Protective Role of *Hsp27* in the Nonylphenol-Induced Locomotory and Longevity Toxicity

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Abstract

Background Gut health is directly proportional to an organism's fitness. Our recent study showed a functional link between oxidative stress and heat shock protein 27 (Hsp27, a stress protein) in the *Drosophila* larval gut, which coordinates the nonylphenol (an endocrine disruptor) allied sub-cellular and developmental adversities.

Objective In continuation with the prior study, the present study aimed to explore the association of *Hsp27* with locomotory and survival against nonylphenol-induced toxicity in the *Drosophila* gut.

Methods and Methodology The freshly emerged adult flies were exposed to nonylphenol (5.0 µg/mL) for 10 to 40 days, and their locomotory performance (climbing activity) and survivability were assessed. ANOVA was used to evaluate the statistical significance of the mean values in control and treated flies.

Results Nonylphenol exposure markedly influenced locomotory activity and survivability after 30 to 40 days. For instance, ∼76% (40 days) declined locomotor behavior, and ∼35% (40 days) reduced survivability was observed. While the overexpression of *Hsp27* in the organism's gut showed improvement in locomotory performance and survivability after 30 to 40 days. No significant alteration in locomotory performance and survivability was observed after 10 to 20 days of nonylphenol exposure.

Conclusion The present study illustrates that *Hsp27* overexpression in the *Drosophila* gut improves the locomotory performance and survivability in the nonylphenol exposed *Drosophila*. This also indicates the possible connection between the gut and organismal fitness.

Keywords
- Nonylphenol
- Hsp27
- locomotion
- survival
- *Drosophila*

Introduction

Nonylphenol is an endocrine-disrupting chemical (EDC) and a persistent alkyl phenolic compound, which is widely used in both industrial and domestic as a surfactant, paints, plastics, herbicides, and pesticide.[1] According to the Environmental Protection Agency (EPA) recommendations, the permissible limit of nonylphenol is 1.7 µg/L in salt water and 6.6 µg/L in freshwater.[2] However, the nonylphenol concentrations of 1.22 to 7.24 µg/L, 3.31 to 30.96 µg/kg, and 18.03 to 23.89 mg/kg have been recorded in water, sediments, and tomato plant, respectively.[3,4] Living organisms are exposed to nonylphenol throughout their lives via the ingestion of contaminated food/water, causing a disturbance in hormonal balance and consequently leading to various health disorders such as behavior disorders, developmental and reproductive abnormalities, and alterations in immune functions.[5–7] In a study by Paolella et al,[8] nonylphenol exposure

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causes ER stress, apoptosis, and mitochondrial dysfunction in the hepatic cell line. Additionally, it was noted that nonylphenol elicits oxidative stress in a dose- and concentration dependent manner. The study also revealed that when exposed to nonylphenol, the Keap1–Nrf2 pathway was activated, reducing the damage caused by oxidative stress at lower doses and shorter exposure. However, at higher doses and chronic exposure, the Keap1–Nrf2 pathway is inhibited, thereby increasing oxidative stress.[9] An organism's gut interfaces between environmental conditions and an individual's fitness. In this line, several studies have underlined toxicants, such as microplastic and pesticide-induced gut toxicity may lead to various health adversities.[10,11] Similarly, our recently published study shows that nonylphenol exposure to *Drosophila* larvae leads to oxidative stress in the gut, which is further associated with poor developmental indices.[12] Therefore, restoring or maintaining gut health might help preserve an organism's fitness against nonylphenol exposure.

The cellular machinery is orchestrated to execute the various defense responses against different stress types, including chemical stress.[13] In this line, the induction of heat shock protein (Hsp) has been established as the primary cellular defense mechanism. Hsp27 is a member of the small Hsp family and is known for its anti-aging and anti-oxidative nature.[14] Our previous results have shown that *Hsp27* overexpression in the midgut of *Drosophila* larvae confers cellular protection against nonylphenol via decreasing oxidative stress.[12] Therefore, in continuation of our previous work, this study aims to investigate the role of *Hsp27* in the midgut against nonylphenol-induced locomotory and survival toxicity using the *Drosophila melanogaster* model system.

