Neurotoxicology 68 (2018) 189–202

Contents lists available at ScienceDirect

Neurotoxicology

journal homepage: www.elsevier.com/locate/neuro

Full Length Article

The cytoplasmic thioredoxin system in *Caenorhabditis elegans* affords protection from methylmercury in an age-specific manner

Joanna A. Ruszkiewicz[a,*], Gabriel Teixeira de Macedo[b], Antonio Miranda-Vizuete[c], João B. Teixeira da Rocha[b], Aaron B. Bowman[d], Julia Bornhorst[e], Tanja Schwerdtle[e], Michael Aschner[a]

[a] Department of Molecular Pharmacology, Albert Einstein College of Medicine Bronx, NY, United States
[b] Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[c] Instituto de Biomedicina de Sevilla, Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla, Sevilla, Spain
[d] Department of Pediatrics, Vanderbilt University Medical Center, Nashville, TN, United States
[e] Department of Food Chemistry, Institute of Nutritional Science, University of Potsdam, Nuthetal, Germany

ARTICLE INFO

Keywords:
Methylmercury
Age
Development
C. elegans
Thioredoxin
Thioredoxin reductase

ABSTRACT

Methylmercury (MeHg) is an environmental pollutant linked to many neurological defects, especially in developing individuals. The thioredoxin (TRX) system is a key redox regulator affected by MeHg toxicity, however the mechanisms and consequences of MeHg-induced dysfunction are not completely understood. This study evaluated the role of the TRX system in *C. elegans* susceptibility to MeHg during development. Worms lacking or overexpressing proteins from the TRX family were exposed to MeHg for 1 h at different developmental stage: L1, L4 and adult. Worms without cytoplasmic thioredoxin system exhibited age-specific susceptibility to MeHg when compared to wild-type (wt). This susceptibility corresponded partially to decreased total glutathione (GSH) levels and enhanced degeneration of dopaminergic neurons. In contrast, the overexpression of the cytoplasmic system TRX-1/TRXR-1 did not provide substantial protection against MeHg. Moreover, transgenic worms exhibited decreased protein expression for cytoplasmic thioredoxin reductase (TRXR-1). Both mitochondrial thioredoxin system TRX-2/TRXR-2, as well as other thioredoxin-like proteins: TRX-3, TRX-4, TRX-5 did not show significant role in *C. elegans* resistance to MeHg. Based on the current findings, the cytoplasmic thioredoxin system TRX-1/TRXR-1 emerges as an important age-sensitive protectant against MeHg toxicity in *C. elegans*.

1. Introduction

Methylmercury (MeHg) is a highly neurotoxic environmental contaminant linked to numerous neurological defects in both humans and experimental animals. It has long been known that brain vulnerability to MeHg is higher during early development. Neurobehavioral effects reported include altered motoric function, memory and learning disabilities of young individuals (Antunes Dos Santos et al., 2016; Llop et al., 2017; Prpic et al., 2017; Johansson et al., 2007). Several mechanisms have been proposed as responsible for MeHg neurotoxicity, such as disturbance of calcium homeostasis, neurotransmitter systems, induction of oxidative stress and disruption of antioxidant protection (Farina and Aschner, 2017; Farina et al., 2011b, a). The thioredoxin (TRX) system together with glutathione (GSH), are key endogenous antioxidants and redox regulators affected by MeHg, as has been shown in numerous *in vitro* and *in vivo* studies (Farina et al., 2011a, b; Meinerz et al., 2017; Kirkpatrick et al., 2015; Ruszkiewicz et al., 2016; Branco et al., 2011; Wagner et al., 2010; Han et al., 2017), however the mechanisms and consequences of this disruption have yet to be completely understood.

Oxidized thioredoxin is reduced by the selenoenzyme – thioredoxin reductase (TRXR), with expense of NADPH. In cells, TRX systems are present in two major compartments, cytoplasm and mitochondria, and are composed of proteins encoded by different genes. The system has wide range of reducing functions, regulates numerous oxidative-sensitive molecules (ribonucleotide reductases, transcription factors or caspases) and repairs oxidized proteins (Ren et al., 2017; Lu and Holmgren, 2014). These probably makes the system essential for mammals – the knockouts (KOs) in mice exhibit lethality during early stages of embryonic development (Cunningham et al., 2015). The differences in embryonic phenotypes of KOs indicate differential physiological roles for individual thioredoxins, but the early lethality

* Corresponding author.
E-mail addresses: joruszkiewicz@gmail.com, joanna.ruszkiewicz@einstein.yu.edu (J.A. Ruszkiewicz).

https://doi.org/10.1016/j.neuro.2018.08.007
Received 11 June 2018; Received in revised form 3 August 2018; Accepted 17 August 2018
Available online 20 August 2018
0161-813X/

associated with them makes it difficult to study.

C. elegans affords a unique model for study, as the TRX system is not essential for survival (Cacho-Valadez et al., 2012; Miranda-Vizuete et al., 2006). Thioredoxins expressed in worms share high similarity with their mammalian orthologs and have been localized in both mitochondria and cytoplasm (Cacho-Valadez et al., 2012; McCallum et al., 2016). While human TRX-1 is ubiquitously expressed in many cell types, *C. elegans* TRX-1 is found exclusively in the two ASJ sensory neurons (Miranda-Vizuete et al., 2006), implicated in the response to stress conditions, regulation of aging and dauer formation (Fierro-Gonzalez et al., 2011a, b; Jee et al., 2005). The non-ubiquitous expression of TRX-1 suggests that despite the high degree of sequence conservation, this is not a functional orthologue of human TRX-1, which might also explain why in worms TRX-1 KOs are non-lethal (Miranda-Vizuete et al., 2006). Nonetheless, depletion of cytosolic thioredoxin affects worms: *trx-1* mutants lacking functional protein exhibit decreased lifespan and fully suppress the lifespan extension induced by dietary restriction or genetic manipulations (Miranda-Vizuete et al., 2006; Jee et al., 2005; Fierro-Gonzalez et al., 2011a, b). In addition, these mutants are more susceptible to oxidative stress (Jee et al., 2005). Cytoplasmic thioredoxin reductase (TRXR-1) is mainly expressed in *C. elegans* pharynx, vulva and intestine (Li et al., 2012) and is the only seleno-cysteine containing protein in *C. elegans* (Stenvall et al., 2011). Mitochondrial thioredoxin TRX-2 expression has been shown in AIYL/R and ASEL neurons, as well as in muscle cells in the head or intestine, whereas TRXR-2 is ubiquitously expressed, especially in pharyngeal and body wall muscles, suggesting that TRXR-2 might have additional substrates other than TRX-2 (Cacho-Valadez et al., 2012). Deletion of *trx-2* and/or *trxr-2*, did not affect longevity, mitochondrial morphology, reactive oxygen species (ROS) production or apoptosis, suggesting that, unlike in higher organisms, in worms this is not a major defense system against stress (Cacho-Valadez et al., 2012).

In the present study, we evaluated the role of the thioredoxin system in *C. elegans* susceptibility to MeHg at different developmental stages. For this purpose, worms without functional cytoplasmic (TRX-1/TRXR-1) and mitochondrial (TRX-2/TRXR-2) thioredoxin systems, as well as newly identified thioredoxin-like proteins, TRX-3, TRX-4 and TRX-5, have been exposed to MeHg. The significance of the thioredoxin systems in worms' lethality, longevity, mercury (Hg) accumulation, total glutathione (GSH) content, as well as dopaminergic neurodegeneration upon MeHg exposure was addressed.

2. Methods

2.1. C. elegans maintenance and MeHg exposure

C. elegans strains used in this work were obtained from the Caenorhabditis Genetic Center (CGC; University of Minnesota), Dr. Antonio Miranda-Vizuete, Dr. Peter Swoboda and Dr. Simon Tuck labs (Table 1). Worms were handled and maintained at 20 °C, and were grown on plates containing nematode growth medium (NGM) or 8 P, seeded with either the *Escherichia coli* (*E. coli*) strain OP50 or NA22, respectively. Synchronous populations were obtained by isolating embryos from gravid worms with a bleaching solution (1% NaOCl and 0.25 M NaOH), and segregating eggs from worms and bacterial debris by flotation in a sucrose gradient. After synchronization eggs were kept in M9 buffer (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 86 mM NaCl, 1 mM $MgSO_4$) for 18–20 h. When the eggs hatched, larvae L1 worms were washed three times with fresh M9, scored and used for experiments, or transferred to NGM plates with concentrated OP50 (without floxuridine, FUDR), where they grew for 48 h to reach the L4 stage, or 96 h to reach adult stage. Synchronized worms at different developmental stages were treated with methylmercury (II) chloride - MeHgCl (MeHg) for 1 h. The 5 mM stock solution was diluted in M9 to reach desirable concentrations of MeHg. Treatments involved combining worms (usually 2500 L1, 1000 L4, or 500 adults), 25 µl MeHg solution and M9 buffer to a total volume of 500 µl. After exposure worms were washed three times with M9 buffer and placed on OP50-containing NGM plates for lethality or lifespan assays, and assayed for Hg accumulation, GSH levels, thioredoxin expression and dopaminergic (DAergic) degeneration. Since most of the worms in the population were hermaphrodites (over 99%), the sex was not considered – when it was possible to easily identify the sex (for L4 and adult worms), only hermaphrodites were analyzed.

2.2. Lethality assay

Lethality tests were performed on synchronized worms at the following developmental stage: L1, L4 and adult. Mutant worms: *trx-1, trxr-1*, *trx-2*, *trxr-2*, *trx-3*, *trx-4*, *trx-5*, *trx-1; trx-2*, *trx-1; trxr-1*, *trx-2; trxr-2*, *trxr-1; trxr-2*, *lin-15 (n765ts) X; ofIs1 [Ptrx-1::trx-1::GFP; pBHL98 (lin-15ab(+))] IV*, *vzEx162 [p558(Pges-1::TRX-1 ORF::trx-1 3'UTR) +p438(Punc-122::DsRed)]*, *svEx889 [(Ptrxr-1::trxr-1::GFP) + (Punc-*

Table 1
Strains used in this study.

Strain name	Genotype	Source
N2	wild type	CGC
VZ1	*trx-1 (ok1449) II*	CGC
VB1414	*trxr-1 (sv47) IV*	Simon Tuck
VZ13	*trx-2 (tm2720) V*	CGC
VZ15	*trxr-2 (ok2267) III*	CGC
VZ130	*trx-1 (ok1449) II; trx-2 (tm2720) V*	CGC
VZ650	*trx-1 (ok1449) II; trxr-1 (sv47) IV*	This study
VZ21	*trxr-2 (ok2267) II; trxr-1 (sv47) IV*	CGC
VZ22	*trxr-2 (ok2267) III; trx-2 (tm2720) V*	CGC
VZ68	*trx-3 (tm2820) IV*	CGC
VZ596	*trx-4 (ok3519) I*	Antonio Miranda-Vizuete
RB1637	*trx-5 (ok2014) V*	Antonio Miranda-Vizuete
OE3265	*lin-15 (n765ts) X; ofIs1 [Ptrx-1::trx-1::GFP; pBHL98 (lin-15ab(+))] IV*	Peter Swoboda
VB2619	*svEx889 [(Ptrxr-1::trxr-1::GFP) + (Punc-122::RFP)]*	Simon Tuck
VZ55	*vzEx8 [trx-2p(4 kb)::trx-2::GFP + pRF4 (rol-6(su1006))]*	CGC
VZ69	*vzEx14 [trxr-2p(int)::trxr-2::GFP + pRF4(rol-6(su1006))]*	CGC
VZ457	*vzEx162 [p558(Pges-1::TRX-1 ORF::trx-1 3'UTR) + p438(Punc-122::DsRed)]*	Antonio Miranda-Vizuete
VZ471	*trx-1 (ok1449) II; vzEx167 [p559(Pssu-1::TRX-1 ORF::trx-1 3'UTR) + p438(Punc-122::DsRed)]*	Antonio Miranda-Vizuete
BY200	*vtIs1 [Pdat-1::GFP]*	CGC
MAB451	*trx-1 (ok1449) II; vtIs1*	This study
MAB452	*trxr-1 (sv47) IV; vtIs1*	This study
MAB453	*lin-15 (n765ts) X; ofIs1; vtIs1*	This study
MAB454	*svEx889; vtIs1*	This study

122::RFP)], trx-1 (ok1449) II; vzEx167 [p559(Pssu-1::TRX-1 ORF::trx-1 3'UTR) + p438 (Punc-122::DsRed)], and similarly treated wildtype (wt) animals were incubated for 1 h in M9 buffer with different final concentration of MeHg: 10–100 μM for L1 and adult, 40–300 μM for L4. Following treatment and washes, 20–40 worms were placed on OP50-seeded 35 mm NGM plates and scored. For strains where transgenes remained as extrachromosomal arrays, a greater number of worms was used (40–60). Both the number of worms expressing the transgene, as well as the total number of worms (transgene + genetic background: gb) were scored. The transgene animals were identified through the presence of mCherry fluorescence. Twenty-four hours later only live worms were scored based on their appearance and ability to move in response to poking with a platinum wire (L1 and L4) and pharyngeal pumping (adults). The experiment was carried out independently 8 times for each strain and age. Results from each dose were calculated as % of control (0 μM MeHg). A sigmoidal dose-response model with a top constraint at 100% was used to draw the curves and determine lethal doses 50% (LD_{50}). Based on these preliminary studies, MeHg dosing for further studies were carried out at 20, 40 μM for L1 and adults; 40, 70, 140 μM at the L4 stage.

