transforms into the aversive conditioned one, which reduces its attracting properties. After a 30-min stay of a naive male with a fertilized female, the intensity of its subsequent courtship of a tester female dramatically decreased. In the latter case, memory was retained for up to 8 h because retrieval of the memory trace is facilitated by the presentation of a fertilized female to the male (Kamyshev et al. 1999). The CCSP is used widely for evaluation of basic courtship behavior and learning ability and memory retention in *Drosophila* (Kuzin et al. 2014; Savvateeva-Popova et al. 2007; Savvateeva-Popova et al. 2008; Godenschwege et al. 2004;

Redt-Clouet et al. 2012; Savvateeva et al. 2000; Fedotov et al. 2020).

At the first stage, the courtship index [CI] was calculated for each male (Fig. S1). A low index in males *white*$^{1118}$ and *hsp70*- strains is apparently associated with a mutation in the gene *white* (Anaka et al. 2008). At the same time, in males *hsp70*- and *w +/hsp70*-, the courtship time of the female immediately and after training is comparable to that of naive males and does not depend on the eye color. Therefore, the males *hsp70*- and *w+/hsp70*- after different times of training maintain a level of courtship comparable to naïve which means that dramatic disturbance of learning, as well as STM and LTM take place in *hsp70*- and *w +/hsp70*- males (Fig. S1).

Figure 1 presents the results of the learning acquisition, short-term (A) analyses under normal conditions. Surprisingly, the mutant flies without *hsp70* genes (*hsp70*- and *w+/hsp70*- ) showed a 4-fold lower 3-h memory and learning ability than control *Canton-S* flies. This result clearly demonstrated the involvement of Hsp70 in the learning process, specifically the formation of short-term memory (STM). Concurrently, *hsp70-1c* and *hsp70-4c* males demonstrated approximately the same learning and short-memory formation as *Canton-S* flies. Thus, the presence of at least one copy of the *hsp70* gene (belonging to either A or B loci) was necessary and sufficient to restore learning ability and STM formation (Fig. 1a). During the next stage, we investigated learning acquisition and long-term memory retention (LTM) in the studied strains (Fig. 1b). The *hsp70*- males, both intact and treated with heat shock (Fig. 2, exhibited an even more drastic, 70-fold reduction in 2-day memory and learning ability compared with Canton-S flies. Thus, the mutant *hsp70*- male flies demonstrated poor ability to learn and to form STM and inability to form LTM in CCSP. Additionally, an increase in the duration of training led to a catastrophic decline in learning of these males (Fig. 2b).

This dramatic effect observed in *hsp70*- males concerning learning acquisition and memory retention may be explained by the presence of the *white* mutation, which makes it difficult for a male to visually find a partner. To test this possibility, we conducted a series of similar experiments using two related strains lacking *hsp70* but differing by eye color (i.e., *hsp70*- and *w+/hsp70*-strains). However, these experiments failed to demonstrate a significant influence of the *white* mutation on learning acquisition and memory retention in (Fig. 1a, b). This observation seems quite logical since CCSP is based predominantly on olfactory rather than visual stimuli. The results obtained in the analysis of learning acquisition and long-term memory retention in the mutant *hsp70-4c* containing four copies of group B were consistent with those obtained for short-term memory. Learning indices immediately and 2 and 8 days after training remained at a high level and did not significantly differ from the wild type (Fig. 1) validating the normal implementation of learning processes and formation of long-term memory in this strain. Thus, the absence of the *hsp70* two copies of group A is not critical for the implementation of learning and memory processes and, hence, we used *hsp70-4c* as a control.

The most intriguing results were obtained in the analysis of the formation of LTM in the mutant strain *hsp70-1c* after HS treatment (Fig. 2). Learning acquisition was not impaired in this strain, regardless of the duration of training (30 min or 5 h) and HS treatment. At a normal temperature, LI remained at a high level for 2 days, but it decreased notably after 8 days, significantly differing from the wild type (Fig. 1B). Finally, in heat-treated males of this strain, LI was dramatically reduced after 2 days (60-fold compared with LI immediately after training and 70-fold compared with the wild type under similar conditions), indicating a disturbance of LTM formation in *hsp70-1c* flies after HS. Our experiments involving the analysis of protein synthesis under normal conditions and after HS treatment help to explain the obtained results. Specifically, it is evident that in *hsp70*- and *hsp70-1c* strains even 5 h after HS treatment, the synthesis of Hsp68 and other Hsps continues at a relatively high rate, in contrast to the strains *hsp70-4c* and *hsp70-6c* where only trace level of Hsps synthesis is observed. At the same time, the *hsp70-6c* strain demonstrates slightly better recovery than *hsp70-4c* one. Interestingly, the level of synthesis of Hsp83 and small Hsps after 5 h of recovery is even higher in *hsp70*- and *hsp70-1c* strains than immediately after HS. This indicates that mild HS (37 °C, 30 min) probably causes serious damage to the brain tissues in *hsp70*- and *hsp70-1c* strains. A more long-lasting synthesis of all Hsps is required to restore the normal functioning of cellular protein synthesis necessary for LTM formation in these strains in contrast to the strains comprising four or six *hsp70* copies (Fig. 3).

Thus, learning acquisition, regardless of the duration of training and effect of stress, was disturbed only in the absence of both groups of *hsp70* genes (A and B). The presence of at least one *hsp70* copy is sufficient to restore the ability to form STM memory and partially LTM under normal conditions (Fig. 1).

## Transcriptome analysis

It is known that the behavioral phenotype and ability to form memory involve complex interactions of various gene networks (Sokolowski 2001). To determine the roles of major stress genes belonging to the *hsp70* family in memory formation and storage, we analyzed the gene expression patterns in the heads of naive males and males after rejection. To achieve this goal, we used a rejection paradigm, which included 5 h of training with a mated female (5h) of several strains with different *hsp70* gene copy numbers (see Methods). We compared the results with those obtained in the *hsp70*- strain lacking all inducible members of the *hsp70* family. We also monitored