Drosophila is widely used as an alternative animal model for toxicological studies.[15] Like higher vertebrates, *Drosophila* also exhibits an age-dependent decline in survival and behavioral function. Locomotory behavior is a reliable parameter to assess motor function in flies. The survival of an organism depicts the overall health of the organism. These characteristics can be affected by an environmental factor or disease etiology, leading to the progression of aging and life-shortening effects. Typically, the life span of healthy *Drosophila* is approximately 65 to 70 days at 25°C.[16] Moreover, *Drosophila* has been previously used to understand the link between *Hsp27*, aging, and locomotory behavior.[17] The European Centre for the Validation of Alternative Methods (ECVAM) recommends *Drosophila* as an alternative model that follows the 3R rule, i.e., reducing, refining, and replacing the use of laboratory animals.[18]

Methods and Methodology

Experimental Organisms and Culture

In the study, the stock w^{1118} and Gal4-UAS transgenic lines of *Drosophila melanogaster*, specifically *Np1-Gal4* (GAL4 expressed in the *Drosophila* midgut) and *UAS-Hsp27* (expressed *Hsp27* under the control of UAS) (►**Fig. 1**). *Drosophila* strains were reared on standard media containing agar-agar, maize powder, sugar, yeast, nipagin, and propionic acid at 24°C with 12 hour light/dark cycles. An additional yeast supplement was given to ensure the organisms' proper growth.

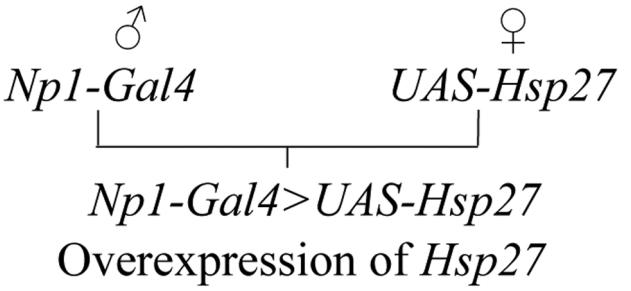

Fig. 1 Schematic representation showing the crossing scheme between *Np1-Gal4* and *UAS-Hsp27*.

Exposure Regime

The study used a nonylphenol concentration of 5.0 µg/mL (Sigma-Aldrich). Nonylphenol concentrations were selected based on the previous study.[12,19] Flies were transferred into nonylphenol mixed food (dissolved in 0.3% dimethyl sulfoxide [DMSO]; final concentration). For control and vehicle control, flies were fed a conventional diet and the diet containing 0.3% DMSO, respectively.

Locomotory Assay

The locomotory behavior of the organism was assessed according to the previously described method with a few modifications.[20] In brief, the newly emerged flies (after 2–3 days post eclosion) were subjected to nonylphenol for 10 to 40 days, and the climbing activity of control and nonylphenol-exposed flies was assessed after each 10 days exposure interval. In brief, the flies were placed in a locomotory chamber marked 15 cm (20 flies/vial). The flies were gently tapped down to the bottom of the locomotory chamber and allowed to move toward the 15 cm mark. The number of flies that crossed the 15 cm mark in 30 seconds in the locomotory chamber was documented. The climbing activity was determined as the average number of flies climbed 15 cm in three replicates (three trials/replicate) and represented as % locomotory performance.

Survival Assay

The newly emerged *Drosophila* were used to assess the effect of nonylphenol on survivability. The organisms (50 flies/vial and 5 vials/group) were transferred to the normal food and food-containing nonylphenol from the first day of their eclosion to 40 days. The control and nonylphenol-exposed flies were transferred to the new respective diet every alternate day, and the mortality was recorded in each vial for 40 days.

Statistical Analysis

The Prism software (GraphPad version 8.4, San Diego, CA, USA) was used for statistical analysis. The analysis of variance (ANOVA) was used to determine the statistical significance in control and treated flies. The *p*-values were ascribed as