2.3. Lifespan assay

Lifespan tests were performed on synchronized worms at the developmental stage L1 (2500 worms per group in treatment) and L4 (1000 worms per group in treatment). N2 and mutant strains: *trx-1*, *trxr-1*, *trx-2*, *trxr-2*, *lin-15 (n765ts) X; ofIs1 [Ptrx-1::trx-1::GFP; pBHL98 (lin-15ab(+))] IV*, *svEx889 [(Ptrxr-1::trxr-1::GFP) + (Punc-122::RFP)]* were incubated for 1 h with MeHg (0, 20, 40 μM for L1 and 0, 70, 140 μM for L4). Following treatment and washing, worms were placed on NGM plates with concentrated OP50 and allowed to grow for 48 h. Then, 30 nematodes from each MeHg concentration group were picked to a fresh OP50-seeded 60 mm NGM/FUDR plates in triplicate (in total 90 worms per group). Live *C. elegans* were transferred to fresh plates every 4–8 days. The worms were scored as live or dead every second day until no live *C. elegans* remained. The worms who escaped from plate or were unable to localize were scored as missing and removed from the calculations. The experiment was carried out independently 3–5 times, dependent upon the strain. The Kaplan-Meier method was used to estimate lifespan curves and determine the median lifespan for each trial.

2.4. Mercury level

Mercury (Hg) accumulation assay was performed on wt and mutant strains: *trx-1*, *trxr-1*, *trx-2*, *trxr-2*, *lin-15 (n765ts) X; ofIs1 IV*. Synchronized worms (20,000 L1, 2000 L4, or 2000 adult) were incubated for 1 h with MeHg (0, 20, 40 μM for L1/adults and 0, 40, 70, 140 μM for L4). Following treatment and washing, worms were placed on NGM plates with concentrated OP50 for 24 h. The next day, all worms (live and dead) were washed off the plates and then washed with M9 three times (centrifuged 1 min, 500xg, 20 °C). The clean pellet was resuspended in 500 μl of ice cold 85 mM NaCl, flashed frozen three times in liquid nitrogen, sonicated (3 x 20 s on ice) and centrifuged (10 min, 9000xg, 4 °C). Next, the supernatant was collected and small aliquot (20 μl) was used for protein measurement, while the rest of the sample was frozen (−80 °C) and subsequently used for inductively coupled plasma-mass spectrometry (ICP-MS) analysis of Hg content. Each experiment was repeated independently three times. 400 μl of the sample was digested in the microwave in the presence of 1.6 ml bidest. H_2O, 250 μl HNO_3 suprapur and 250 μl HCl suprapur. The analysis of the Hg content by ICP-MS was performed with the No gas mode. 0.01 μg/l Rhodium was used as internal standard and the calibration was prepared in 10% HNO_3 suprapur and 10% HCl suprapur (concentration range: 1–300 ng/l). The washout solution contained 1 ppm Gold in 5% HNO_3 and 5% HCl (Lohren et al., 2015). The results were calculated per sample protein content and presented as ng Hg/mg protein.

2.5. Total glutathione level

The total glutathione (GSH) level was analyzed in wt and mutant strains: *trx-1*, *trxr-1*, *trx-2*, *trxr-2*, *trx-1; trx-2*, *trx-1; trxr-1*, *trx-2; trxr-2*, *trxr-1; trxr-2*, *lin-15 (n765ts) X; ofIs1 IV*. Synchronized worms (40,000 L1, 10,000 L4, or 5000 adult) were incubated for 1 h with MeHg (0, 20, 40 μM for L1/adults and 0, 40, 70, 140 μM for L4). Following treatment and washing, worms were placed on NGM plates with concentrated OP50 for 24 h. The next day, sample preparation was performed as previously described (Caito and Aschner, 2015). Briefly, all worms (live and dead) were harvested from the plate and washed with M9 three times (centrifuged 1 min, 500x*g*, 20 °C). The clean pellet was re-suspended in 110 μl of extraction buffer and transfer to a chilled eppendorf tube, then vortexed (5 s), frozen with liquid nitrogen and thawed in a water bath (37 °C), all steps were repeated three times. Subsequently the sample was sonicated (20 s on ice), centrifuged (10 min, 9200 × *g*, 4 °C) and supernatant was collected into a new chilled tube and frozen (−80 °C) until analysis of GSH and protein content. Each experiment was repeated independently four times. GSH was assayed by spectrophotometric method using 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) with the presence of GSH standards (0.0625–40 μM) (Caito and Aschner, 2015). Results were calculated per protein content and presented as nmol GSH /mg protein.

2.6. Total protein level

Glutathione and Hg contents were standardized to total protein content, determined with the bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific), according to the manufacturer's protocol.

2.7. Expression of thioredoxins

Transgenic lines expressing translational green fluorescent protein (GFP) reporters were used to evaluate the protein expression of TRX-1 (*lin-15 (n765ts) X; ofIs1 [Ptrx-1::trx-1::GFP; pBHL98 (lin-15ab(+))] IV*), TRXR-1 (*svEx889 [(Ptrxr-1::trxr-1::GFP) + (Punc-122::RFP)]*, TRX-2 (*vzEx8 [trx-2p(4 kb)::trx-2::GFP + pRF4(rol-6(su1006))]* and TRXR-2 (*vzEx14 [trxr-2p(int)::trxr-2::GFP + pRF4(rol-6(su1006))]*. Synchronized worms were incubated for 1 h with MeHg (0, 20, 40 μM for L1 and 0, 70, 140 μM for L4). Following treatment and washing, worms were placed on NGM plates with concentrated OP50. Twenty-four and forty-eight hours following exposure worms were manually picked and mounted on 4% agarose pads and anaesthetized with 1 mM levamisole. The imaging settings were determined based on control worms. A PerkinElmer Spinning Disk Confocal microscope was used for the imaging head neurons expressing TRX-1 and TRX-2 (60× magnification, scanning every 100 nm for XZ sections). GFP fluorescence intensity was measured in pixels inside neuron-surrounding equal-size sphere using Volocity software (PerkinElmer). At least 10 worms per group were analyzed in each of these strains. An epifluorescence microscope Nikon Eclipse 80i was used for imaging expression of TRXR-1 (4× magnification) and TRXR-2 (20× magnification). Fluorescence intensity was measured in pixels from a square region of the anterior-most region of the intestine, behind the pharynx of animal, and analyzed using Image-J software. 20–40 worms per group were analyzed from these strains. Data are presented as control-normalized for fold change mean ± SEM of 3–5 independent trials for each of the strains.

2.8. Dopaminergic neurodegeneration

Worms expressing GFP in dopaminergic (DAergic) head neurons: 4 cephalic (CEPs) and 2 anterior deirid (ADEs) from the strain BY200

(*Pdat-1::GFP*) were used to evaluate DAergic neurodegeneration after MeHg treatment (*dat-1* is coding a presynaptic dopamine transporter). The *Pdat-1::GFP* male worms were crossed into *trx-1*, *trxr-1*, *lin-15 (n765ts) X; ofIs1 [Ptrx-1::trx-1::GFP;pBHL98 (lin-15ab(+))] IV* and *svEx889 [(Ptrxr-1::trxr-1::GFP) + (Punc-122::RFP)]* hermaphrodites. Synchronized L1 and L4 worms were treated as described above and placed onto concentrated OP50-seeded NGM plates for 96 h (L1) or 48 h (L4), thus for both treatments final analysis was performed at the same developmental stage – adult. Thirty worms per condition were mounted onto 4% agar pads and anaesthetized with 1 mM levamisole. Worms were observed under an Olympus BX41 fluorescent microscope and scored for DAergic degeneration. Each group was scored for absence (intact) or presence of any of the following morphological changes, representing degeneration: dendritic puncta formation, shrunken soma, loss of dendrites, or loss of soma. Each experiment was repeated independently 4 times. Representative confocal images of intact and degenerated neurons were taken using the PerkinElmer Spinning Disk Confocal Microscope.

2.9. Statistical analysis

The statistical analyses of significance were performed using Prism 7 (GraphPad software) and SPSS 25 (IBM). A sigmoidal dose-response model with a top constraint at 100% was used to draw the curves and determine the LD_{50} in lethality assays. The Kaplan-Meier method was used to estimate survival curves in lifespan experiments and to determine the median lifespan for each treatment. Data were analyzed by multifactorial ANOVA followed by Tukey's multiple comparisons tests, to compare the mean values of various experimental groups, $P < 0.05$ was considered statistically significant. The results were expressed as mean ± SEM.

3. Results

3.1. MeHg-induced lethality of single and double thioredoxin KOs

trx-1 and *trxr-1* mutants showed significantly higher susceptibility to MeHg than wt *C. elegans*, especially when treated at L4 stage. The *trx-1* worms were substantially more susceptible to the toxicant than wt when treated at L4 ($P = 0.0003$, $F = 43$) (Fig. 1B) and adult stage ($P = 0.0029$, $F = 19.9$), with 31% and 36% decrease in LD_{50}, respectively (Fig. 1C). Whereas the *trxr-1* worms were more susceptible to MeHg than wt at L1 ($P < 0.0001$, $F = 90.67$) (49% decrease in LD_{50}) (Fig. 1D) and L4 ($P = 0.0036$, $F = 18.49$) (17% decrease in LD_{50}) (Fig. 1E). Moreover, significantly greater number of *trx-1* L1 worms died when treated with 40 μM MeHg when compared to wt worms (Fig.1A). Susceptibility of other null mutants remained similar to the wt: mitochondrial thioredoxin *trx-2* and mitochondrial thioredoxin reductase *trxr-2* (Fig. 1G–L), as well as thioredoxin-like proteins, *trx-3, trx-4, trx-5* (Fig.1M–W). Of note, *trxr-2* showed slight, but significant resistance to 40 μM MeHg at the L1 stage (Fig. 1J), but higher susceptibility to 140 μM MeHg at the L4 stage (Fig. 1K). Double mutants *trx-1; trxr-1* were more susceptible to the MeHg; however the effect was somewhat different from the one observed for single mutants. The strain was more susceptible than wt only at the L4 stage ($P < 0.0001$, $F = 62.73$) (35% decrease in LD_{50}) (Fig. 2B), whereas at L1 mutants showed more resistance to 40 μM MeHg (Fig. 2A). Similarly, *trx-1; trx-2* worms were more susceptible to MeHg than wt at L4 ($P < 0.0001$, $F = 62.49$) (38% decrease in LD_{50}) (Fig. 2E), but also at L1 ($P = 0.0485$, $F = 5.687$) (28% decrease in LD_{50}) (Fig. 2D). Surprisingly, given the increased sensitivity of the single mutant, worms lacking both thioredoxin reductases *trxr-1; trxr-2* were more resistant to 40 μM MeHg when at the L1 stage (Fig. 2G), and to 140 μM MeHg when at the L4 stage (Fig. 2H), but the strain effect was not statistically different from the wt. The MeHg effect in *trxr-1; trxr-2* L1 was analogous to the one observed in *trxr-2* worms (Fig. 1J). *C. elegans trx-2; trxr-2* lacking mitochondrial system were more resistant to 40 μM MeHg when at L1 (Fig. 2J), but less resistant to 140 μM MeHg when at L4 (Fig. 2K), analogous to what was observed for *trxr-2* worms (Fig. 1J, K). The differences in MeHg susceptibility for wt and mutants were age-dependent, with L4 worms being the most resistant to MeHg – LD_{50} ~140 μM MeHg for wt, whereas for L1 and adults LD_{50} approximated 40 μM (Figs. 1 and 2). With results given above, further experiments were carried out with 20, 40 μM MeHg for L1 and adult worms, and 70, 140 μM MeHg in L4; 40 μM was also used in select L4 experiments, to provide comparable treatment conditions for all developmental stages.