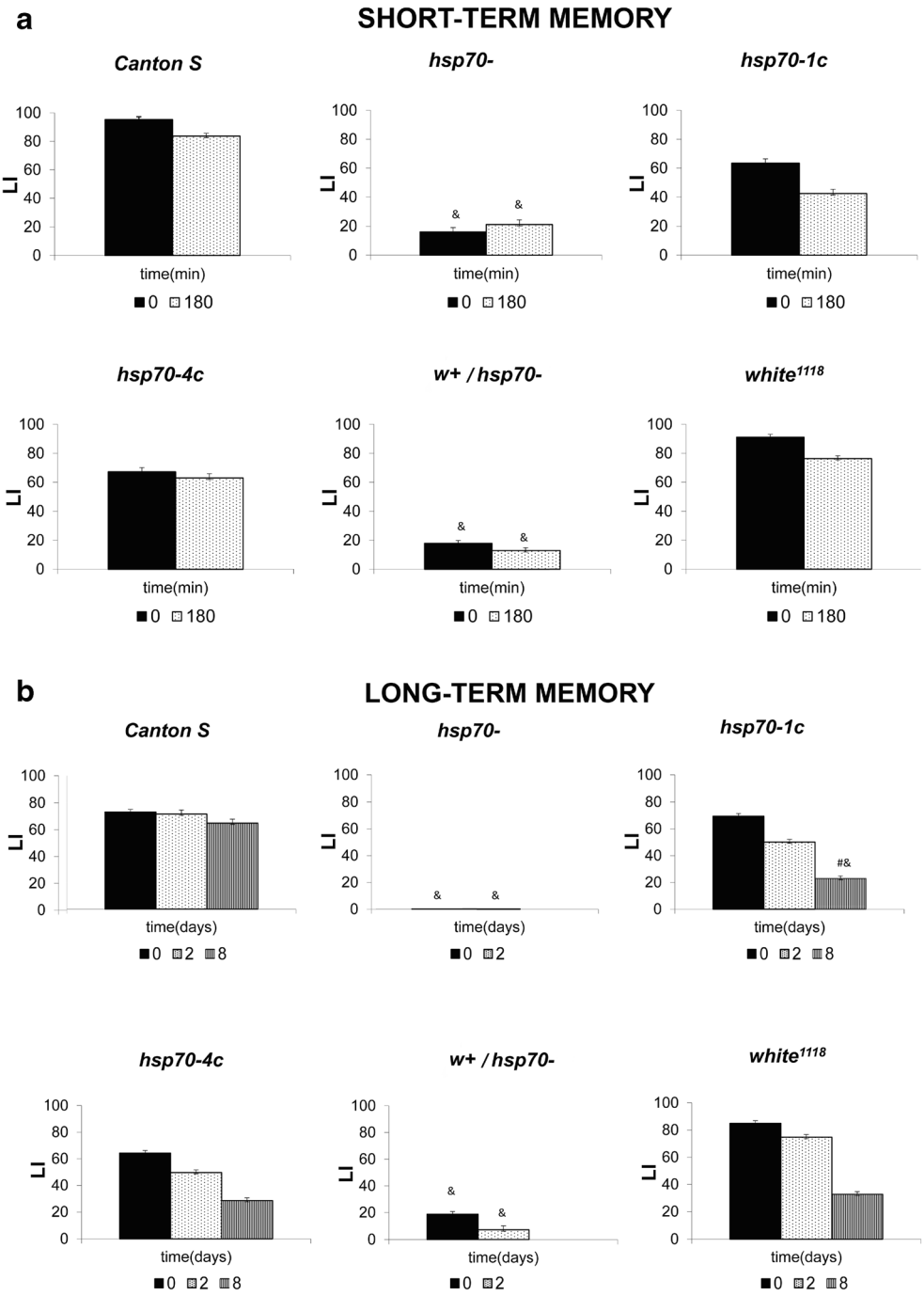


**Fig. 1** Dynamics of learning acquisition, short-term (**a**) and long-term memory retention (**b**) as revealed by conditioned courtship suppression in mutant males under normal conditions. Males of the *Canton-S*, *hsp70-*, *hsp70-1c*, *hsp70-4c, w+/hsp70-*, and *white*$^{1118}$ strains were tested. Abscissa: time after training (min or days); ordinate: LI - learning index, standard units (see M&M). The sample size for each time point was 20 males. & - LI significantly lower than the wild type *Canton-S* strain under similar conditions (two-sided randomization test, $\alpha_R$<0.05); # - LI in the delayed test was significantly lower than in the test immediately following training (two-sided randomization test, $\alpha_R$<0.05)

the transcriptomic changes in the investigated strains (*hsp70-4c*; *hsp70-1c*; *w+/hsp70* and *hsp70-*) that occurred due to the stress of rejection after 5h. The pairwise analysis of differentially expressed genes between all experimental groups and strains is depicted in Table S2.

Analysis of transcriptomic data revealed a low basal level of *hsp70* transcription in the *hsp70-1c* strain, which was not upregulated after 5 h of training. In the strain carrying 4 copies of *hsp70,* the basal level of transcription was higher and slightly increased after 5h of training ( RT-PCR analysis confirmed the upregulation of *hsp70* expression after 1h of training in the male heads of *hsp70-4c* strain (Fig. S2A) and in the male heads of *Canton-S* strain, which contains 6 copies of the *hsp70* gene (Fig. S2B). These results were corroborated by our Western analysis using 7FB antibodies that recognize inducible Hsp70 in the heads of *Drosophila* naïve males (Fig. S3). We analyzed the difference in the Hsp70 expression level in naïve males at normal temperature (Fig. S3A) and after 30 min of recovery following HS 37$^0$C (Fig. S3B) in the investigated strains. Our analysis confirmed that the basal and inducible level of Hsp70 in the

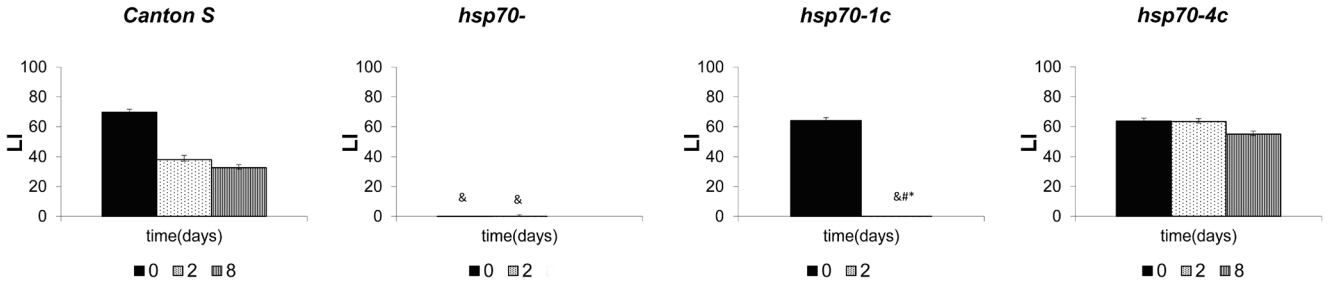


**Fig. 2** Dynamics of learning acquisition and long-term memory retention as revealed by conditioned courtship suppression in mutant males under heat shock (37°C 30 min). Males of the *Canton-S*, *hsp70-*, *hsp70-1c* and *hsp70-4c* strains were tested. Abscissa: time after training (days); ordinate: LI - learning index, standard units. The sample size for each time point was 20 males. * - LI after heat shock significantly differed from LI at 25°C (two-sided randomization test, $\alpha_R<0.05$); & - LI was significantly lower than the wild type *Canton-S* strain under similar conditions (two-sided randomization test, $\alpha_R<0.05$); # - LI in the delayed test was significantly lower than in the test immediately following training (two-sided randomization test, $\alpha_R<0.05$)

studied strains exhibited a clear-cut correlation with the number of *hsp70* copies present in the genome. Slight induction of Hsp70 after rejection was revealed in *hsp70-4c* and *hsp70-6c* strains (Fig. 3, Fig. S3).

The comparative transcriptome analysis demonstrated significantly fewer differences in terms of differentially expressed gene numbers after 5h of training in the two strains lacking *hsp70* copies (Table 1).Tables 1 and 2). By contrast, the *hsp70-4c* strain carrying a maximal number of *hsp70* copies exhibited the largest number of significantly changed genes (up or downregulation) at the level of transcription after rejection (5h). Characteristically, the introduction of a single copy of the *hsp70* gene into the *hsp70-* strain increased the differentially expressed gene number (Tables 1 and 2).

The pairwise analysis of differentially expressed genes using all studied strains exhibited in Table 2 is depicted in Table S2.

## Genes involved in reproduction and memory processes

The performed analysis demonstrated characteristic differences in the expression of gene fruitless -*fru* that play important role in the courtship behavior of male fruit flies (Billeter et al. 2006). Thus, while the *fru* gene was expressed at higher levels in the heads of naive males containing *hsp70* copies (*hsp70-4c* and *hsp70-1c* strains) compared with those lacking representatives of this family (i.e., *w+/hsp70-* and *hsp70-* strains) (Fig. 5a), the rejection resulted in a significant drop in *fru* expression in all the studied strains (Figs. 5b and 6).