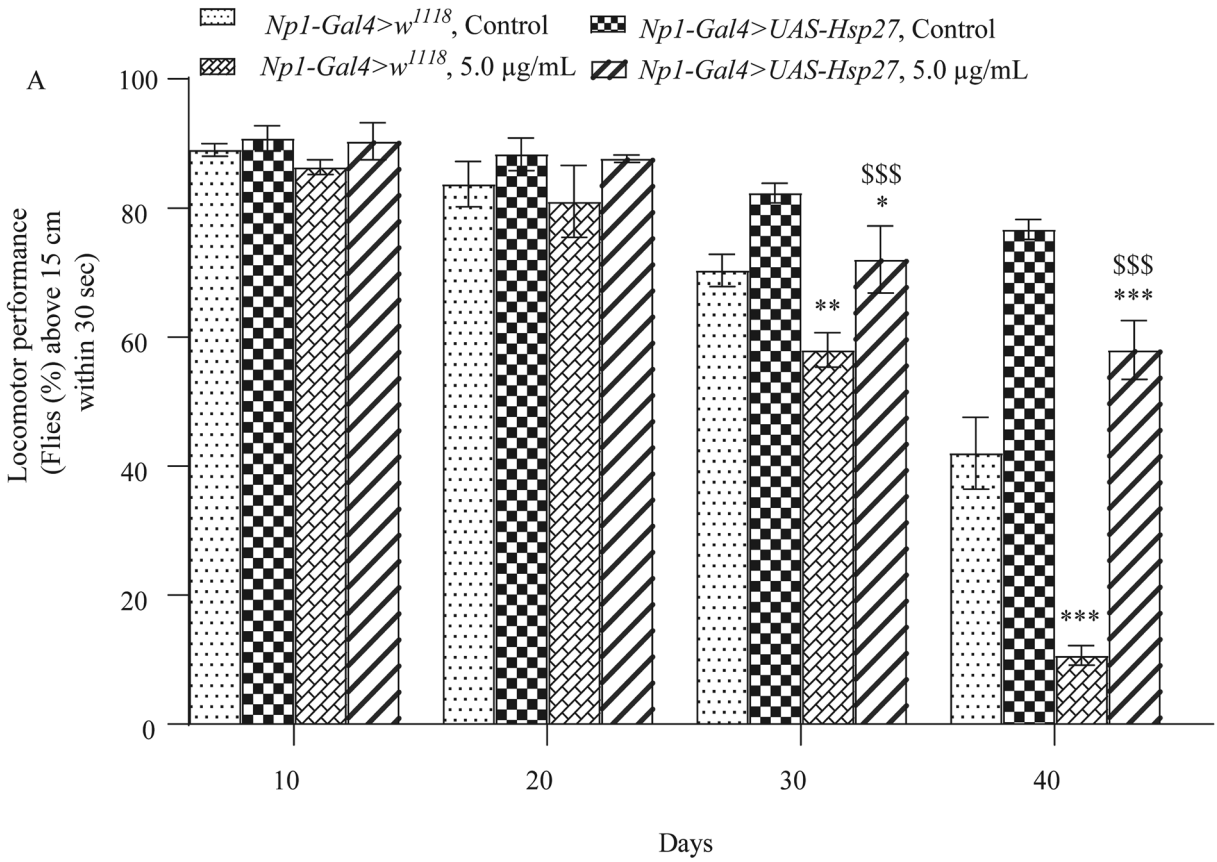

Fig. 2 Overexpression of *Hsp27* improves the locomotory performance in nonylphenol-exposed adult *Drosophila* emerged. Significance is ascribed to $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). While the comparison with overexpressed *Np1-Gal4 > UAS-Hsp27* flies to *Np1-Gal4 > w*^{1118}, the significance is represented as $p < 0.001$ ($^{\$\$\$}$). Data represent mean ± SD ($n = 3$).

$p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). While the comparison with overexpressed *Hsp27* flies to wild-type flies, the significance is represented as $p < 0.001$ ($^{\$\$\$}$).

Results

The experiments were performed using *Drosophila* adult flies, and the overexpression of *Hsp27* in the *Drosophila* gut was achieved through a standard genetic cross between *Np1-Gal4* and *UAS-Hsp27* strains. The study found no overt harmful effects of nonylphenol exposure in *Drosophila* flies. *Drosophila* flies fed with 0.3% DMSO (vehicle control) showed no significant change. Therefore, the data presented in the study were compared against the biological control.