3.2. The lifespan of thioredoxin KOs treated with MeHg

Worms which survived MeHg treatment when at L1 or L4 stage, at doses close to their LD_{50}, did not show affected lethality later in life – the median lifespan of wt, *trx-1*, *trxr-1*, *trx-2* and *trxr-2* mutants was not affected by MeHg (Fig. 3). Some strains showed variability in median lifespan, when compared between L1 and L4 stage – there was a significant age effect for *trx-1* ($P = 0.016$, $F = 6.263$) and *trxr-1* ($P = 0.001$, $F = 11.946$), with slightly higher median lifespan for worms treated with MeHg at the L4 stage (0 μM MeHg), however the multiple comparisons test does not show significant difference.

3.3. The mercury levels 24 h after MeHg treatment

The accumulation of mercury (Hg) 24 h post-treatment was dose-, age-, and strain-dependent (Fig. 4). Mercury levels detected in control, untreated worms were between 0.069 and 3.23 ng/mg of protein. Mean Hg levels were higher with higher MeHg dosages; however, in some groups the statistical significances between groups were not attained, probably due to a low number of replicates (n = 3). L4 worms commonly accumulated the least Hg, and adults the most, when comparing the 40 μM MeHg-treated groups (Fig. 4). For *trx-1* worms, both age ($P < 0.0001$, $F = 32.253$), dose ($P < 0.0001$, $F = 52.974$) and age x dose ($P < 0.0001$, $F = 28.226$) effects were significant. Similarly, for *trxr-1* significant effect was observed for age ($P < 0.0001$, $F = 11.324$), dose ($P < 0.0001$, $F = 27.655$), and age x dose ($P < 0.0001$, $F = 11.032$). In worms lacking mitochondrial *trx-2* significant effects were observed for age ($P < 0.0001$, $F = 13.427$), strain ($P = 0.003$, $F = 10.171$) dose ($P < 0.0001$, $F = 35.697$), and age x dose ($P < 0.0001$, $F = 11.415$), with mutant worms accumulating significantly more Hg than wt, especially when at the L4 stage. In *trxr-2* mutants, significant effect was observed for age ($P < 0.0001$, $F = 19.184$), dose ($P < 0.0001$, $F = 45.159$), age x strain ($P < 0.0001$, $F = 10.527$), age x dose ($P < 0.0001$, $F = 13.411$), and age x strain x dose ($P < 0.0001$, $F = 10.954$), with mutants accumulating higher levels of Hg in L1, but less in adult when compared to wt worms.

3.4. The glutathione levels 24 h after MeHg treatment

The total glutathione (GSH) level was usually higher in L1 than L4 and adult worms and did not significantly change upon MeHg treatment in wt worms, but decreased in some mutants (Fig. 5A, B). For worms lacking cytoplasmic thioredoxin *trx-1* significant effect of age ($P = 0.04$, $F = 3.410$), strain ($P = 0.001$, $F = 13.356$), dose ($P = 0.031$, $F = 2862$), dose x age interaction ($P = 0.049$, $F = 2.769$), as well as age x strain interaction ($P < 0.0001$, $F = 10.656$) were observed, with lower GSH levels upon MeHg treatment in L1 (40 μM) and L4 (140 μM) stage, when compared to the control group (0 μM), and with generally lower GSH content when compared to wt worms (Fig. 5A). For *trxr-1* worms only age factor was significant ($P < 0.0001$, $F = 18.533$), with the highest levels of GSH in L1. In L4 stage *trxr-1* worms showed decreased GSH levels upon 140 μM MeHg treatment, however, no significance for strain ($P = 0.054$, $F = 3.870$) nor dose ($P = 0.211$, $F = 1.509$) was observed in these worms. The *trx-*

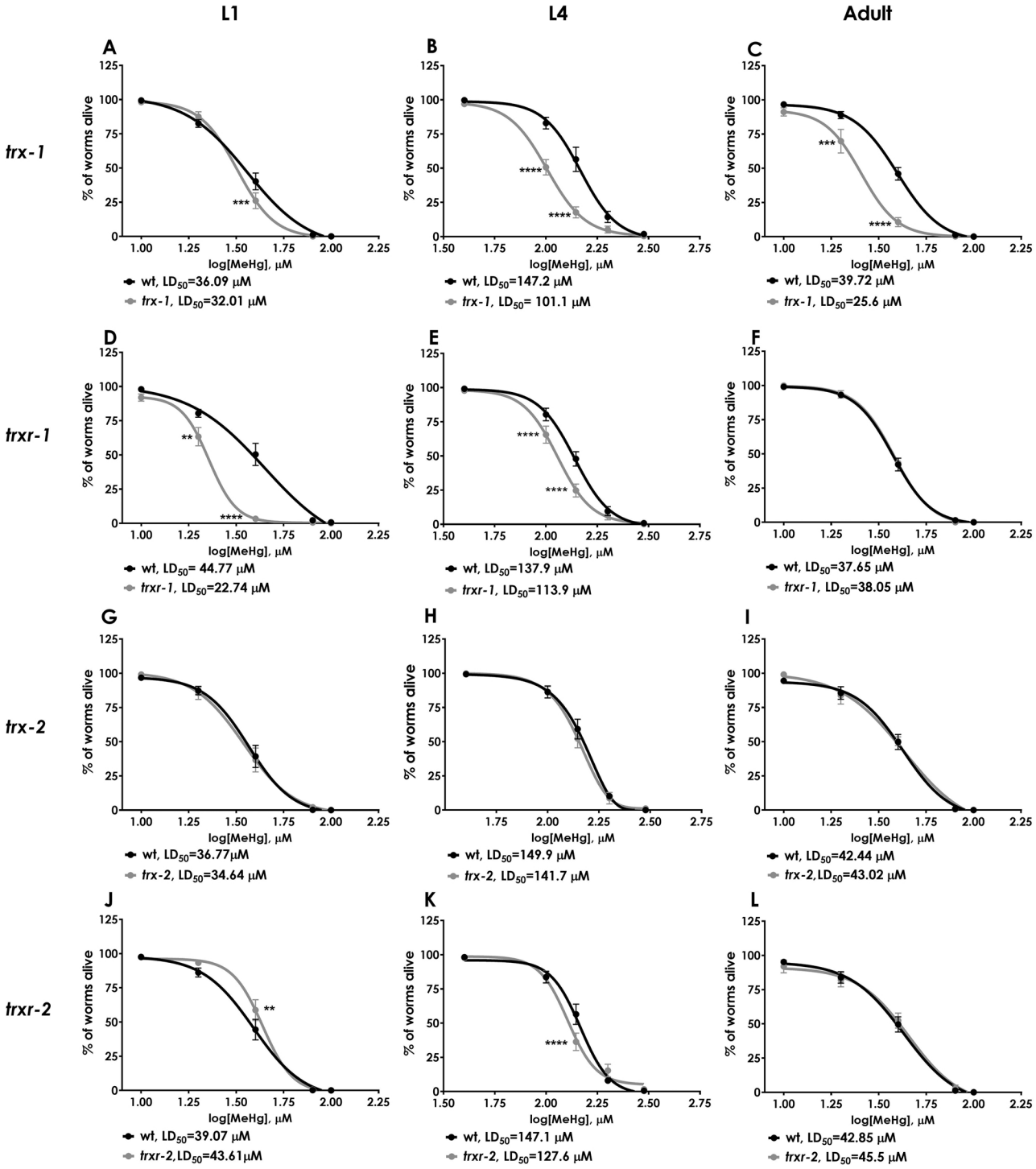

Fig. 1. Lethality of single null mutants from the thioredoxin family exposed to MeHg. Synchronized wt and transgenic worms at different developmental stage (L1, L4 and adult) were treated with MeHg for 1 h. Twenty–four hours later alive worms were scored and based on the sigmoidal dose-response curves lethal doses 50% (LD_{50}) were determined. Worms without functional cytoplasmic thioredoxin system showed higher and age-dependent susceptibility to MeHg than wt. The *trx-1* worms were more susceptible at L4 ($P = 0.0003$, $F = 43$) (B) and adult stage ($P = 0.0029$, $F = 19.9$) (C), whereas *trxr-1* worms at L1 ($P < 0.0001$, $F = 90.67$) (D) and L4 ($P = 0.0036$, $F = 18.49$) (E). Susceptibility of other null mutants remained generally unaffected (G–W) (n = 8). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.0001$ *vs.* wt (post-hoc Tukey's test)..

2 worms exhibited significant age effect ($P < 0.0001$, $F = 20.944$), while *trxr-2*: age ($P < 0.0001$, $F = 11.527$) and age x strain effect ($P = 0.016$, $F = 4.452$), with higher levels of GSH found in L4 when compared to wt (Fig. 5A). For double mutants *trx-1; trxr-1* statistically significant age ($P < 0.0001$, $F = 9.943$) and dose ($P = 0.025$, $F = 3.020$) effects, as well as age x strain interaction ($P = 0.004$, $F = 5.979$) were observed. These worms showed decreased GSH levels upon MeHg treatment (140μM) at L4 stage. For *trx-1; trx-2* worms significant age effect ($P < 0.0001$, $F = 9.838$), strain ($P = 0.027$, $F = 5.145$), dose ($P = 0.048$, $F = 2551$) and age x strain interaction ($P = 0.001$, $F = 7.631$) were observed. This strain showed significantly lower GSH levels in L4 worms upon 140 μM MeHg, but also, higher GSH levels after 70 μM MeHg exposure. In *trxr-1; trxr-2* strain significant age effect ($P < 0.0001$, $F = 11.178$), as well as age x strain interaction

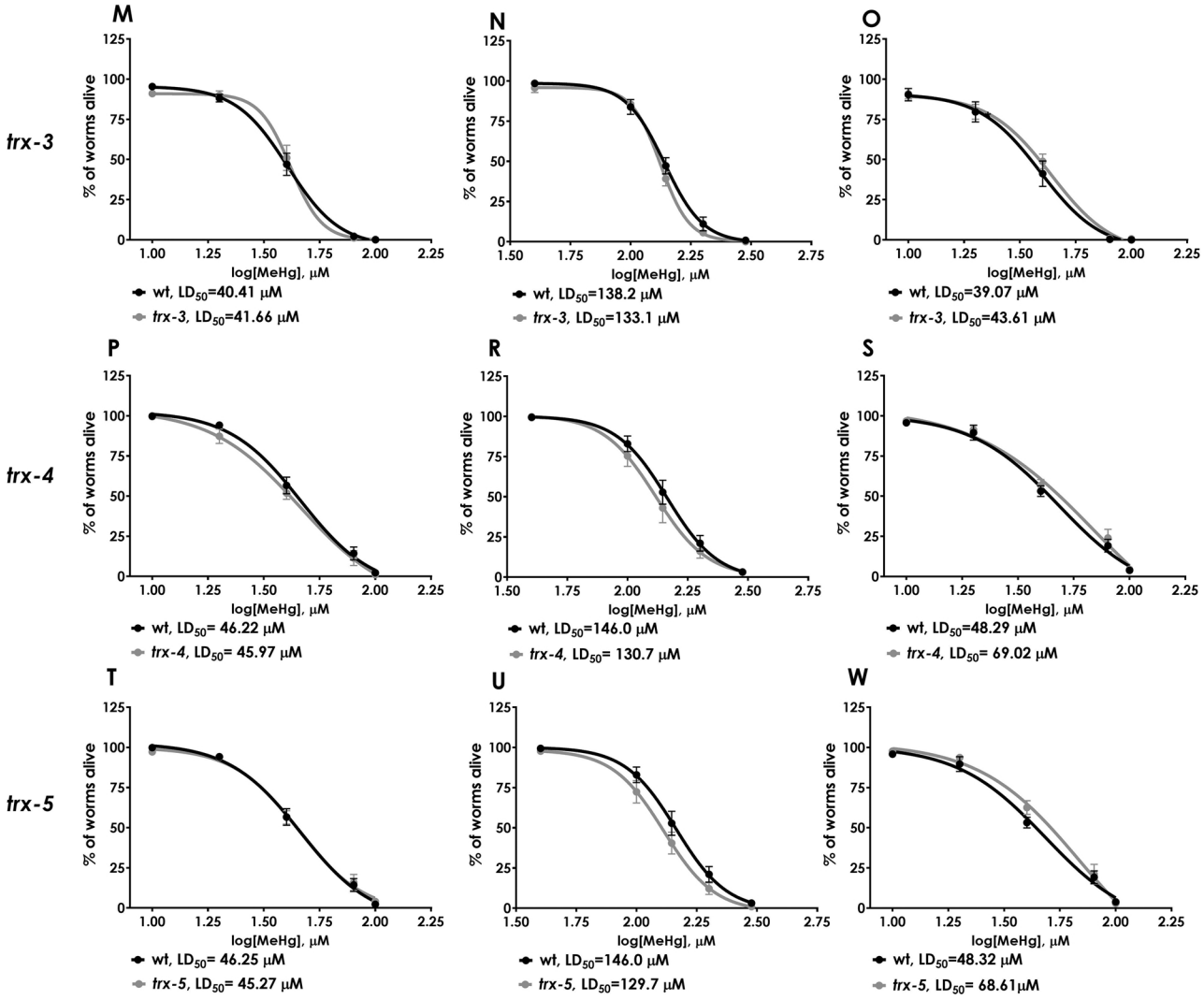

Fig. 1. (*continued*)

($P = 0.007$, $F = 5.404$) was observed, analogous to *trx-2; trxr-2* worms, where a significant age effect ($P < 0.0001$; $F = 12.885$), and age x strain interaction were noted ($P = 0.016$, $F = 4.408$) (Fig. 5B).