Our transcriptome analysis revealed several genes that play an important role in mating and reproductive behavior (*Obp56h* and *Est-6*), as well as in memory formation (*dunce* and *ss*), that were expressed at higher levels in the heads of naive males of *hsp70-1c* and *hsp70-4c* strains than in the *hsp70-* and *w+/hsp70-* strains (Fig. 5a). It is noteworthy that the *Oamb* gene has higher expression in the heads of naive and trained males of the *hsp70-4c* strain carrying the maximal number of *hsp70* copies (Fig. 5a Fig. S3). The neurotransmitter octopamine (OA) and its receptor OAMB are expressed in the neurons of mushroom bodies and are necessary for courtship conditioning (Zhou et al. 2012). It is assumed that the LTM formed during courtship is not under the influence of most clock genes (Sakai and Kitamoto 2006). Interestingly, a significant increase in the expression of *per, tim, vri,* and *Usp8* after 5-h training was observed in all red-eyed strains carrying the normal allele of the *white* gene. By contrast, these genes were downregulated after 5-h training in the white-eyed *hsp70-* strain (Fig. 6).

Interestingly, the expression of the Clockwork orange gene (cwo), which activates transcription of the *period* and *timeless* genes (Gerstner and Yin 2010), was upregulated after 5-h training only in the strains containing *hsp70* genes.

The historical genes rutabaga (*rut*) and dunce (*dun*) that regulate the level of cAMP and are expressed in mushroom bodies (MBs), one of the main structures in the adult insect brain, play a critical role in olfactory learning and memory (Guan et al. 2011; Livingstone et al. 1984; Levin et al. 1992). In the *hsp70-1c* and *hsp70-4c* strains, the basal level in naïve males of *rut* transcription was reduced in comparison to both strains lacking *hsp70* (Fig. 5a). Characteristically, an increase in *rut* gene expression after 5-h training is observed (Fig. 6) only in the strains containing *hsp70* copies (i.e., *hsp70-1c* and *hsp70-4c* strains), characterized by normal memory formation. Similarly, the *dunce* gene, which is involved in regulating the level of the cAMP-specific phosphodiesterase that degrades cAMP slightly increases its expression after 5-h training in the strains lacking *hsp70* copies in the genome (Fig. 6).

The genes encoding the subunits of protein kinase A (Pka) represent another group of critical genes expressed in mushroom body neurons. In the strains containing copies of the *hsp70* gene, the Pka-C3 catalytic subunit was expressed at a higher level in the heads of trained males in comparison to the *hsp70-* strain (Fig. 5b). It is known that activated Pka phosphorylates the transcriptional activator CREB. The level of CrebB isoform expression was increased after 5-h training in all strains

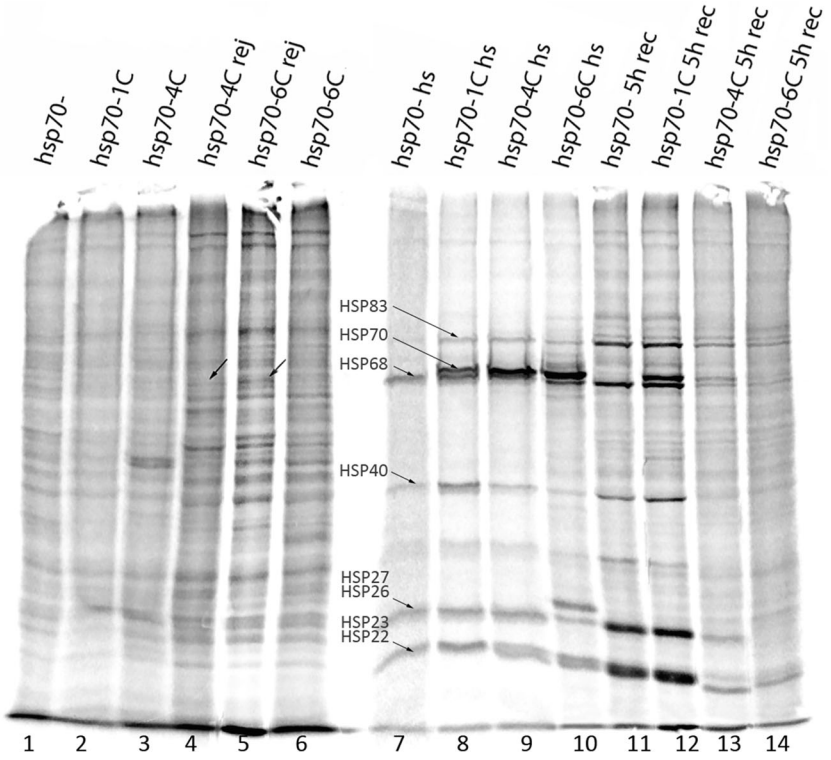


**Fig. 3** Incorporation of $S^{35}$ methionine in *Drosophila melanogaster* imago brains. Strains containing different *hsp70* copy numbers *hsp70-, hsp70-1c, hsp70-4c,* and *Canton-S* (*hsp70-6c*) were used. Lanes 1–6: brains were dissected from male flies kept at 25 °C. Lanes 4–5: brains were dissected from males after 5-h training (rejection experiments) with a fertilized female, lanes 1–3, 6–10 from naïve males. Lanes 7–10: brains were dissected just after HS 37 °C 30min. Lanes 11–14: brains were dissected after 4 h of recovery at 25 °C after HS. The labeling period lasted 1 h in all cases. Arrows indicate Hsp70 induction after rejection

used in the study with the prominent exception of *w+/hsp70-* flies (Fig. 6). Such changes in gene expression may play critical roles in long-term memory formation. It is known that activated Pka modulates the activity of $K^+$ channels (Yao and Wu 2001). Our analysis demonstrated that the relative expression level of the *eag* gene, which exhibits voltage-gated potassium channel activity and is involved in learning (Cowan and Siegel 1986), was slightly higher in the heads of naïve males of the strains with *hsp70* copies (Fig. 4a). A similar expression pattern was revealed for the Shaker (*Sh)* gene, which encodes the A potassium ion channel (Timpe et al. 1988).

It is well-known that epigenetic post-translational modifications (PTMs) of histones that control gene expression patterns play important role in memory formation (Peixoto and Abel 2013; Xu et al. 2014). Our transcriptome analysis demonstrated an increase in the expression level of the *Naa40* (N (alpha)-acetyltransferase 40) gene in the strains containing *hsp70* after 5-h training (Fig. 6) This gene plays an important role in epigenetic protein modifications that may be involved in the processes of learning and memory formation (Gupta et al. 2010; Zovkic et al. 2013).

It was shown that LTM but not STM requires *Notch* (Presente et al. 2004) signaling in courtship conditioning, and *Notch* is also required for memory consolidation, a process believed to require remodeling of existing neurons in adults. In our experiments, the expression of *Notch* has a tendency for upregulation after 5-h training only in the strains containing *hsp70* copies (Fig. 6). We also detected downregulation of expression of gene *Murashka* in the *hsp70-4c* strain after 5-h training. The selective translational repression of gene *Murashka* during LTM formation was earlier observed (Mastushita-Sakai et al. 2010).