Hsp27 Overexpression in the *Drosophila* Gut Resists the Nonylphenol-induced Locomotory Decline

To examine the *Hsp27* role in the locomotory behavior of the organism, we have assessed the climbing activity of nonylphenol-exposed flies. An insignificant change was observed in the climbing activity of *Np1-Gal4 > w*^{1118,} and *Np1-Gal4 > UAS-Hsp27* flies after 10 to 20 days of nonylphenol exposure. However, a significant reduction in the climbing activity was observed after 30 (~20%, $p < 0.01$) and 40 (~76%, $p < 0.001$) days of nonylphenol exposure in *Np1-Gal4> w*^{1118} as compared with the control (►**Fig. 2**). While *Np1-Gal4 > UAS-Hsp27* showed a comparatively lesser reduction of ~13% (30 days) to ~23% (40 days) in the climbing performance in exposed flies as compared with *Np1-Gal4 > UAS-Hsp27* flies. Moreover, *Np1-Gal4 > UAS-Hsp27* flies exposed to 5.0 µg/mL nonylphenol showed~ 35% to~ 69% improvement climbing than flies *Np1-Gal4 > w*^{1118} to 30 and 40 days nonylphenol exposure, respectively (►**Fig. 2**).

Hsp27 Overexpression Improves the Survivability of the Nonylphenol-exposed Organism

To analyze the impact of *Hsp27* overexpression in the organism's gut on longevity, a survival assay was performed in nonylphenol-exposed flies. An insignificant change was observed in the survival of flies until 25 days of nonylphenol exposure in *Np1-Gal4 > w*^{1118} and *Np1-Gal4 > UAS-Hsp27* (►**Fig. 3**). A significant ($p < 0.001$) reduction in survival was observed after 35 to 40 days of nonylphenol exposure in *Np1-Gal4 > w*^{1118} compared with the control. The maximum decrease in survival of the organism was observed after 40 days (~35%) of nonylphenol exposure in *Np1-Gal4> w*^{1118}. Under similar exposure conditions *Np1-Gal4 > UAS-Hsp27,* flies displayed a reduction of ~20% after nonylphenol exposure compared with the respective control flies (►**Fig. 3**). Altogether *Np1-Gal4 > UAS-Hsp27* flies showed ~40% survival benefit compared with *Np1-Gal4 > w*^{1118} after nonylphenol exposure.

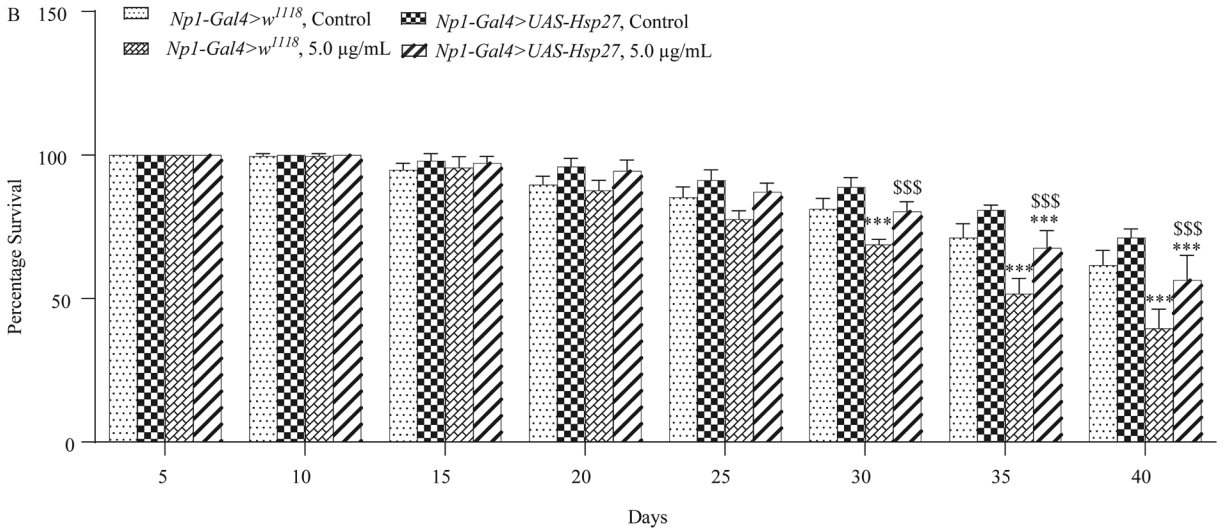

Fig. 3 *Hsp27* overexpression diminishes the nonylphenol-induced survival toxicity in adult *Drosophila*. The data are represented in the form of percentage survival. Significance is ascribed to $p < 0.01$ (**), and $p < 0.001$ (***). While the comparison with overexpressed *Np1-Gal4 > UAS-Hsp27* flies to *Np1-Gal4 > w*^{1118}, the significance is represented as $p < 0.001$ ($^{\$\$\$}$). Data represent mean ± SD ($n = 5$).