3.5. The expression of cytoplasmic and mitochondrial thioredoxin systems after treatment with MeHg

Transgenic lines expressing translational GFP reporters were used to evaluate the effect of MeHg on expression of major proteins from the thioredoxin family. The expression of TRXR-1 protein decreased significantly in MeHg-treated worms, when exposed at L1 and L4 stage, both 24 and 48 h post-treatment, suggesting a permanent change (Fig. 6B). Expression of other analyzed proteins: TRX-1 (Fig. 6A), TRX-2 (Fig. 6C) and TRXR-2 (Fig. 6D) remained unaffected upon treatment with MeHg. While expression of TRXR-2 decreased upon MeHg exposure, the results did not attain statistical significance (Fig. 6D).

3.6. Overexpression of cytoplasmic thioredoxin and thioredoxin reductase in MeHg toxicity

Transgenic lines expressing translational GFP reporters were also used to investigate the effect of overexpression (oe) of TRX-1 and TRXR-1 on worms' resistance to MeHg. *C. elegans* expressing TRX-1 in ASJ neurons (wt-like): *lin-15 (n765ts) X; ofIs1 [Ptrx-1::trx-1::GFP; pBHL98 (lin-15ab(+))] IV*), and in the intestine: *vzEx162 [p558(Pges-1::TRX-1 ORF::trx-1 3′UTR) + p438(Punc-122::DsRed)]* were used, to evaluate the role of neuronal localization of TRX-1 in protection against MeHg. Worms overexpressing TRXR-1 in different tissues (wt-like): *svEx889* [(*Ptrxr-1::trxr-1::GFP*) + (*Punc-122::RFP*)] were also analyzed. Since in the last two strains transgenes remained as extrachromosomal arrays, for lethality assay the number of worms expressing the transgene (solid line), as well as the total number of worms – transgene + genetic background (gb) (dotted lines) – were scored for L4 and adults (for L1, due do difficulties with identification, only the total number of worms was scored). Resistance to MeHg increased significantly in L1 worms oe TRX-1 in ASJ neurons ($P = 0.0443$, $F = 5.986$) (Fig. 7A) and intestine ($P = 0.0296$, $F = 7.415$) (Fig. 7D). This effect was not preserved in older animals, L4 (Fig. 7B, E) and adults (Fig. 7C, F). Interestingly, L4 worms with TRX-1 oe in neurons showed lower resistance to 100 and 140 μM MeHg, when compared to the wt (Fig. 7B). TRXR-1 oe did not improve the resistance of worms to MeHg (Fig. 7G–I). For L4, group of all worms, with and without array, exhibited significantly lower resistance to MeHg than wt ($P = 0.0235$, $F = 9.104$) (Fig. 7H).

Similar to wt and other analyzed strains, the lifespan of TRX-1 oe (in ASJ neurons) and TRXR-1 oe worms was not affected by MeHg exposure, both at L1 and L4 stage (Fig. 8A). Worms which survived 48 h post-treatment did not show changed lethality later in life. However, the median lifespans of mutant animals were decreased, when compared with wt, and the effect was observed for both developmental stages, with greater difference at L4 (Fig. 8A). For TRX-1 oe, both strain

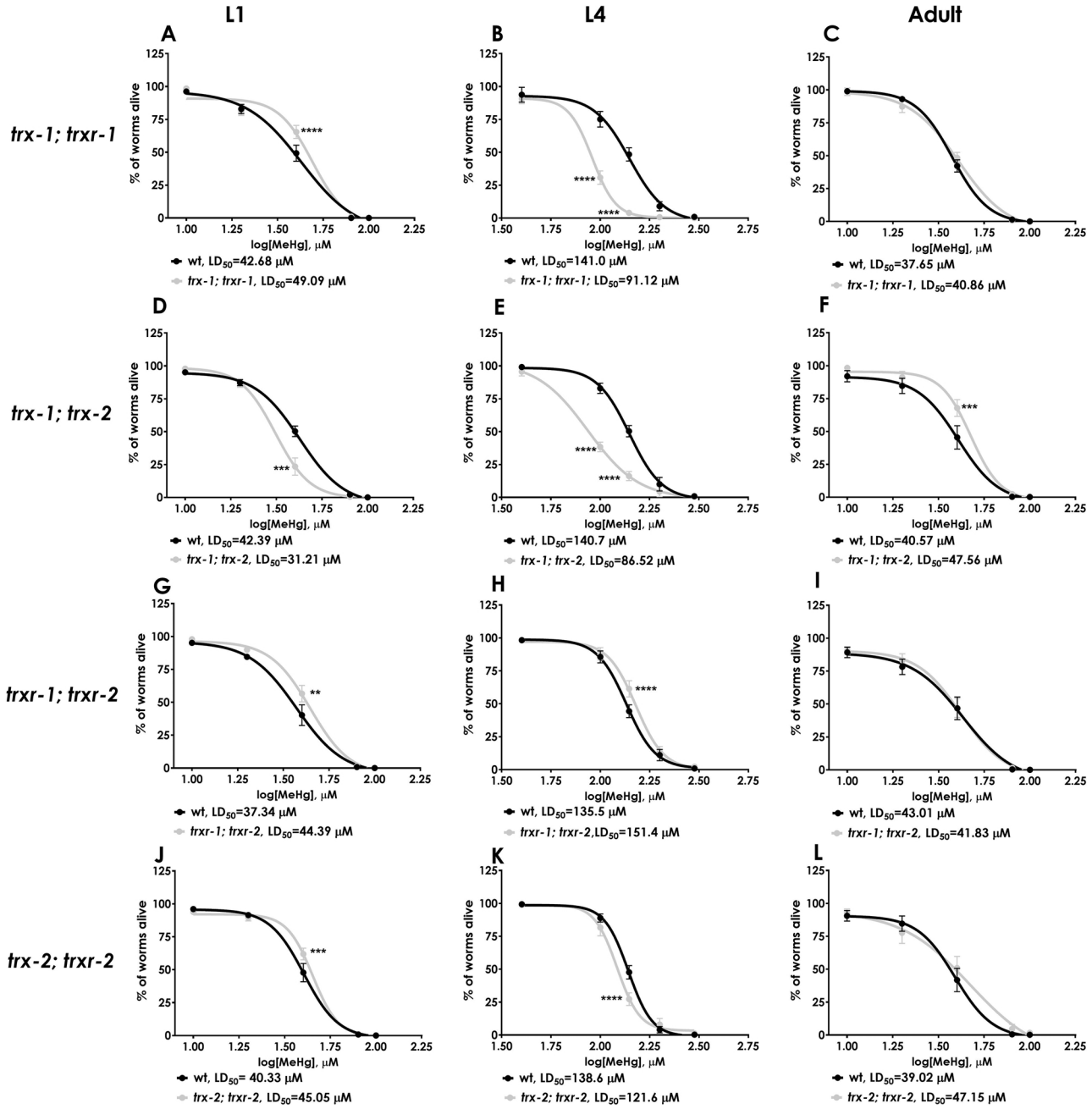

Fig. 2. Lethality of double null mutants from the thioredoxin family exposed to MeHg. Synchronized wt and transgenic worms at different developmental stage (L1, L4 and adult) were treated with MeHg for 1 h. Twenty-four hours later alive worms were scored and lethal doses 50% (LD_{50}) were determined. Double mutants without the whole cytoplasmic thioredoxin system *trx-1; trxr-1* were more susceptible to the MeHg than wt only at L4 stage ($P < 0.0001$, $F = 62.73$) (B), similarly, *trx-1; trx-2* worms were more susceptible to MeHg at L4 ($P < 0.0001$, $F = 62.49$) (E), but also at L1 ($P = 0.0485$, $F = 5.687$) (D). Other double mutants were less sensitive to MeHg (G–L) (n = 8). $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.0001$ *vs.* wt (post-hoc Tukey's test).

($P < 0.0001$, $F = 88.783$), age ($P = 0.011$, $F = 6.981$), as well as strain x age factor ($P = 0.036$, $F = 4.673$) were statistically significant. Similarly, for TRXR-1 oe: strain ($P = 0.003$, $F = 9.908$) and age ($P = 0.047$, $F = 4.205$) factors were significant.

With given data, further studies have been performed in order to get more insight into the significance of TRX-1 oe in an age-dependent MeHg resistance. For Hg levels 24 h post-treatment, statistically significant effect was observed for dose ($P < 0.0001$, $F = 26.447$), strain ($P = 0.003$, $F = 9.966$) and age ($P < 0.0001$, $F = 9.452$), as well as dose x age ($P = 0.002$, $F = 6.176$) and dose x strain x age interactions ($P = 0.038$, $F = 3.095$), suggesting higher than in wt Hg accumulation in TRX-1 oe worms, especially when at early stages of development (L1 and L4) (Fig. 8B). For the GSH content, the strain ($P < 0.0001$, $F = 16.386$) and age ($P < 0.001$, $F = 18.210$) effects, as well as age x strain interaction ($P = 0.002$, $F = 6.971$) were statistically significant (Fig. 8C). The GSH levels in TRX-1 oe nematodes were lower when compared with the wt and decreased upon MeHg treatment (in L1 and L4), however did not reach significant difference when compared with control group (0 μM MeHg). The GSH levels in both wt and transgenic worms were the highest at the L1 stage (Fig. 8C).

The effect of rescue transgene in *trx-1* mutants was also evaluated. Worms with *trx-1* genetic background and transgene carrying TRX-1 oe in ASJ neurons: *trx-1 (ok1449) II; vzEx167 [p559(Pssu-1::TRX-1 ORF::trx-1 3'UTR)+p438(Punc-122::DsRed)]* were evaluated for MeHg resistance at L4 larval stage (due to the high susceptibility of *trx-1* to the MeHg at L4) (Fig. 8D). TRX-1 rescue provided partial protection – the

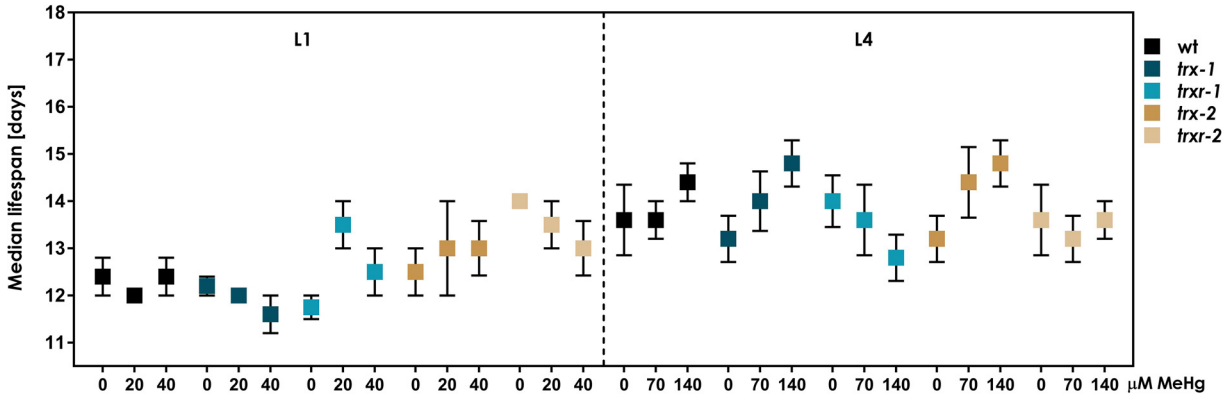

Fig. 3. The median lifespan of null mutants from the thioredoxin family exposed to MeHg. Synchronized L1 and L4 worms were treated with MeHg for 1 h. Forty-eight hours later, 90 alive nematodes from each group were picked to fresh plates and every second day were scored as live or dead until no live *C. elegans* remained. The Kaplan-Meier method was used to estimate lifespan curves and determine the median lifespan for each trial. Worms longevity was not affected by MeHg. Statistically significant age effect was observed for *trx-1* ($P = 0.016$, $F = 6.263$) and *trxr-1* ($P = 0.001$, $F = 11.946$), with slightly higher median lifespan for L4 group (n = 3–5).

susceptibility was still significantly higher than in wt animas: all animals ($P = 0.0055$, $F = 8.218$) and transgene arrays only ($P = 0.0007$, $F = 12.64$), however was also significantly lower when compared with *trx-1* worms: a transgene only ($P = 0.0043$, $F = 8.723$) (12% increase in LD_{50}) and the whole strain ($P = 0.0015$, $F = 10.88$) (Fig. 8D).