Collectively, our experiments demonstrated that 5-h training upregulated the transcription of a certain set of genes involved in memory formation and consolidation, such as *cwo*,

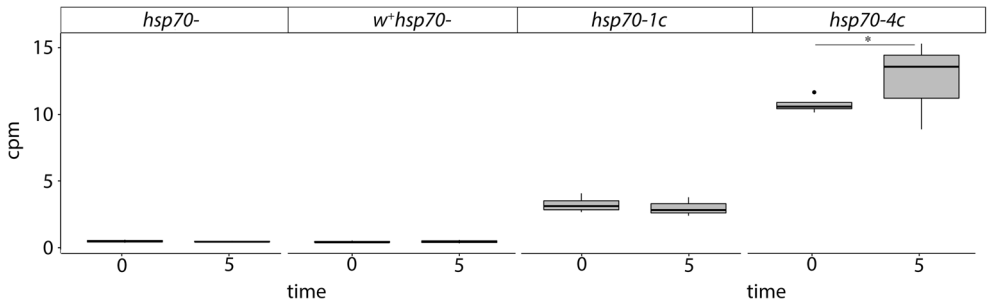


**Fig. 4** The expression level of *hsp70* genes in the heads of naïve males (point 0) and after 5-h training (point 5) in the following strains: *hsp70- 4c*, *hsp70-1c*, *w+/hsp70-*and *hsp70-*. * - $p < 0.05$ (Fisher exact test)

**Table 1** The number of differentially expressed genes in the strains with different *hsp70* copy numbers after 5-h training. p value <0.05

| Drosophila strain (after 5-h training) | Number of genes | |
|---|---|---|
| | Upregulated | Downregulated |
| *hsp70-4c* | 1111 | 1191 |
| *hsp70-1c* | 758 | 939 |
| *w+hsp70* | 521 | 527 |
| *hsp70-* | 248 | 266 |

*Gclm*, *Desat1*, *cer*, *spin*, and *ru*, only in strains containing *hsp70* copies (Fig. 6).

## Immune system gene expression depends on the presence of hsp70 genes

In the last decade, several groups have demonstrated that putative antimicrobial peptides expressed in the brain of flies and other organisms influence LTM (Bozler et al. 2017; Barajas-Azpeleta et al. 2018). Mating appears to switch on the immune system in preparation for the effects of external agents, including various pathogens, as well as possible stress of rejection by females.

Comparison of immune response gene expression in the heads of naive males and males after 5-h training from the studied strains demonstrated similar patterns of expression in the strains lacking *hsp70* genes (i.e., *hsp70-* and *w+/hsp70-*) suggesting a role of constitutive Hsp70 expression in this vital process (Fig. S6). Interestingly, in the heads of naive males of the strain with four copies of *hsp70,* the relative level of several immune genes (i.e., *LyzE, Mtk*, *CecA2 CecA1, Def, DptA*, and *DptB*) was lower than in strain *hsp70-1c* containing only one copy of *hsp70* (Fig. S5). On the other hand, the relative expression level of genes such as *CecA1*, *CecA2*, *Mtk, Im1, Im3*, and *GNBP-like3* after 5-h training was higher in the *hsp70-4c* strain (Fig. S6).

Only two genes belonging to the innate immune system (CecA1 and CecA2) were upregulated in all the studied strains after 5-h training. Figs. 7 and 8). Notably, in both strains containing *hsp70* copies, the induction of *Im1*, *Mtk*, and *Pgrp-SB1* after 5-h training was evident (Fig. 8). Moreover, the maximal upregulation of all differentially expressed genes was observed in strain *hsp70-4c* (Fig. 8), which was the only strain that showed a significant upregulation of the genes *Dro*, *Cg43055*, *GNBP-like3*, and *IM3*. These data suggest that the basal level of Hsp70 expression plays an important role in modulating the immune response and in the function of certain immune antibacterial peptides that participate in LTM formation.

Interestingly, after 5-h training, the expression of the *LysE* gene, functioning in the negative regulation of the innate immune response, was decreased in strains carrying *hsp70* genes and upregulated in *hsp70-* strains.

It is of note that differentially expressed genes *Dro, PGRP-SB1, Mtk,* CecA1, and *CecA2* (Fig. 8) belong to a category of genes with a cyclic pattern of transcription in the heads of *D. melanogaster* (McDonald and Rosbash 2001), which corroborates their involvement in the function of the *Drosophila* central nervous system, including LTM formation and consolidation (Gerstner and Yin 2010).

**Table 2** The number of up- and down-regulated genes in naïve males in the strains with different *hsp70* copy numbers compared to the strain without *hsp70* genes *(hsp70-)* in control conditions. p value <0.05

| Drosophila strain (naïve males) | Number of genes | |
|---|---|---|
| | Upregulated | Downregulated |
| *hsp70-1c* vs *hsp70-* | 1046 | 713 |
| *hsp70-4c* vs *hsp70-* | 1387 | 1192 |
| *w+hsp70-* vs *hsp70-* | 286 | 218 |

## Genes involved in methionine metabolism and glutathione metabolic processes showed altered expression in trained males

Genes involved in methionine metabolism (transsulfuration pathway) comprise another distinct group that exhibited characteristic changes after 5-h training in all strains used in the study (Fig. 9). Interestingly, maximal induction of expression (especially in the *hsp70-4c* strain) was demonstrated for the glycine N-methyltransferase (*Gnmt*) gene, which controls the amount of the methyl donor S-adenosylmethionine (Obata et al. 2014) and plays a key role in life extension in *Caenorhabditis elegans* and fruit flies (Obata and Miura 2015). In the *hsp70-4c* strain, we also observed a strong induction of the genes *Sardh* and *vermillion* (*v*). *Sardh* plays an important role in the S-adenosylmethionine cycle. *Vermillion* gene participates in tryptophan metabolism and plays a role in memory performance, as revealed in a courtship-conditioning paradigm (Savvateeva et al. 1999).

All strains with the prominent exception of *hsp70-* were also characterized by upregulation of the *cbs* gene encoding cystathionine beta-synthase and representing one of the three major genes responsible for H2S production in *Drosophila* and other organisms including humans (Kabil et al. 2011; Kimura 2014). Additionally, training of the males (5h) resulted in the induction of ecdysone-induced genes (i.e., *Eip55E*; *Eip71CD*), as well as several other methionine metabolism genes including *Sam-S*, *Ahcy*, and *GstE3* (Fig. 8). Eip55E encodes cystathionine γ-lyase (*cse*) and together with *cbs,* is responsible for H2S production in various organisms (Kabil et al. 2011; Zars 2017).

