Cytoprotective responses in learned systemic stress defenses in *Caenorhabditis elegans*

PhD thesis

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Table of Contents

Abbreviations, Definitions

AM	antimycin A
ANOVA	Analysis of variance
BA	benzaldehyde
BS	*Bacillus subtilis*
ccBA	"concentratus" benzaldehyde, undiluted benzaldehyde
ccDA	"concentratus" diacetyl, undiluted diacetyl
CI	chemotaxis index
DA	diacetyl
GFP	green fluorescent protein
gst-4	glutathione S-transferase 4
hsp-6	heat shock protein 6
LI	learning index
NGM	Nematode Growth Medium
ns	non-significant
PA14	*Pseudomonas aeruginosa* PA14
PC	pre-conditioned
PF	*Pseudomonas fluorescens*
PQ	paraquat

1 Introduction

1.1 Imprinted memory and early life stress

Learning and memory allow animals to navigate, find food and thus survive in a changing environment. Associative learning is defined as a process by which an association occurs between two or more different stimuli, and it supports individuals to access resources or help to defend effectively against dangers. In addition, an adequate, coordinated interaction of intracellular molecular defense mechanisms and complex behavioral responses is also required for survival. Associative learning provides quick and efficient adaptation to already experienced, re-emerging circumstances[1,2]. Re-encountering sensory signals associated with a relevant past experience evokes the memory and elicits a complex response corresponding to the past event. Associative memories can be short-, medium-, and long-term memories. A peculiar learning process takes place in early life during a specific time window, called the critical or sensitive period. This special form of associative learning during early age is called imprinting, which forms especially persistent memories and develops lasting patterns of behavior. These early life stimuli create a lifelong response that may fundamentally affect the life of individuals in adulthood. In the course of imprinting, newly born animals acquire various sensory inputs about the external world and behavioral characteristics of their parents. Freshly hatched ducks and geese immediately begin to follow the first largest object that moves around them, i.e. the parent. A lasting attachment develops to individuals of the species they first saw. Imprinting was first described in the 19th century by an amateur biologist, Douglas Spalding, on domestic chickens. This phenomenon was re-discovered by Oskar Heinrot, and then his student, Konrad Lorenz began to study the phenomenon more deeply in summer geese. Lorenz observed that the goose that hatched in the incubator also created a strong bond with the first moving object seen during the critical period, which is roughly 13-16 hours after hatching[3]. In the goslings, for example, it was Lorenz himself who left a mark, more precisely his boots. The little birds found it difficult to distinguish between Lorenz and his assistant, as they both wore the same boots. Therefore, if on the first day of their lives they see not their mother, but a moving object of roughly similar size, they begin to follow it, and its properties are memorized very quickly and permanently.

Besides visual cues, olfactory memories shaping adult behaviors have been recognized in several vertebrate species, including the homing of salmons to reproduce in the stream they were born[4] and preference for odors of food experienced perinatally in mammals[5,6]. In the critical period, sensory cues and various environmental effects are imprinted together to form adaptive behavioral responses. In adulthood, the re-encounter with the sensory cues can trigger a characteristic pattern of behavior *per se* (Fig. 1). Hence, imprinting serves as the biological basis of secure, long lasting attachment to qualities essential for individual and/or species' survival.

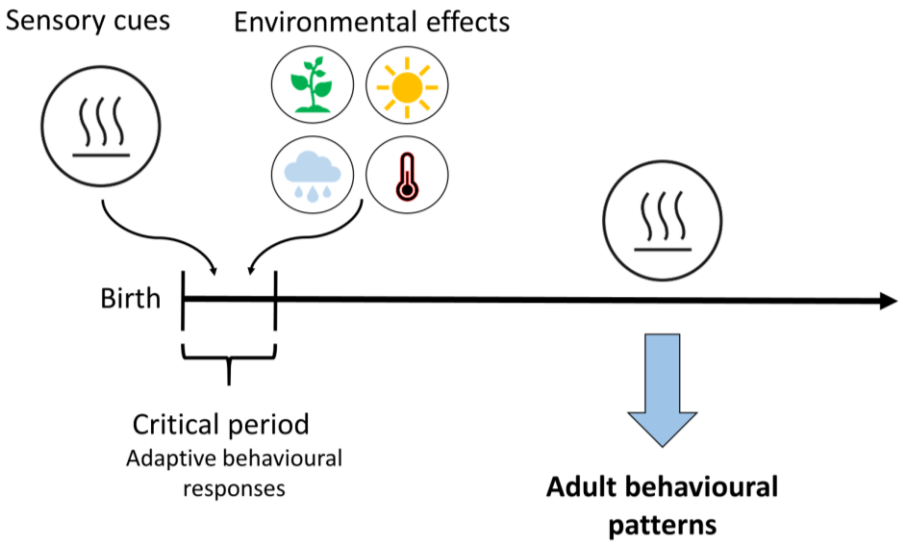

Figure 1. Schematic of the imprinting mechanism

In further support of the profound, life-long impact of early life memories, a growing body of evidence shows that facing adversity in the critical period is connected to different cognitive and affective disorders and are accompanied by brain epigenetic, structural and endocrine alterations[7, 8, 9]. Early life stress cause long-term changes, however it is not known whether these alterations are due to the destructive effects of stress or a neuronal learning mechanism, more specifically, to imprinting. Recent studies suggest that specific neuroendocrine responses caused by early life stress are stored in the long run. Stress response genes in the hypothalamic-pituitary-adrenal (HPA) axis are epigenetically modified by early life trauma. FKBP5 (Hsp90 co-chaperone), MAO (monoamine