3.7. Degeneration of dopaminergic neurons after MeHg exposure

The relative number of worms with intact DAergic neurons did not change significantly upon MeHg treatment in wt and most of analyzed mutant strains, both for worms treated at L1 and L4 stage (Fig. 9C). Only *trxr-1* worms exhibited higher susceptibility to MeHg-induced DAergic neurodegeneration (significant dose effect: $P = 0.001$, $F = 6.222$) – the number of worms with intact neurons decreased in this phenotype of 26% in 70 μM MeHg and 32% in 140 μM MeHg-treated groups when compared to the control (0 μM MeHg). The effect was only observed when worms were treated at the L4 stage. Interestingly, the strain effect was significant for TRXR-1 oe worms ($P = 0.003$, $F = 9.883$), with more intact neurons in this strain when compared to the wt (Fig. 9C).

4. Discussion

4.1. The cytoplasmic thioredoxin system is an important protectant against MeHg-induced toxicity

Data presented in this study establish the age-specific role of the cytoplasmic thioredoxin system TRX-1/TRXR-1 in affording MeHg resistance in *C. elegans*. This emerges from the higher susceptibility of *trx-1* and *trxr-1* KO mutants to the toxicant, characterized by higher lethality (Figs. 1A–E, and 2 A–E), lower levels of GSH (Fig. 5), higher number of degenerated neurons (Fig. 9C), as well as downregulated TRXR-1 protein expression upon MeHg exposure (Fig. 6B). Other members of the thioredoxin family did not afford significant protection against MeHg toxicity. For instance, mitochondrial system TRX-2/TRXR-2 was not affected by MeHg as evidenced by analyses of protein expression (Fig. 6C, D), and *trx-2* and/or *trxr-2* mutants overall did not show higher susceptibility to MeHg (Figs. 1–3), with the exception of *trx-2* accumulating more Hg (Fig. 4) and *trxr-2* exhibiting higher levels of GSH (Fig. 5A) than wt worms. This was a surprising outcome, given that in higher organisms mitochondrial thioredoxin system has been

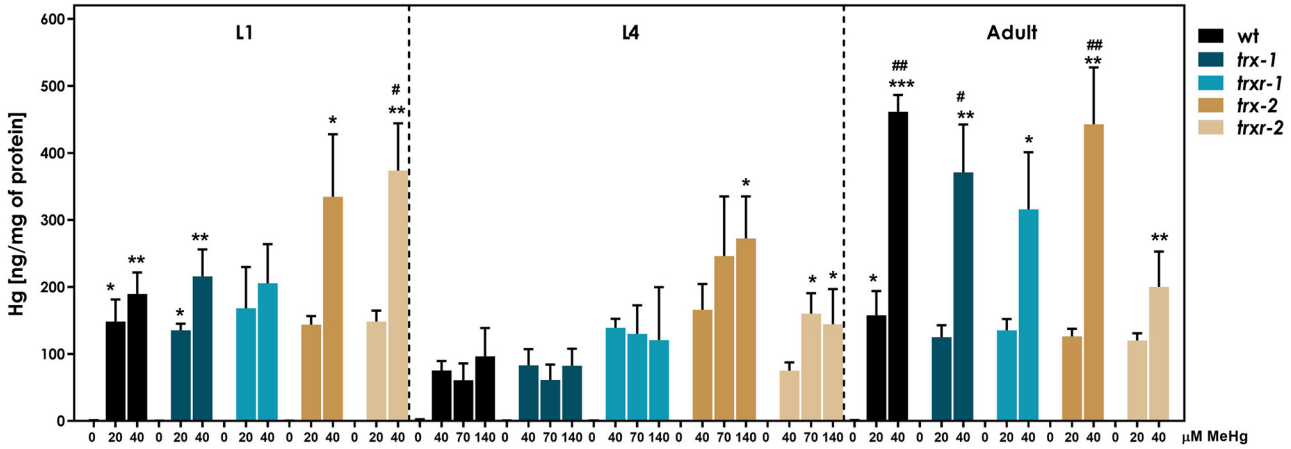

Fig. 4. Mercury (Hg) content in null mutants from the thioredoxin family exposed to MeHg. Synchronized worms at different developmental stage (L1, L4 and adult) were treated with MeHg for 1 h. Twenty-four hours later worms were collected and extracts for Hg assay were prepared. The accumulation of Hg was dose-, age-, and strain-dependent. For *trx-1* worms, both age ($P < 0.0001$, $F = 32.253$), dose ($P < 0.0001$, $F = 52.974$) and age x dose ($P < 0.0001$, $F = 28.226$) effects were statistically significant. Similarly, for *trxr-1* significant effect was observed for age ($P < 0.0001$, $F = 11.324$), dose ($P < 0.0001$, $F = 27.655$), age x dose ($P < 0.0001$, $F = 11.032$). In worms lacking mitochondrial *trx-2*, significant effects were observed for age ($P < 0.0001$, $F = 13.427$), strain ($P = 0.003$, $F = 10.171$) dose ($P < 0.0001$, $F = 35.697$), and age × dose ($P < 0.0001$, $F = 11.415$). In *trxr-2* mutants, significant effect was for age ($P < 0.0001$, $F = 19.184$), dose ($P < 0.0001$, $F = 45.159$), age x strain ($P < 0.0001$, $F = 10.527$), age x dose ($P < 0.0001$, $F = 13.411$), and age x strain x dose ($P < 0.0001$, $F = 10.954$) (n = 3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, *vs.* 0 μM MeHg; #$P < 0.05$, ##$P < 0.01$ *vs.* 20 μM MeHg (post-hoc Tukey's test).

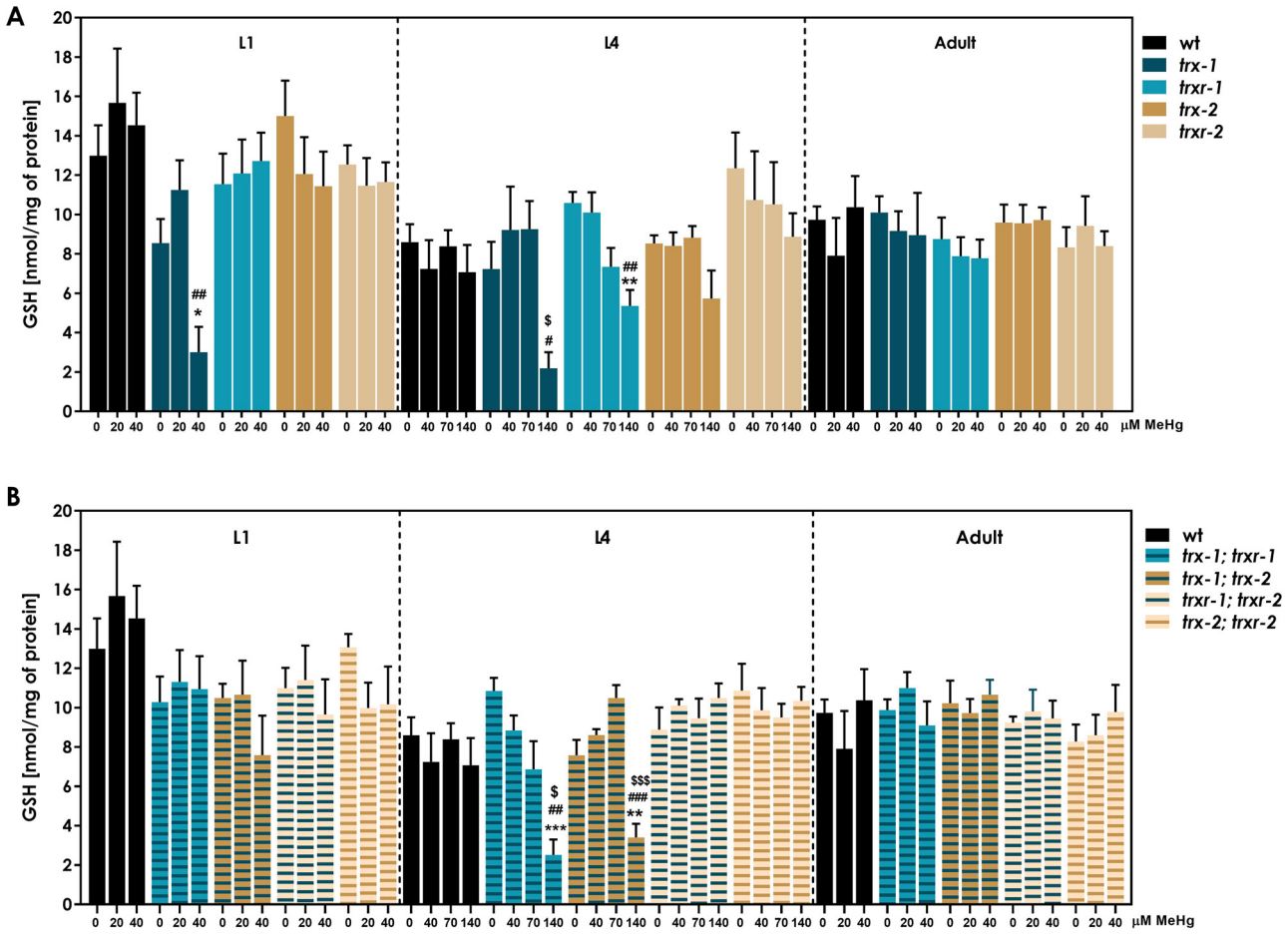

Fig. 5. Total glutathione (GSH) levels in null single (A) and double (B) mutants from the thioredoxin family exposed to MeHg. Synchronized worms at different developmental stage (L1, L4 and adult) were treated with MeHg for 1 h. Twenty-four hours later worms were collected and extracts for GSH assay were prepared. GSH was not changed upon MeHg treatment in wt worms but decreased in some mutants in an age-specific manner. Multifactorial ANOVA revealed for *trx-1* significant effect of age ($P = 0.04$, $F = 3.410$), strain ($P = 0.001$, $F = 13.356$), dose ($P = 0.031$, $F = 2862$), dose × age ($P = 0.049$, $F = 2.769$), as well as age × strain ($P < 0.0001$, $F = 10.656$) interaction, with lower GSH levels in control group (L1) or upon MeHg treatment. In *trxr-1* age factor was significant ($P < 0.0001$, $F = 18.533$), in *trx-2* too ($P < 0.0001$, $F = 20.944$), while for *trxr-2* age ($P < 0.0001$, $F = 11.527$) and age x strain interaction ($P = 0.016$, $F = 4.452$) (A). For *trx-1; trxr-1* statistically significant age ($P < 0.0001$, $F = 9.943$) and dose ($P = 0.025$, $F = 3.020$) effects, as well as age x strain interaction ($P = 0.004$, $F = 5.979$) were observed. For *trx-1; trx-2* significant age ($P < 0.0001$, $F = 9.838$), strain ($P = 0.027$, $F = 5.145$), dose ($P = 0.048$, $F = 2551$) effects, and age x strain interaction ($P = 0.001$, $F = 7.631$) have been observed. In *trxr-1; trxr-2* significant age effect ($P < 0.0001$, $F = 11.178$), as well as age x strain interaction ($P = 0.007$, $F = 5.404$) were observed, similarly in *trx-2; trxr-2*: age ($P < 0.0001$; $F = 12.885$), age x strain ($P = 0.016$, $F = 4.408$) (B) (n = 4). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ *vs.* 0 µM MeHg; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.005$ *vs.* 20 µM MeHg; $$P < 0.05$, $$$P < 0.005$ *vs.* 70 µM MeHg (post-hoc Tukey's test).

often described as key target and/or protectant in mechanism of oxidative stress, including mercury toxicity (Branco et al., 2014; Hansen et al., 2006a), mostly due the mitochondrial origin of many reactive oxygen species (Hansen et al., 2006b). This was however consistent with a previous study, where the mitochondrial thioredoxin system was not required to maintain general redox homeostasis in *C. elegans* even under different stress conditions: *trx-2* and *trxr-2* single and double mutants did not show enhanced sensitivity to stressors such as arsenite, juglone and sodium azide, moreover, *trx-2* and *trx-2; trxr-2* showed a slightly higher resistance to paraquat treatment (Cacho-Valadez et al., 2012). This indicates the existence of redundant system which counterbalances the absence of TRX-2/TRXR-2. In higher organisms cytosolic thioredoxin reductase gene *trxr-1* produce an isoform which under some conditions is targeted to mitochondria (Rundlof et al., 2004), which might supplement the lack of *trxr-2*; however since *trxr-1; trxr-2* double mutants were fully viable (Cacho-Valadez et al., 2012) and showed no changes in susceptibility to MeHg (Fig. 2G–I), this possibility unlikely exists in *C. elegans*.