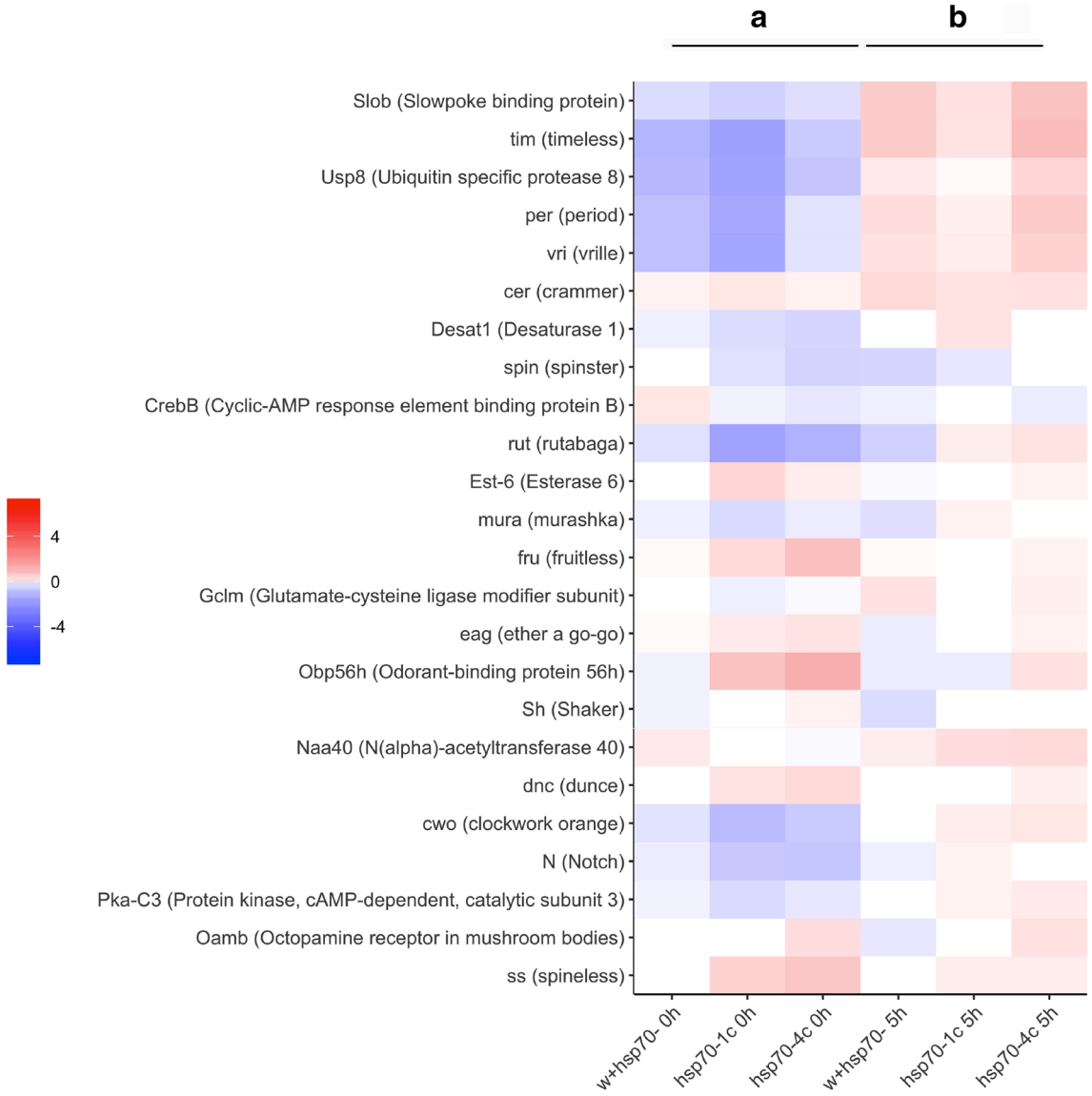


**Fig. 5** Heatmap illustrating the comparative analysis of differentially expressed genes involved in reproduction and memory processes. Differentially expressed genes in the heads of naive males – 0 h (**a**) and the males after 5 h of training with fertilized females – 5 h (**b**) in strains *hsp70-4c*, *hsp70-1c*, *w+/hsp70-* in comparison to *hsp70-*. Genes with $p < 0.05$ (quasi-likelihood F-test) in at least one comparison were considered significant. (Statistic data for described genes are presented in Table S3 and Fig. 7)

Analysis of the transcriptome data revealed a significant effect of 5-h training on the expression of the gene system participating in sulfur metabolism and glutathione metabolic processes.

Glutathione transferases (GSTs), represented by 40 genes in *Drosophila,* are ubiquitous key enzymes that catalyze the conjugation of glutathione to xenobiotic compounds in the detoxification process and play a critical role in insect chemo detection by modifying odorant molecules (Gonzalez et al. 2018). When we compared the expression levels of "sulfur" and GST genes in naive males in the studied strains, it became clear that strains without *hsp70* copies were characterized by comparatively lower expression levels of these genes (Figs. S6 and S7). By contrast, after 5-h training, the minimal relative expression level of these genes was detected in *hsp70-4c* flies and maximal level in the *w+/hsp70-* (Figs. S6, S7). Glutathione (GSH) is a downstream critical metabolite of the transsulfuration pathway (TSP), and hence, it is not surprising that genes making up these two olfactory-related systems exhibit a rather similar pattern of expression after 5-h training (Durand et al. 2018). In our experiments, the expression


**Fig. 6** Heatmap illustrating the comparative analysis of differentially expressed genes involved in reproduction and memory processes. Differentially expressed genes in the heads of males after 5 h of training (5h) with fertilized females in comparison to corresponding naive males of the *hsp70-4c*, *hsp70-1c*, *w+/hsp70-* and *hsp70-* strains. Genes with $p < 0.05$ (quasi-likelihood F-test) in at least one comparison were considered significant. (Statistic data for described genes are presented in Table S3 and Fig. 7)

levels of the number of genes belonging to this category increased after 5-h training: *GstE3*, *GstE7*, *GstE1*, and *Eip55E* decreased in all the studied strains: *GstZ1*, *gfzf*. It should be noted that maximum induction of these genes was observed in the *w+/hsp70-* strain (Fig. S8). Only strains carrying *hsp70* copies were characterized by an upregulation of *GstD2* and *Gclm* gene expression (Fig. S8). *GstD2* participates in the modulation of chemoperception in insects (Gonzalez et al. 2018) while glutamate–cysteine ligase modifier subunit (*Gclm*) is involved in glutathione biosynthesis, and its overexpression was shown to extend the mean life span (Orr et al. 2005). Additionally, only in the *hsp70-4c* strain, the *GstD9* and *GstZ2* genes were induced after 5-h training.

The observed differences in the learning and memory formation as well as in brain transcription patterns characteristic for strains lacking *hsp70* genes in principle may be due to some developmental abnormalities in the brains of the strains with deletions accumulated in the process of *hsp70* deletion generation. To check this possibility, we obtained a new *hsp70-* strain with GFP gene coding region controlled by the Elav gene promoter (see Materials and methods). We examined brain samples from third instar larvae and adult flies by confocal microscopy. We monitored the GFP fluorescence caused by the activity of the Elav gene promoter in the neurons of the mushroom body. In the course of this analysis, we failed to detect any visible gross deviations in the structure of the mushroom body in the *hsp70-* strain (Fig. S9).

## Discussion

At present, a plethora of reports has described numerous genes that are somehow involved in the learning process and STM and LTM in fruit flies and other organisms including humans (Griffith and Ejima 2009; Winbush et al. 2012; Keleman et al. 2007; Akalal et al. 2011; Kahsai and Zars 2011; Sokolowski 2001; Guan et al. 2011). Many circuits and signaling pathways involved in memory formation are rather conservative because memory exists at all levels of organization from worms to humans. Learning and long-term memory formation in *D. melanogaster* represent important neuronal functions that are controlled by various interacting gene systems. Early stages of memory formation do not require de novo protein synthesis and are achieved by covalent modifications of preexisting target proteins in the neurons responsible for cellular membrane conductivity and neuronal excitation (Bailey et al. 1996; Tully 1996; Kandel 2012). Long-term memory requires the synthesis of new proteins and the formation of new synapses (Kandel 2012). The regulation of gene expression during the formation of long-term memory partially is due to the phosphorylation of the transcription factor CREB (CRE-binding protein) and the induction of transcription of genes having CRE motifs (CREB targets) in the promoter region (Flexner et al. 1963; Kandel 2012). Notably, the level of CrebB isoform expression was increased after 5-h training in all strains used in our studies except for *w+/hsp70-* flies (Fig. 6).