Discussion

The gut is the primary organ for ingestion/oral exposure. Several first-line defense mechanisms, such as induction of stress proteins, activation of metabolic reactions, and tissue repair process, are orchestrated in the gut in response to biological, physical, and chemical stress, which eventually provide the organism's short- or long-term protection.[21–23] In addition, the gut harbors microorganisms, which play a critical role in regulating several organisms' responses/physiological processes, such as immune response, xenobiotics metabolism, digestion, and absorption.[24,25]

Nonylphenol enters the organism's system mainly through water and food. It readily gets absorbed in an organism's gut and affects gut homeostasis.[26] Our previous study revealed that exposure to nonylphenol via food hampers the emergence pattern and causes cellular adversities such as increased oxidative stress and cell death in the midgut *Drosophila* larvae.[12] However, modulation of *Hsp27* by overexpressing in *Drosophila* larvae midgut improves the emergences pattern and reduces cellular toxicity. In the study, we aimed to explore the role of *Hsp27* overexpression in the gut against nonylphenol-caused locomotory and survival toxicity in exposed adult *Drosophila.*

Overexpression of *Hsp27* Diminishes the Locomotory Toxicity due to Nonylphenol Exposure

The gut is the second brain of the organism, as it contains a series of enteric nervous system which runs from the esophagus, through the stomach and intestines to the anus. This enteric nervous system depends on the same kind of neurons and neurotransmitters found in the brain. The neuronal cross-talk between the gut and brain is known as the gut–brain axis. Prior studies have shown that a disturbed gut is linked with mental health.[27] The disturbing gut homeostasis causes an increase in the inflammation and the permeability of the gut barrier, which hampers the blood–brain barriers and promotes the progression of neurodegeneration diseases. Prior study has shown that exposure to bisphenol A hampers the tight intestinal junctions, leading to gut barrier dysfunction. Its exposure also decreases the level of serum neurotransmitters, affects the hippocampus, and tryptophan, 5-hydroxytryptamine (5-HT), and 5-hydroxy indole acetic acid and microbial community, which later promotes impaired mental capabilities and inflammation in the intestine and brain.[28] Therefore, restoring gut health could be the primary way to improve neurological issues. Hsp27 functions as an antioxidant by lowering reactive oxygen species (ROS) and increasing the intracellular glutathione.[12] In this aspect, our study has shown that overexpression of *Hsp27* (*Np1-Gal4 > UAS-Hsp27*) rescued locomotor insufficiency on exposure to nonylphenol. With this, previous research has also demonstrated the protective role of *Hsp27* in different organ systems. For example, previous studies have shown that overexpression of Hsp27 in the brain ameliorates symptoms of neurological diseases.[17,29]

Hsp27 Overexpression Improves the Nonylphenol-Induced Survival Toxicity

The gut has a permeable wall that restricts the passage of bacteria and harmful chemicals while permitting nutrients and immunological signaling. The dysfunction in the gut causes leaking of the gut, inflammation, and premature aging. Prior study has shown that dietary restriction improves intestinal integrity by modulating *dMyc* and decreasing cell death, increasing the organisms' life span.[30] Similarly, upregulation in the Hsp27 has been shown to inhibit apoptosis.[14] Our previous study demonstrated that *Hsp27* overexpression in the midgut of *Drosophila* larvae reduces oxidative stress and cell death and improves host fitness upon nonylphenol exposure.[12] In the present study, we have shown a significant improvement in life span in nonylphenol-exposed *Np1-Gal4 > UAS-Hsp27* flies compared with *Np1-Gal4> w*^{1118} flies. Alexander et al[31] showed that