Alternative protection might come from the GSH system. For instance, GSH-dependent glutaredoxin has been shown to reduce TRXs in the absence of functional TRXRs (Du et al., 2012); however this interaction remains to be confirmed in *C. elegans* (Cacho-Valadez et al., 2012). Glutathione reductase (GR) is encoded in *C. elegans* by *gsr-1* gene which is essential and required for *C. elegans* early embryonic development – *gsr-1* loss of function mutation resulted in embryonic lethality of worms, prevented by restoring GSR-1 activity in the cytoplasm, but not in mitochondria, suggesting prominent role of the GSH system in maintaining redox homeostasis in the nematode (Mora-Lorca et al., 2016). This effect in context of MeHg toxicity will be addressed in later parts of the discussion.

The exclusive role of the cytoplasmic TRX system has been previously observed – the loss of TRX-1 promoted nuclear localization of intestinal SKN-1 in a redox-independent, cell non-autonomous fashion, and this regulation was not general to the thioredoxin family, as TRX-2 and TRX-3, did not play a role in this process (McCallum et al., 2016). Other studies showed that in *C. elegans trxr-1* and *trxr-2* function differently at organismal level: deficiency of *trxr-2* caused defects in longevity and development under stress conditions, while *trxr-1* resulted in impaired acidification of lysosomal compartment in intestine; however for both TRXR-1 and TRXR-2, the gene expression was induced by heat shock, followed by production of reactive oxygen species (Li et al., 2012).

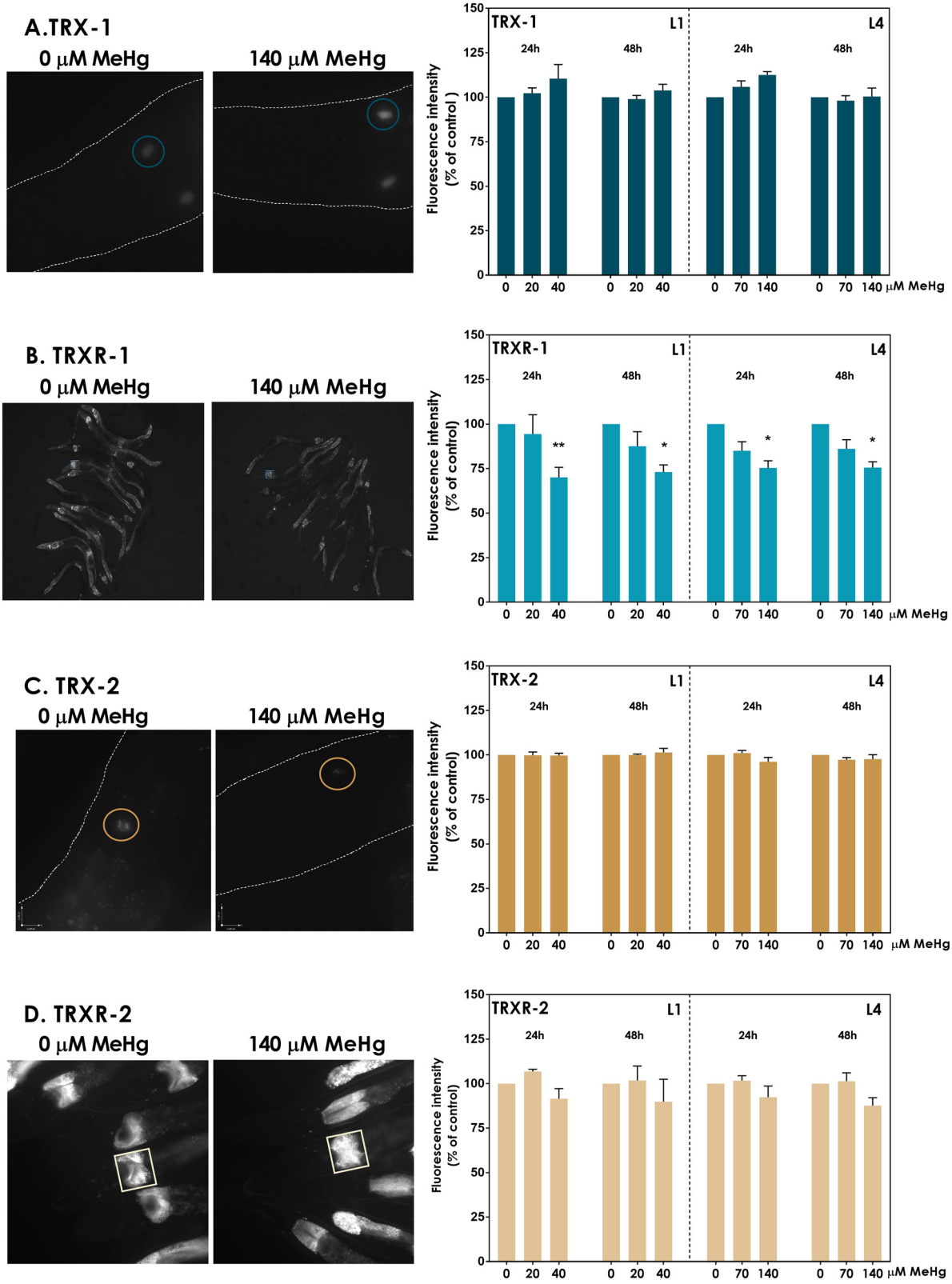

Fig. 6. The expression of TRX-1 (A), TRXR-1 (B), TRX-2 (C), TRXR-2 (D) upon MeHg exposure. Synchronized transgenic worms at L1 and L4 stage were treated with MeHg for 1 h. Twenty-four and forty-eight hours later alive worms were picked for imaging. (A, C) 10–20 worms per group were analyzed and GFP fluorescence GFP intensity was measured in pixels inside neuron-surrounding equal-size sphere. (B, D) 20–40 worms per group were analyzed and GFP fluorescence intensity were measured in pixels from a square region of the anterior-most region of the intestine. Data were control-normalized. The expression of TRXR-1 protein decreased significantly in MeHg-treated worms, when exposed at L1 and L4 stage, both 24 and 48 h post-treatment (B), while other analyzed proteins remained unaffected (n = 4). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* 0 μM MeHg (post-hoc Tukey's test).

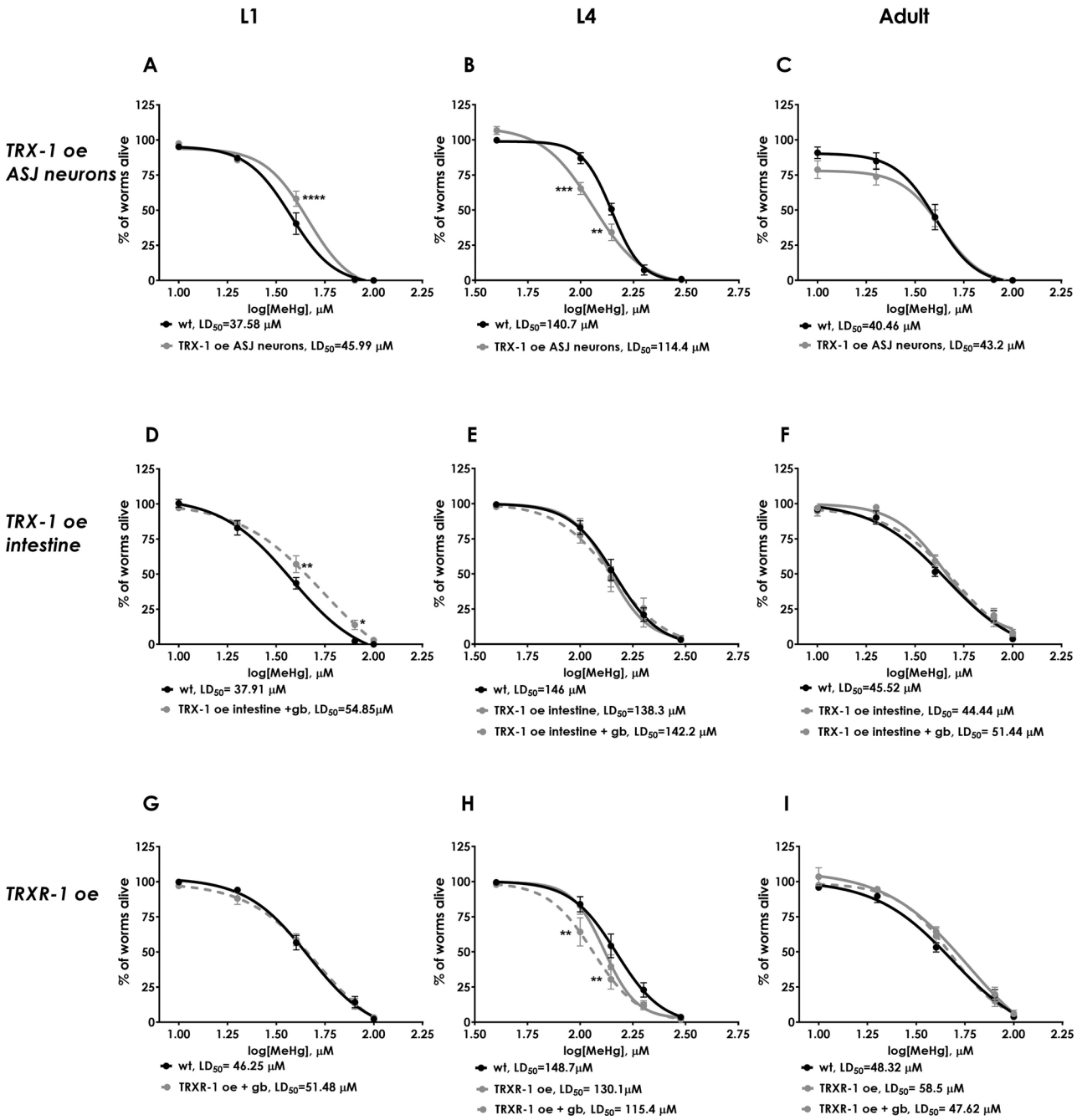

Fig. 7. Lethality of worms overexpressing TRX-1 and TRXR-1 exposed to MeHg. Synchronized worms at different developmental stage (L1, L4 and adult) were treated with MeHg for 1 h. Twenty-four hours later alive worms were scored (including those lacking transgenic arrays - genetic background: gb) and based on the sigmoidal dose-response curves lethal doses 50% (LD_{50}) were determined. The resistance to MeHg increased significantly in L1 worms with TRX-1 overexpression (oe) in ASJ neurons ($P = 0.0443$, $F = 5.986$) (A) and intestine ($P = 0.0296$, $F = 7.415$) (D), but the effect was not preserved in older animals. TRXR-1 oe worms did not show improved resistance to MeHg (G–I), of note L4 exhibited significantly lower resistance to MeHg than wt ($P = 0.0235$, $F = 9.104$) (with and without transgenic array) (H) (n = 8). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.0001$ *vs.* wt (post-hoc Tukey's test).

Interestingly, while previous studies showed that *trx-1* mutants display decreased (19%) lifespan when compared to wt worms (Miranda-Vizuete et al., 2006), we failed to note such a difference, probably due to substantial differences in the handing protocols – where we used first generation of worms after bleaching, which might affect longevity. The unchanged lifespan in response to MeHg is analogous to observations from an earlier study (Helmcke et al., 2009).

4.2. The thioredoxin system and age-specific susceptibility to MeHg

Susceptibility of wt worms to MeHg was age-dependent, with L4 worms being the most resistant to the toxicant. These results are consistent with earlier observations, where worms treated with MeHg for 0.5 h at L1 stage were more sensitive to the toxicant that worms treated at the L4 stage (Helmcke et al., 2009). Enhanced susceptibility of worms non-expressing cytoplasmic thioredoxin system was also not uniform for all stages of development (Figs. 1 and 2), indicating an age-dependent role of this proteins in development and/or protection. Several factors might affect this, including age-dependent: protein expression, protection from alternative antioxidant systems or Hg deposition.