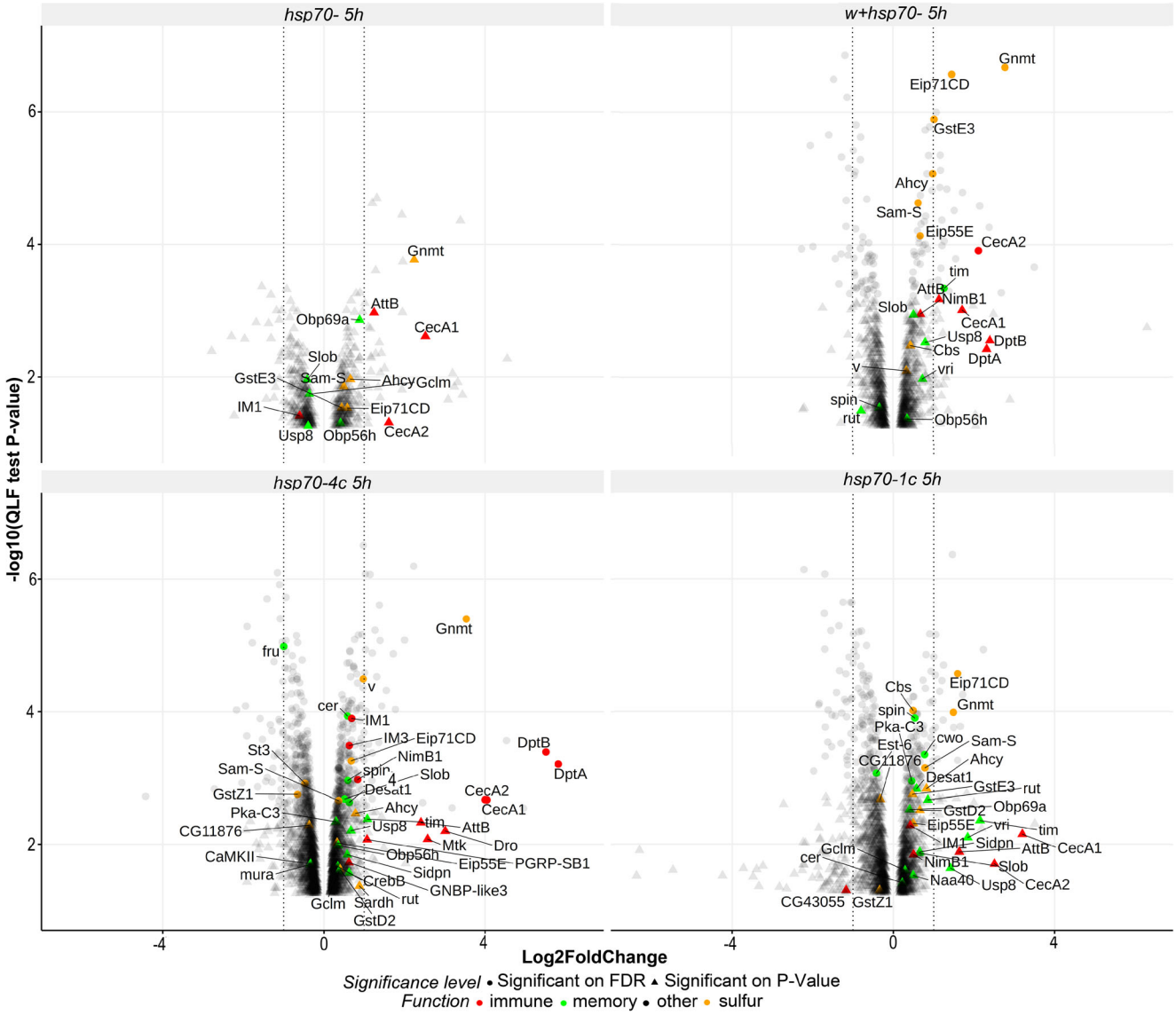


**Fig. 7** Volcano plot. Differentially expressed genes in the heads of males after 5 h of training (5h) with fertilized females in comparison to corresponding naive males of the *hsp70-4c*, *hsp70-1c*, *w+/hsp70-*, and *hsp70-*strains. Only genes that exhibited statistically significant differences are included in the plot (p value <0.05 or FDR<0.05). LogFC values are displayed on the abscissa. Upregulated genes are depicted on the right side of the plot, and downregulated genes are shown on the left side of the plot

Several groups studying LTM using conditioned courtship or courtship suppression in males that have been repeatedly rejected by mating females have used RNA sequencing and described a large number of differentially expressed genes with known functions, including those that function in cyclic AMP, neuronal development, chromatin biology, translation, and various aspects of behavior (Ge et al. 2004; Winbush et al. 2012; Keleman et al. 2007; Ishimoto et al. 2009). However, it is clear that LTM formation is an extremely complex process and includes contributions from additional mechanisms. Thus, here we used several *D. melanogaster* strains with different copy numbers of *hsp70* genes to determine the role of this major stress protein in STM and LTM formation. Available data suggest a coupled evolution of stress response and memory formation (Hooper et al. 2016; Porto et al. 2018; Scaccianoce et al. 2003; Thekkuveettil and Lakhotia 1996).

This is also confirmed by the fact that psychophysiological stress can cause the induction of proteins belonging to the Hsp70 family. Thus, Hsp70 and Hsp60 levels were elevated in daphnia exposed to a predator (fishes) (Pijanowska and Kloc 2004). Similarly, stress caused by predators induces Hsp70 expression in goldfish and cats (Kagawa et al. 1999; Fleshner et al. 2004). Immersion of rats in water caused a significant increase in the level of Hsp70 and Hsc70 mRNA in the hippocampus (Fukudo et al. 1997; Fukudo et al. 1999).

Heat shock proteins and, in particular, Hsp70 is important in the processes of protein folding and degradation (Hartl et al. 2011), and, hence, they participate in the

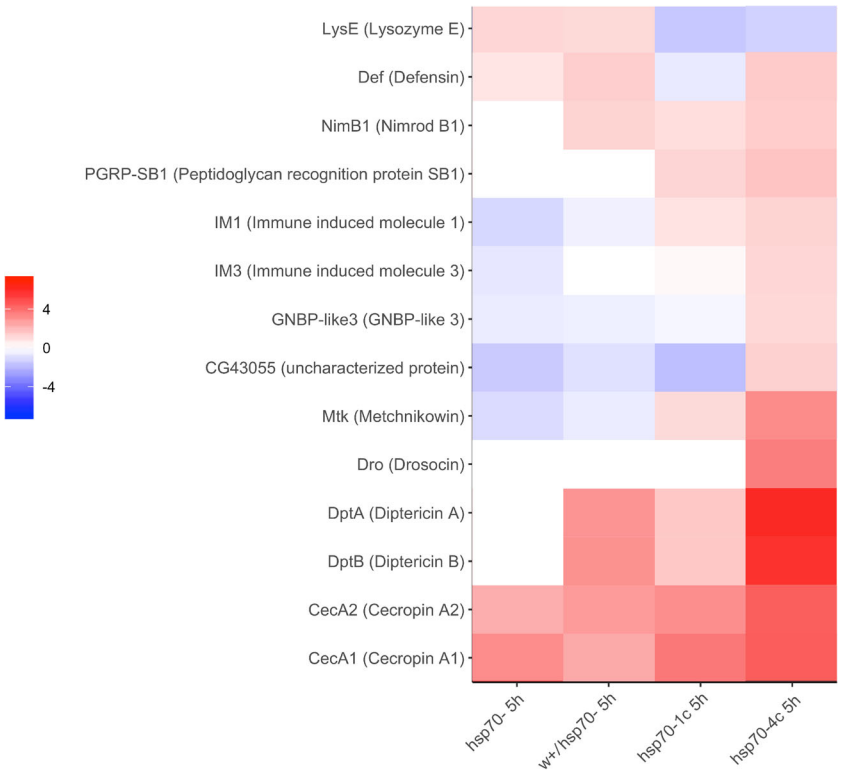


**Fig. 8** Heatmap illustrating the comparative analysis of differentially expressed genes involved in immune processes. Differentially expressed genes in the heads of males after 5 h of training with fertilized females in comparison to corresponding naive males of the *hsp70-4c*, *hsp70-1c*, *w+/hsp70-*, and *hsp70-*strains. Genes with $p < 0.05$ (quasi-likelihood F-test) in at least one comparison were considered significant. (Statistic data for the described genes are summarized in Table S3 and Fig. 7)

processes of protein synthesis and transport, that are necessary for the maintenance of existing synapses and the formation of new synapses during memory formation. In the promoter region of mammalian *hsp70* genes, the CRE-motifs were identified. Furthermore, in the promoter region of *Drosophila hsp70* genes, sites for binding of transcriptional factor FOXO were found (Donovan and Marr 2nd 2016). It was shown that FOXO-DAF-16 regulates learning and memory, regeneration, and stress resistance in *C. elegans* neurons (Kaletsky et al. 2016: Kim and Webb 2017). Collectively, these data suggest a possible role of Hsp70 in the memory processes.