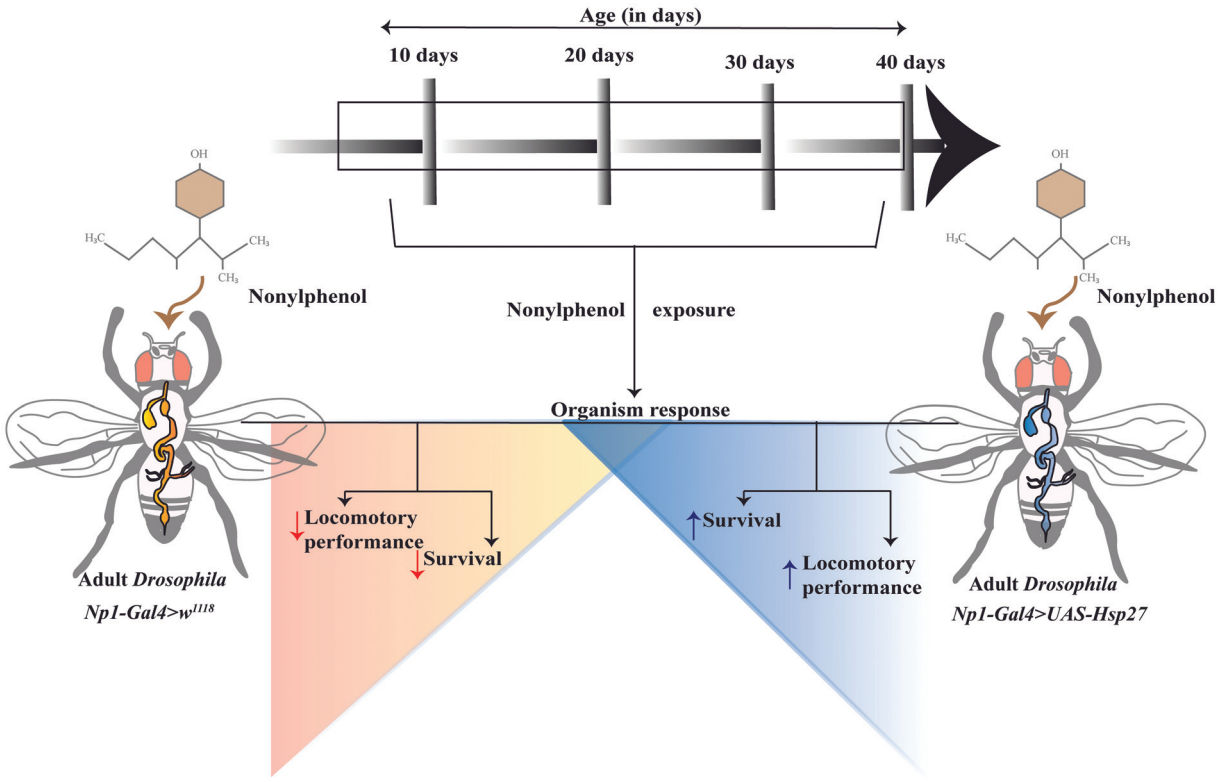

Fig. 4 Schematic representation of the protective function of *Hsp27* against prolonged nonylphenol exposure-induced locomotory and survival toxicity.

Hsp27 (HspB1) improves the life span of the organisms by decreasing the oxidative stress in exposed *Caenorhabditis elegans*.

Considering the foregoing, the overexpression of *Hsp27* in the gut of adult *Drosophila* exposed to nonylphenol improves the life span and locomotory behavior of exposed *Drosophila* (►**Fig. 4**). The overexpression of this chaperone might decreases oxidative stress due to nonylphenol exposure. This focuses on therapeutic attention as the target moiety toward organismal (including human) health in xenobiotic-contaminated environments. Due to the genetic and functional homology between *Drosophila* and mammalians, the resultant information generated in this study aids in understanding the nonylphenol-induced toxicity in a higher organism.

Conclusion

In conclusion, the present study indicates that nonylphenol 5.0 µg/mL exposure to adult *Drosophila* induces deficits in locomotory activity and lifespan. We showed that prolonged exposure to nonylphenol causes a significant reduction in climbing activity and survival after 30 to 40 days of exposure. Interestingly, *Hsp27* overexpression in the midgut of *Drosophila* reverses the impact of nonylphenol-induced locomotory and survivability toxicity, which highlights the role of gut health in organisms' fitness. The study shows that because higher vertebrates and *Drosophila* share many genetic and functional characteristics, Hsp27 may represent a significant therapeutic target against nonylphenol exposure.

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Conflict of Interest

None declared.

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