The expression of TRX/TRXR proteins might change with age and

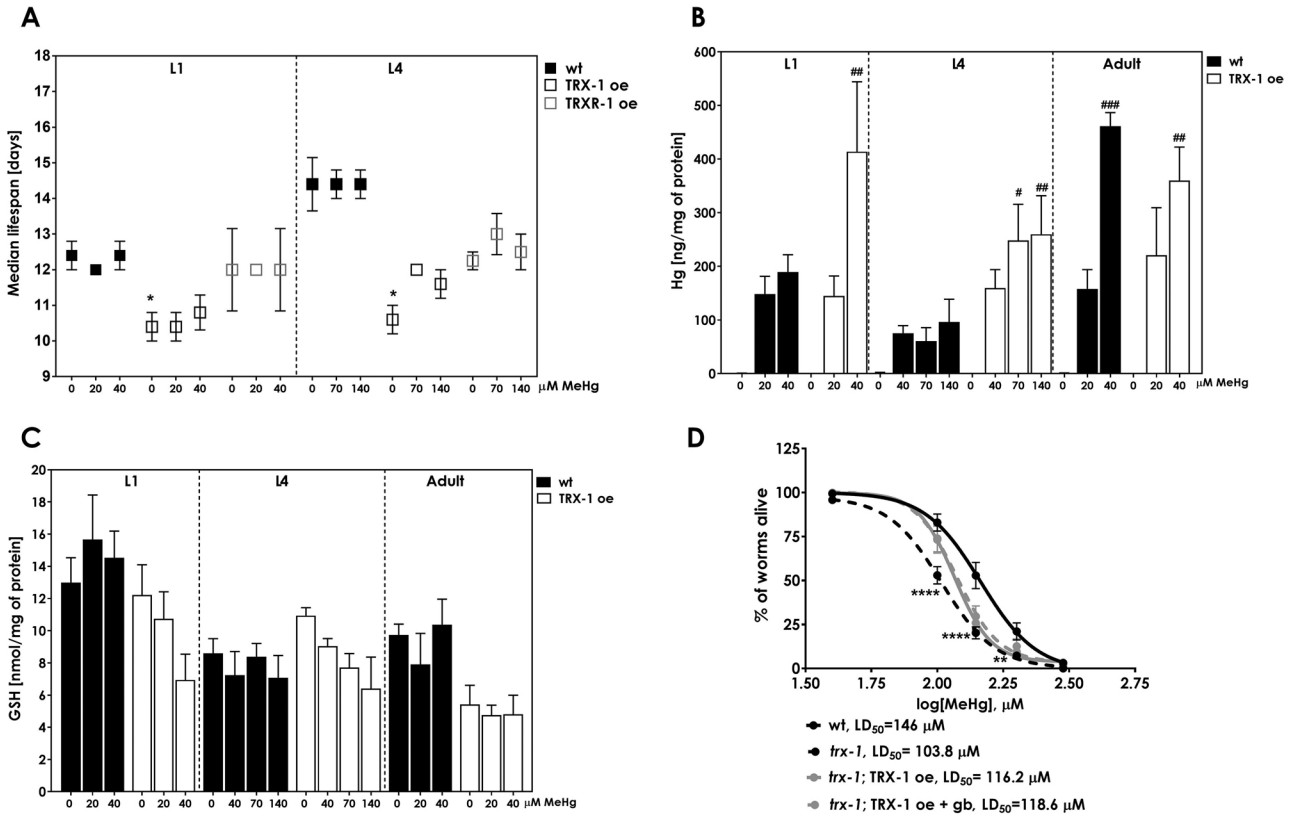

Fig. 8. (A) Lifespan of worms overexpressing TRX-1 and TRXR-1 exposed to MeHg. The longevity of TRX-1 oe and TRXR-1 oe worms was not affected by MeHg exposure. For TRX-1 oe, both strain ($P < 0.0001$, $F = 88.783$), age ($P = 0.011$, $F = 6.981$), as well as strain x age factor ($P = 0.036$, $F = 4.673$) were statistically significant. Similarly, for TRXR-1 oe: strain ($P = 0.003$, $F = 9.908$) and age ($P = 0.047$, $F = 4.205$) factors were significant (n = 5). (B) Mercury (Hg) content in TRX-1 oe worms exposed to MeHg. Statistically significant effect was observed for dose ($P < 0.0001$, $F = 26.447$), strain ($P = 0.003$, $F = 9.966$) and age ($P < 0.0001$, $F = 9.452$), as well as dose x age ($P = 0.002$, $F = 6.176$) and dose x strain x age($P = 0.038$, $F = 3.095$) interactions, suggesting higher than in wt Hg accumulation, especially when at early stages of development (n = 3). (C) Total glutathione (GSH) levels in TRX-1 oe worms exposed to MeHg. Significant strain ($P < 0.0001$, $F = 16.386$) and age ($P < 0.001$, $F = 18.210$) effects, as well as age x strain interaction ($P = 0.002$, $F = 6.971$) were observed, with lower GSH levels in TRX-1 oe upon MeHg treatment (n = 4). (D) Lethality of rescue *trx-1* strain expressing TRX-1 in ASJ neurons. TRX-1 rescue provided partial protection - worms susceptibility was significantly higher than in wt animas: all animals ($P = 0.0055$, $F = 8.218$) and transgene arrays only ($P = 0.0007$, $F = 12.64$), but also significantly lower when compared with *trx-1* worms: a transgene only ($P = 0.0043$, $F = 8.723$) and the whole strain ($P = 0.0015$, $F = 10.88$) (n = 8). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.0001$ *vs.* wt, #p < 0.05, ##p < 0.01, ###p < 0.005 *vs.* 0 µM MeHg (post-hoc Tukey's test).

thus their significance in protection against MeHg might be age-dependent. There are no studies evaluating expression of cytoplasmic thioredoxin system in details, including changes during development, however from clues in previous papers and our own observations appears that the expression, especially for TRXR-1, increases with age. Second possibility is that during aging other antioxidant systems are

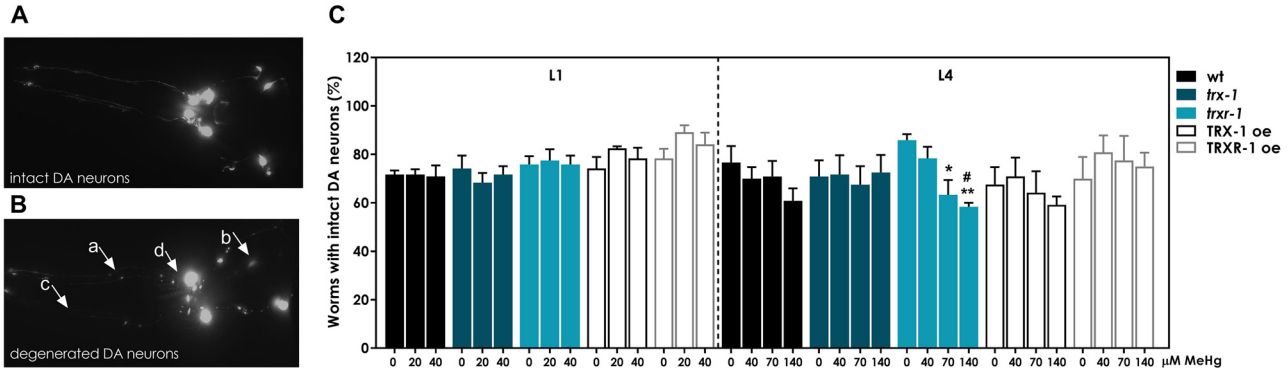

Fig. 9. Degeneration of DAergic neurons in worms lacking or overexpressing TRX-1 or TRXR-1. Synchronized worms at L1 or L4 stage were treated with MeHg for 1 h. Ninety-six (L1) or forty-eight (L4) hours later alive worms (30 per group), were scored for degeneration of six DAergic head neurons. Data were expressed as percentage of worms with intact neurons. Representative confocal images of normal (intact) (A) and degenerated (B) neurons, which exhibit dendritic puncta (a), shrunken soma (b) and/or loss of dendrites (c) and soma (d) are shown. *trxr-1* worms exhibited higher susceptibility to MeHg-induced DAergic neurodegeneration ($P = 0.001$, F = 6.222) when worms were treated at L4 stage and analyzed 48 h later. The strain effect was significant for TRXR-1 oe worms ($P = 0.003$, $F = 9.883$), indicating on more intact neurons in this strain, when compared to wt (C) (n = 4). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* 0 µM MeHg, #$P < 0.05$ *vs.* 40 µM MeHg (post-hoc Tukey's test).

induced, providing alternative protection against MeHg, while thioredoxins are missing. This refers to the most abundant antioxidant system, based on the reducing power of the GSH. In a previous study, when worms were collected immediately after acute treatment (L1, 0.5 h, 0.4 mM MeHg,) no changes in total GSH was observed in wt animals, while a decrease upon chronic exposure was noted (L4, 15 h, 0.4 mM MeHg) (Helmcke and Aschner, 2010). In our study the samples were collected 24 h after treatment (a timepoint for lethality assays) to analyze more permanent effects of MeHg, including adaptation. Glutathione levels were significantly downregulated by MeHg in *trx-1* (L1 and L4), *trxr-1* (L4), *trx-1; trxr-1* (L4) and *trx-1; trx-2* (L4) worms (Fig. 5), which correspond at high degree (but not completely) to decreased susceptibility of these strains at respective stages of development. Furthermore, *trx-1* (L1) exhibited significantly lower GSH levels when compared to the wt, also in untreated group (Fig. 5A). This is consistent with other data, demonstrating age-specific GSH status and adaptational response. GSSG/GSH ratio has been found to decrease exponentially during worms larval development (Back et al., 2012). Modest decrease in GSH levels in young adult worms promoted stress resistance and increased lifespan, whereas depletion of GSH (both chemical and through RNAi attenuating expression of γ-glutamylcysteine synthetase) was detrimental to freshly hatched and developing worms, indicating that dependent upon the developmental stage, GSH depletion may be either damaging or beneficial to the nematode (Urban et al., 2017). Adaptation to stress by increasing content of other antioxidant - superoxide dismutase (SOD) in young nematodes but not in mature or old individuals has been also observed (Darr and Fridovich, 1995). Nevertheless, with the highest MeHg resistance observed for L4 worms (LD_{50} ≈140 *vs*. 40 μM MeHg for L1 or adult) and the highest GSH levels in L1, decreasing with age (*trx-1* was an exception) (Fig. 5), the GSH content do not appear as dominant factor in age-dependent protection against MeHg. Alternatively, age-specific MeHg uptake and/or Hg metabolism and accumulation might contribute to differential susceptibility of nematodes to the toxicant. For most strains, L4 accumulated less Hg than L1 or adult (Fig. 4), which coincide with the highest resistance of L4 to MeHg toxicity (Figs. 1 and 2). These results differ from previous data, where higher Hg levels were found in L4 worms than in L1 (Helmcke et al., 2009); however, the doses used in that study were generally higher, and the exposure time differed as well. The probable explanation here is that to penetrate the cuticle of the more developed worm, a higher dose is required. However, in our study we observed an increase in Hg content for adults. This may be due to morphological changes – *e.g.* egg laying open the vulva which provides an additional route of MeHg ingestion – lower metabolism, or excretion capacity – *e.g.* GSH levels were often lower in adult than in L4 worms from respective group (Fig. 5A). Nonetheless, our data revealed no differences in Hg levels in *trx-1* and *trxr-1* when compared to the wt, suggesting that the susceptibility of these worms to MeHg was not related to differences in Hg accumulation. Interestingly, *trx-2* accumulate more Hg than wt, especially at L4 stage (significant age x strain interaction), but this is without an effect on susceptibility (lethality, lifespan or neurodegeneration).

Finally, we must acknowledge the possibility that using deletion mutants might not provide us with the full picture of TRX-dependent resistance to MeHg. If in nematode MeHg inhibits TRXs, as it has been shown in higher organisms (Ruszkiewicz et al., 2016; Branco et al., 2011; Wagner et al., 2010), such disrupted system might produce in wt animals outcomes similar to worms missing these proteins, and thus masking the differences between wt and mutant animals. This might be also dependent upon stage of development – age-specific susceptibility of *trx-1/trxr-1* mutants suggest that the scale of this inhibition might be also age-sensitive. In fact, expression of TRXR-1 protein has been downregulated in worms upon MeHg treatment, which indicate on MeHg-induced impairment of native TRX system in *C. elegans*, however with no relation to age (Fig. 6B). The lack of response of other proteins (Fig. 6A,C,D) is consistent with observations in mammals, with TRXR-1 being the primary target among the TRX family to various toxicants, including MeHg (Ruszkiewicz et al., 2016).