Similar to our results (Fig. 4) in several rodent models, the induction of *hsp70* was detected in postsynaptic neuronal structures, hippocampus, and cerebellum during learning, as well as memory formation and consolidation (Porto et al. 2018; Ambrosini et al. 2005; Pizarro et al. 2003; Suzuki et al. 1999; Igaz et al. 2004). It was also demonstrated that an optimal level of neuronal Hsp70 is required for normal memory formation (Ambrosini et al. 2005; Ammon-Treiber et al. 2008; Pizarro et al. 2003). These results explain the observed severe memory disturbances observed in strain *hsp70-* (Figs. 1 and 2).

Taking into account the observation that LTM formation and consolidation require the synthesis of new proteins and various modifications of existing proteins in the brain regions, the presence and induction of Hsp70 during memory formation are not surprising. However, most of the abovementioned studies are correlative, and, therefore, the investigators were able to only speculate regarding specific gene systems and signal pathways that require the presence of Hsp70 for memory formation.

Thus, the *Drosophila* strains used in this study either lacking or carrying different numbers of *hsp70* copies represent a unique tool to pin-point gene systems that interact with Hsp70 during memory formation. Generally speaking, the dramatic memory deficit observed in *hsp70-* flies may be a consequence of the deletion of all copies of *hsp*70 per se because the deletion may have some adverse neurodevelopmental effects and indirectly affects learning and memory formation. However, when comparing the LI in different training schemes (30 min or 5 h), it is evident (Fig. 1) that a low level of learning is still observed in *hsp70-* and *w+/hsp70-* flies after 30 min of training. This does not allow us to explain the low ability of the *hsp70-* strain in learning by possible neuronal developmental disturbances inflicted by deletion per se. Besides, the introduction of a single *hsp70* copy into the strain with the deletion significantly restored learning and memory parameters at normal temperatures. The performed analysis of GFP fluorescence in the neurons of third instar larvae and the brains of adult flies revealed no visible gross abnormalities in the development of the nervous system in the *hsp70-* strain. Therefore, we assume that the absence of memory formation in *hsp70-* flies most likely is not due to some defects in the development of the nervous system, but is associated with the deficit of Hsp70 necessary for the induction of several

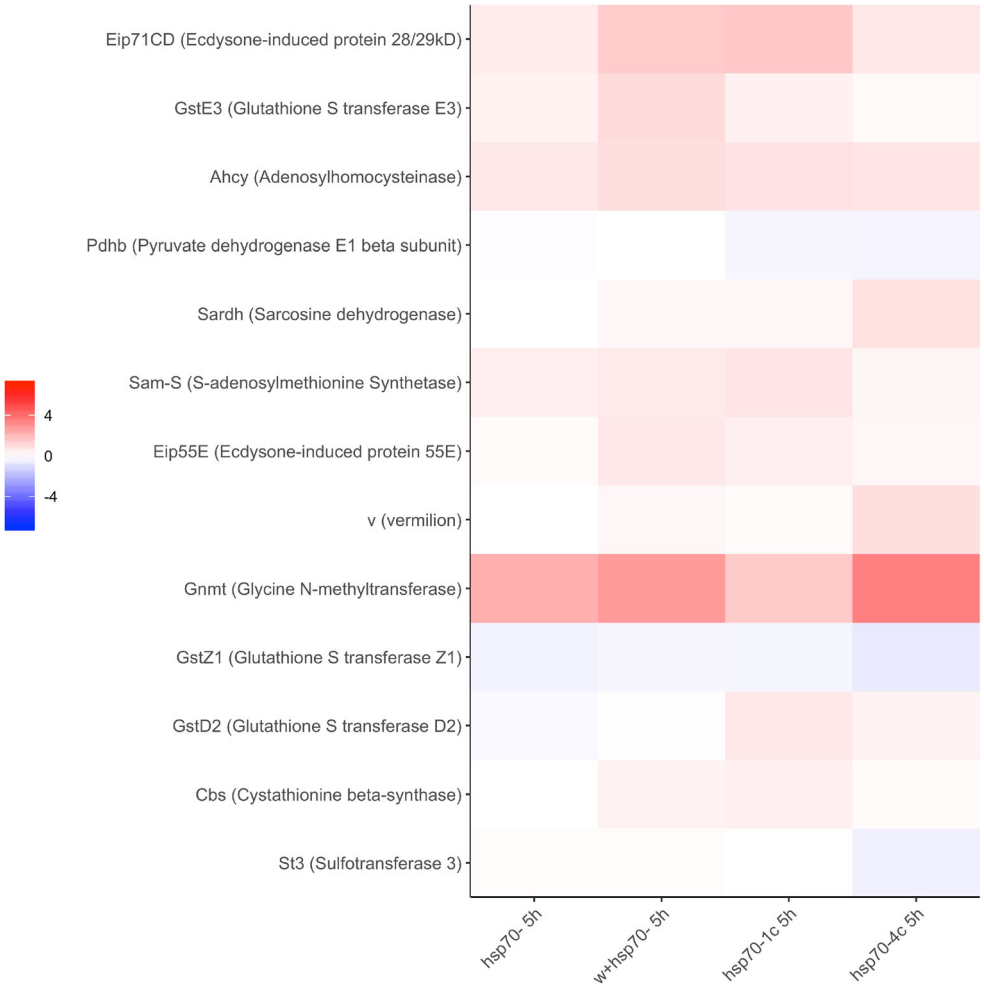


**Fig. 9** Heatmap illustrating the comparative analysis of differentially expressed genes involved in the processes of sulfur metabolism. Differentially expressed genes in the heads of males after 5-h training with fertilized females in comparison to corresponding naive males of the *hsp70-4c*, *hsp70-1c*, *w+/hsp70-*, and *hsp70-* strains. Genes with $p < 0.05$ (quasi-likelihood F-test) in at least one comparison were considered significant. (Statistic data for described genes is represented in Table S3 and Fig. 7)

transcription cascades participating in memory formation. It is also clear that this problem requires more in-depth study. In our experiments, we also used red-eyed flies lacking all *hsp70* genes but containing a normal allele of the *white* gene ("mini-white" construct). This strain developed by us from the white-eyed strain *hsp70-* was used as an additional control to eliminate the effect of the *white* gene, which has been previously shown to significantly affect synaptic transmission and memory in fruit flies (Anaka et al. 2008; Sitaraman et al. 2008).

Specifically, our transcriptomic analysis revealed several major pathways that depend on the presence of *hsp70* copies in the genome and are involved in memory formation. Thus, it is well-known that the cAMP signaling cascade, which changes the excitability of mushroom body neurons, plays a critical role in learning, LTM, and STM formation (Levin et al. 1992; Livingstone et al. 1984). Adenylate cyclase (Ac) is encoded by *rutabaga* (*rut*), while cAMP-specific phosphodiesterase is encoded by *dunce,* which degrades cAMP. Additionally, cAMP signaling (*rut-dnc*) modulates synaptic morphology and physiology (Renger et al. 2000), a requirement for learning and memory formation (Zhong and Wu 1993). Characteristically, here we established that the expression of these two "historical" genes in response to 5-h training depends on the presence of *hsp70* copies in the fly genome (Figs. 5 and 6) Thus, in both strains carrying *hsp70* copies (i.e., *hsp70-1c* and *hsp70-4c*), 5-h training led to the upregulation of *rut* expression while the transcription of gene *dnc* was not changed. By contrast, in the strains lacking *hsp70* copies (*hsp70-*and *w+/hsp70-)* the expression of the *rut* was downregulated in parallel with a slight increase in *dnc* transcription, which should result in a drop in the cAMP level and, hence, may interfere with memory formation.

In our studies, we also observed an upregulation of critical components of CREB-responsive transcription such as the *Pka-C3* catalytic subunit, preferentially in the strains with *hsp70* copies. Phosphorylated CREB binds to cAMP-responsive elements (CRE) in the regulatory regions of cAMP-inducible genes. This system has an essential and well-established role in long-term memory formation throughout a diverse set of organisms (Presente et al. 2004; Zhang et al. 2013). It was shown that exogenous Hsp70 stimulates CREB phosphorylation in mice hippocampus, and improves memory (Kwon et al. 2019).

The effect of *hsp70* deletions herein is not surprising since LTM requires protein synthesis, and, hence, normal CREB

activity and intact signaling through CaMKII (Yu et al. 2006; Akalal et al. 2010).

We observed differential expression of many immune response genes in the strains with different *hsp70* copy numbers. Earlier, it was found that Diptericin B, an immune peptide with antimicrobial activity, and Gram-Negative Bacteria Binding Protein like 3 (*GNBP-like3*), are upregulated following behavioral training (Barajas-Azpeleta et al. 2018). Deletion and knockdown experiments revealed that *Diptericin B*, *GNBP-like3*, and *IM18* (Barajas-Azpeleta et al. 2018; Bozler et al. 2017) regulate long-term but not short-term memory in *Drosophila.* In our results, most of the immune genes related to LTM formation exhibited significantly higher induction after 5-h training in the strains containing *hsp70* copies, i.e., *hsp70-1c* and especially *hsp70-4c*, confirming the need for a certain basal level of Hsp70 synthesis (Figs. 7 and 8). This result confirms that Hsp70 modulates the immune response (Kim and Yenari 2013) and that *hsp70* genes may be involved in the activation of immune peptides in the nervous system during LTM formation. These data help to understand how some immune peptides may have been repurposed to influence the function of the nervous system (Harris et al. 2015; Stevens et al. 2007).

Furthermore, there is growing evidence in favor of crosstalk between the stress gene system comprising *hsp* genes and another ancient and highly conserved $H_2S$-producing system which also provides an antioxidant defense in all organisms (Kimura 2014; Paul and Snyder 2015; Stevens et al. 2007; Yurinskaya et al. 2020). It is known that $H_2S$ acts as a neuromodulator and promotes long-term potentiation and regulates intracellular calcium levels, necessary for learning and memory (Nagai et al. 2004; Yong et al. 2010). It has been proposed that $H_2S$ is essential for visual memory (Zars 2017). Also, it was shown that mutant flies containing an insertion in the first exon of *cbs* gene are characterized by memory deficit (Kuntz et al. 2017).

Our transcriptomic analysis demonstrated that naive males from the strains without *hsp70* copies but differing by eye color were very similar in terms of sulfur and GSH metabolism (Figs. S6, S7). However, the presence of the wild-type allele of the *white* gene significantly modulated the response of genes participating in sulfur metabolism, courtship behavior, and the circadian rhythm in response to the 5-h training procedure (Figs. 6 and 9).

It is of note, that we observed comparatively higher induction of the most sulfur metabolism genes in the *w+/hsp70-* and *hsp70-1c* strains after 5-h training. These findings suggest that the presence of the *hsp70* gene can modulate the activation pathways of sulfur metabolism genes.

We constructed the network for protein–protein interaction using genes differentially expressed in the process of memory formation (5-h training) described in the article (Fig. S10). It is seen that Hsp70 proteins (A and B) interact with a few definite protein groups and are located in the center of the interactome.

Our experiments exploring strains with different *hsp70* copy numbers demonstrated a positive correlation between the number of *hsp70* copies and LTM formation (Fig. 1). However, when we applied a heat shock treatment (37°C, 30 min) to the studied strains, we obtained seemingly paradoxical effects. Thus, in the strain with four *hsp70* copies, HS treatment slightly improves memory formation, but in the strain containing only one copy of *hsp70* (*hsp70-1c*) and characterized by comparatively lower LTM, HS treatment dramatically disturbed LTM formation (Fig. 2). We speculate that here we have some trade-off and in *hsp70-4c* strain, the induced Hsp70 amount is optimal and sufficient to perform chaperone properties eliminating stress consequences and participate in LTM formation. By contrast, in the case of the *hsp70-1c* strain, a low level of Hsp70 is insufficient to protect the brain tissues from the deleterious effect of HS which results in strong memory impairment in this strain after HS.

Our experiments exploring $^{35}$S-methionine labeling of proteins synthesized in the brain shed new light on this issue. It was shown that in *hsp70-* and *hsp70-1c* strains even 5 h after HS, the synthesis of all Hsps and especially small ones continues at a high rate. It is known that low molecular weight chaperones after HS associate with misfolded proteins and facilitate their folding or degradation by other chaperones (Sun and MacRae 2005). To this end, it was shown that Hsp23 and Hsp26 show high expression levels in CNS during fly ontogenesis, suggesting a role in neural system development (Santana et al. 2020). Probably in the case of strains *hsp70-* and *hsp70-1c*, small Hsps are required for recovery of neuronal structures after HS. Higher level of small Hsps observed in strains *hsp70-* and *hsp70-1c* probably represents compensation for the absence or low level of Hsp70 synthesis in the brains of strains *hsp70-* and *hsp70-1c* that are not able to recover 5 h after HS (Figs. 2 and 3).

Our experiments also demonstrated that the rejection accompanied by antiaphrodisiac excretion represents serious stress for *Drosophila* males and results in memory formation and modulation of multiple gene expression. The constitutive level of *hsp70* gene expression in the brain and probably a modest induction of these genes by 5h training (Figs. 3 and 4, Figs. S2, S3) are necessary to cope with the stress consequences and provide conditions for memory formation.

## Conclusions

This is the first report describing an important role of constitutive *hsp70* expression in learning and memory formation in *D. melanogaster* males in CCSP. In this study, exploring the courtship rejection paradigm and *D. melanogaster* strains with

different *hsp70* copy numbers including strains carrying a deletion of all six *hsp70* genes, we demonstrated that a low constitutive level of Hsp70 is required for learning and the formation of short and long-term memories in males. The courtship-dependent genes interacting with the *hsp70* system that we identified contribute to many vital biological processes, including behavior and reproduction, stress, and immune responses as well as the production of gaseous signaling molecules such as $H_2S$. Furthermore, our results revealed a positive correlation between the number of *hsp70* copies present in Drosophila genome and the number of differentially expressed genes that responded to training with a fertilized female. Further studies are necessary to dissect in detail the molecular mechanisms underlying the demonstrated role of *hsp70* expression in memory formation and consolidation in flies and other organisms.

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**Author contribution** Study conception and design were performed by (Evgen'ev M.B., Zatsepina O.G., Nikitina E.A.). Material preparation, data collection, and analysis were performed by (Shilova V.Y., Chuvakova L.N., Sorokina S., Tokmacheva E.V.). Libraries for RNA-seq were prepared by (Chuvakova L.N., Funikov S.Y.). Differential gene expression analysis was performed by (Rezvykh A.P.). Construction of Elav GFP/FM7; Hsp70-/Hsp70- strain for confocal analysis was done by Vorontsova J.E. The first draft of the manuscript was written by (Zatsepina O.G., Nikitina E.A., Evgen'ev M.B.). All authors read and approved the final manuscript.

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## Declarations

**\Consent to participate** Consent for participation was obtained from all authors of this paper.

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