4.3. Overexpression of the cytoplasmic thioredoxin system and MeHg toxicity

With deletion mutants displaying MeHg-sensitive phenotype, the possibility that overexpression (oe) or rescue of the TRX-1 and TRXR-1 provide protection has been investigated. TRXR-1 oe resulted in lower number of worms with degenerated DAergic neurons (Fig. 9C), which validate the protective role of this protein in neurodegeneration – earlier study demonstrated that *trxr-1* mutants are more susceptible to DAergic neurodegeneration induced by 6-hydroxydopamine (6-OHDA) (Arodin et al., 2014). It is noteworthy, *trxr-1* were also more susceptible to neurodegeneration induced by MeHg (Fig. 9C). While none of oe strains provide wide resistance to MeHg-induced lethality, some protective effects have been observed. The higher resistance to MeHg has been noted for TRX-1 oe at L1 (Fig. 7A, D), but not in older animals. Moreover, *trx-1* rescue through TRX-1 oe in ASJ neurons provided partial, but not complete, protection at L4 stage (Fig. 8D). On the other hand, TRX-1 oe (in ASJ neurons) resulted in higher than in wt levels of Hg (L1, L4) (Fig. 8B), lower levels of GSH (L1, adult) (Fig. 8C) and slightly decreased lifespan (Fig. 8A). Interestingly, the latter result is inconsistent with previously reported observation that overexpression of TRX-1 resulted in a moderate lifespan increase (Miranda-Vizuete et al., 2006) – this discrepancy might be attributed to different protocols for worm handing and lifespan assay. Nevertheless, these phenomena might contribute to the questionable aid of TRX-1 oe in MeHg toxicity. Also probable is that MeHg inhibited excessive proteins and thus resulting in outcomes similar, or only slightly different from the wt.

5. Conclusions

In summary, worms lacking cytoplasmic TRX system exhibited age-specific increase in susceptibility to MeHg, which corresponded partially to decreased GSH levels or enhanced degeneration of dopaminergic neurons upon MeHg exposure. Surprisingly, overexpression of the TRX-1/TRXR-1 did not provide sufficient protection against MeHg. This is probably due to the inhibition of the TRX-1/TRXR-1 system by MeHg, as it has been shown through downregulation of TRXR-1 protein expression. Based on the current findings, the cytoplasmic thioredoxin system TRX-1/TRXR-1 emerges as a target, but also an important age-sensitive protectant against MeHg toxicity in C. *elegans*. These finding will help elucidate the role of cytoplasmic thioredoxin in MeHg toxicity during early development.

Conflicts of interest

The authors declare no conflict of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

This work has beensupported by the National Institutes of Health [NIEHS R01ES07331 to MA and ABB]. We thank the German Research Foundation (DFG) for the financial support of BO 4103/2-1 as well as the DFG Research Unit TraceAge (FOR 2558). *C. elegans* strains were provided by the Caenorhabditis Genetics Center (CGC, University of Minnesota, USA), which is funded by NIH Office of Research Infrastructure Programs [P40 OD010440]. We would like to acknowledge Hillary Guzik for expert assistance in microscopy. The imaging

was conducted in the Analytical Imaging Facility, which is funded by the NCI Cancer Grant P30CA013330.

References

Antunes Dos Santos, A., Appel Hort, M., Culbreth, M., Lopez-Granero, C., Farina, M., Rocha, J.B., Aschner, M., 2016. Methylmercury and brain development: a review of recent literature. J. Trace Elem. Med. Biol. 38, 99–107.

Arodin, L., Miranda-Vizuete, A., Swoboda, P., Fernandes, A.P., 2014. Protective effects of the thioredoxin and glutaredoxin systems in dopamine-induced cell death. Free Radic. Biol. Med. 73, 328–336.

Back, P., De Vos, W.H., Depuydt, G.G., Matthijssens, F., Vanfleteren, J.R., Braeckman, B.P., 2012. Exploring real-time in vivo redox biology of developing and aging Caenorhabditis elegans. Free Radic. Biol. Med. 52, 850–859.

Branco, V., Canario, J., Holmgren, A., Carvalho, C., 2011. Inhibition of the thioredoxin system in the brain and liver of zebra-seabreams exposed to waterborne methylmercury. Toxicol. Appl. Pharmacol. 251, 95–103.

Branco, V., Godinho-Santos, A., Goncalves, J., Lu, J., Holmgren, A., Carvalho, C., 2014. Mitochondrial thioredoxin reductase inhibition, selenium status, and Nrf-2 activation are determinant factors modulating the toxicity of mercury compounds. Free Radic. Biol. Med. 73, 95–105.

Cacho-Valadez, B., Muñoz-Lobato, F., Pedrajas, J.R., Cabello, J., Fierro-González, J.C., Navas, P., Swoboda, P., Link, C.D., Miranda-Vizuete, A., 2012. The characterization of the Caenorhabditis elegans mitochondrial thioredoxin system uncovers an unexpected protective role of thioredoxin reductase 2 in β-amyloid peptide toxicity. Antioxid. Redox Signal. 16, 1384–1400.

Caito, S.W., Aschner, M., 2015. Quantification of Glutathione in Caenorhabditis elegans. Curr. Protoc. Toxicol. 64 6.18.1–6.

Cunningham, G.M., Roman, M.G., Flores, L.C., Hubbard, G.B., Salmon, A.B., Zhang, Y., Gelfond, J., Ikeno, Y., 2015. The paradoxical role of thioredoxin on oxidative stress and aging. Arch. Biochem. Biophys. 576, 32–38.

Darr, D., Fridovich, I., 1995. Adaptation to oxidative stress in young, but not in mature or old, Caenorhabditis elegans. Free Radic. Biol. Med. 18, 195–201.

Du, Y., Zhang, H., Lu, J., Holmgren, A., 2012. Glutathione and glutaredoxin act as a backup of human thioredoxin reductase 1 to reduce thioredoxin 1 preventing cell death by aurothioglucose. J. Biol. Chem. 287, 38210–38219.

Farina, M., Aschner, M., 2017. Methylmercury-induced neurotoxicity: focus on pro-oxidative events and related consequences. Adv. Neurobiol. 18, 267–286.

Farina, M., Aschner, M., Rocha, J.B., 2011a. Oxidative stress in MeHg-induced neurotoxicity. Toxicol. Appl. Pharmacol. 256, 405–417.

Farina, M., Rocha, J.B., Aschner, M., 2011b. Mechanisms of methylmercury-induced neurotoxicity: evidence from experimental studies. Life Sci. 89, 555–563.

Fierro-Gonzalez, J.C., Cornils, A., Alcedo, J., Miranda-Vizuete, A., Swoboda, P., 2011a. The thioredoxin TRX-1 modulates the function of the insulin-like neuropeptide DAF-28 during dauer formation in Caenorhabditis elegans. PLoS One 6, e16561.

Fierro-Gonzalez, J.C., Gonzalez-Barrios, M., Miranda-Vizuete, A., Swoboda, P., 2011b. The thioredoxin TRX-1 regulates adult lifespan extension induced by dietary restriction in Caenorhabditis elegans. Biochem. Biophys. Res. Commun. 406, 478–482.

Han, J., Yang, X., Chen, X., Li, Z., Fang, M., Bai, B., Tan, D., 2017. Hydrogen sulfide may attenuate methylmercury-induced neurotoxicity via mitochondrial preservation. Chem. Biol. Interact. 263, 66–73.

Hansen, J.M., Zhang, H., Jones, D.P., 2006a. Differential oxidation of thioredoxin-1, thioredoxin-2, and glutathione by metal ions. Free Radic. Biol. Med. 40, 138–145.

Hansen, J.M., Zhang, H., Jones, D.P., 2006b. Mitochondrial thioredoxin-2 has a key role in determining tumor necrosis factor-α–induced reactive oxygen species generation, NF-κB activation, and apoptosis. Toxicol. Sci. 91, 643–650.

Helmcke, K.J., Aschner, M., 2010. Hormetic effect of methylmercury on Caenorhabditis elegans. Toxicol. Appl. Pharmacol. 248, 156–164.

Helmcke, K.J., Syversen, T., Miller 3rd, D.M., Aschner, M., 2009. Characterization of the effects of methylmercury on Caenorhabditis elegans. Toxicol. Appl. Pharmacol. 240, 265–272.

Jee, C., Vanoaica, L., Lee, J., Park, B.J., Ahnn, J., 2005. Thioredoxin is related to life span regulation and oxidative stress response in Caenorhabditis elegans. Genes Cells 10, 1203–1210.

Johansson, C., Castoldi, A.F., Onishchenko, N., Manzo, L., Vahter, M., Ceccatelli, S., 2007. Neurobehavioural and molecular changes induced by methylmercury exposure during development. Neurotox. Res. 11, 241–260.

Kirkpatrick, M., Benoit, J., Everett, W., Gibson, J., Rist, M., Fredette, N., 2015. The effects of methylmercury exposure on behavior and biomarkers of oxidative stress in adult mice. Neurotoxicology 50, 170–178.

Li, W., Bandyopadhyay, J., Hwaang, H.S., Park, B.J., Cho, J.H., Lee, J.I., Ahnn, J., Lee, S.K., 2012. Two thioredoxin reductases, trxr-1 and trxr-2, have differential physiological roles in Caenorhabditis elegans. Mol. Cells 34, 209–218.

Llop, S., Ballester, F., Murcia, M., Forns, J., Tardon, A., Andiarena, A., Vioque, J., Ibarluzea, J., Fernandez-Somoano, A., Sunyer, J., Julvez, J., Rebagliato, M., Lopez-Espinosa, M.J., 2017. Prenatal exposure to mercury and neuropsychological development in young children: the role of fish consumption. Int. J. Epidemiol. 46, 827–838.

Lohren, H., Bornhorst, J., Galla, H.J., Schwerdtle, T., 2015. The blood-cerebrospinal fluid barrier–first evidence for an active transport of organic mercury compounds out of the brain. Metallomics 7, 1420–1430.

Lu, J., Holmgren, A., 2014. The thioredoxin antioxidant system. Free Radic. Biol. Med. 66, 75–87.

Mccallum, K.C., Liu, B., Fierro-Gonzalez, J.C., Swoboda, P., Arur, S., Miranda-Vizuete, A., Garsin, D.A., 2016. TRX-1 regulates SKN-1 nuclear localization cell non-autonomously in Caenorhabditis elegans. Genetics 203, 387–402.

Meinerz, D.F., Branco, V., Aschner, M., Carvalho, C., Rocha, J.B.T., 2017. Diphenyl diselenide protects against methylmercury-induced inhibition of thioredoxin reductase and glutathione peroxidase in human neuroblastoma cells: a comparison with ebselen. J. Appl. Toxicol. 37, 1073–1081.

Miranda-Vizuete, A., Fierro Gonzalez, J.C., Gahmon, G., Burghoorn, J., Navas, P., Swoboda, P., 2006. Lifespan decrease in a Caenorhabditis elegans mutant lacking TRX-1, a thioredoxin expressed in ASJ sensory neurons. FEBS Lett. 580, 484–490.

Mora-Lorca, J.A., Saenz-Narciso, B., Gaffney, C.J., Naranjo-Galindo, F.J., Pedrajas, J.R., Guerrero-Gomez, D., Dobrzynska, A., Askjaer, P., Szewczyk, N.J., Cabello, J., Miranda-Vizuete, A., 2016. Glutathione reductase gsr-1 is an essential gene required for Caenorhabditis elegans early embryonic development. Free Radic. Biol. Med. 96, 446–461.

Prpic, I., Milardovic, A., Vlasic-Cicvaric, I., Spiric, Z., Radic Nisevic, J., Vukelic, P., Snoj Tratnik, J., Mazej, D., Horvat, M., 2017. Prenatal exposure to low-level methylmercury alters the child's fine motor skills at the age of 18 months. Environ. Res. 152, 369–374.

Ren, X., Zou, L., Zhang, X., Branco, V., Wang, J., Carvalho, C., Holmgren, A., Lu, J., 2017. Redox signaling mediated by thioredoxin and glutathione systems in the central nervous system. Antioxid. Redox Signal. 27, 989–1010.

Rundlof, A.K., Janard, M., Miranda-Vizuete, A., Arner, E.S., 2004. Evidence for intriguingly complex transcription of human thioredoxin reductase 1. Free Radic. Biol. Med. 36, 641–656.

Ruszkiewicz, J.A., Bowman, A.B., Farina, M., Rocha, J.B.T., Aschner, M., 2016. Sex- and structure-specific differences in antioxidant responses to methylmercury during early development. Neurotoxicology 56, 118–126.

Stenvall, J., Fierro-Gonzalez, J.C., Swoboda, P., Saamarthy, K., Cheng, Q., Cacho-Valadez, B., Arner, E.S., Persson, O.P., Miranda-Vizuete, A., Tuck, S., 2011. Selenoprotein TRXR-1 and GSR-1 are essential for removal of old cuticle during molting in Caenorhabditis elegans. Proc. Natl. Acad. Sci. U. S. A. 108, 1064–1069.

Urban, N., Tsitsipatis, D., Hausig, F., Kreuzer, K., Erler, K., Stein, V., Ristow, M., Steinbrenner, H., Klotz, L.O., 2017. Non-linear impact of glutathione depletion on C. elegans life span and stress resistance. Redox Biol. 11, 502–515.

Wagner, C., Sudati, J.H., Nogueira, C.W., Rocha, J.B., 2010. In vivo and in vitro inhibition of mice thioredoxin reductase by methylmercury. Biometals 23, 1171–1177.