oxidase), NR3C1 (glucocorticoid receptor) and BDNF (neurotrophin growth factor) genes show altered methylation associated with childhood trauma [10]. Other studies reported that early life stress, especially in the limbic system, induces enduring changes in neuronal circuits, neurotransmitter systems, neuronal architecture and plasticity that are further associated with inadequate emotional and cognitive information processing later in life[11]. Moreover, the environment, particularly early life severe stress or trauma, can lead to lifelong molecular alterations in the form of epigenetic modifications that can set the organism to health or disease trajectories[12]. On the other hand, early life stress also predispose to increased telomere erosion, metabolic and cardiovascular diseases in adulthood ultimately affecting healthy lifespan[13, 14, 15].

1.2 *Caenorhabditis elegans* nematode as a model organism

C. elegans is a 1 mm long 959-cell nematode that lives on overripe and rotting fruits. In 1963, Sydney Brenner introduced *C. elegans* as a model system for genetics, development and neuroscience research for which he received the Nobel Prize[16]. Since then, its development, genetics, and molecular biology has been extensively studied and today *C. elegans* is one of the most important, most commonly used model organism in biology due to its beneficial properties.

Laboratory maintenance of *C. elegans* is simple and cheap, nematodes are cultured on agar plates at 15-25°C and fed with *Escherichia coli* OP50 strain. More than 99% of the animals are hermaphrodite providing stable genetic lines by producing isogenic progenies. A small fraction of males also supplements the population, which allows crossing different lines. A hermaphrodite lays about 250-300 eggs during the reproductive period, which takes about 3-4 days from reaching adulthood. Its rapid, temperature-dependent 3-5-day generation time and the high number of offsprings make *C. elegans* suitable for frequent testing of large numbers of samples, and thousands of worms examined in each test provide statistically reliable data. The life cycle of *C. elegans* is comprised of the embryonic stage, four larval stages (L1-L4) and adulthood. The lifespan of wild type animals in favorable conditions is 2-3 weeks. *C. elegans* can also endure adverse environmental conditions by entering to a facultative diapause stage called dauer larva, which is extremely resistant and can survive, for example hunger, for months. At the end of the harsh circumstances, they resume their normal life cycle and complete it.

Young animals in the L1 larval stage can also be frozen to form strain banks from lines with different genetic backgrounds.

C. elegans possesses an entirely mapped, invariant network of 302 neurons including a highly sensitive chemosensorium[17], and the localization and connectivity of neurons are considered to be largely conserved among individuals. Due to its transparent and simple structure, *C. elegans* cells can also be investigated by light microscopy and subjected to gene expression and protein localization studies by fluorescence microscopy[18]. The *C. elegans* genome was fully sequenced and approximately 40% of its genes show some degree of homology with human genes. In addition, the lineage of all cells of the worm is also known[19], making *C. elegans* a preferred model organism in developmental biology research. Another advantage of *C. elegans* is that the *Caenorhanditis elegans* research community shares a great knowledge in free databases. Wormbase (wormbase.org), Wormbook (wormbook.org) and Wormatlas (wormatlas.org) websites provide information about *C. elegans* genes; molecular biology methods, discoveries, and an extensive international strain collection is also available for researchers on the Caenorhabditis Genetics Center website (cgc.umn.edu).

1.3 Learning and memory in *C. elegans*

Learning is inherently connected to the appearance of the nervous system. In *C. elegans*, the nervous system is by far the most complex organ, almost third of all cells in the body are neurons (302 neurons out of 959 cells). 20 neurons are located inside the pharynx forming a smaller pharyngeal nervous system. The remaining 282 neurons constitute a larger somatic nervous system and are located in different ganglia of the head and the tail region, as well as along the main longitudinal axon tract called the ventral cord. Most of the neurons develop during embryogenesis, but there are 80 neurons (mainly motoneurons) which develop post-embryonically[20]. The structure of the nervous system has been described in extraordinarily detail by electron microscopic reconstruction[17]. The high resolution obtained with electron microscopy images allowed White and colleagues to identify all synapses (about 5000 chemical synapses, 2000 neuromuscular junctions and approximately 500 gap junctions), map all connections and define the entire neural circuit (Fig. 2).

Despite its simple nervous system, *C. elegans* is capable of surprisingly complex behavior and exhibits various forms of learning based on prior experiences[21]. It can distinguish several forms of environmental stimuli and is able to respond adaptively to them. Besides chemosensory adaptation, and habituation, a form of non-associative learning, *C. elegans* is characterized by a remarkable ability for associative learning, capable of chemotaxis, thermotaxis and aerotaxis to achieve more favorable environmental conditions. Both larval-stage and adult worms are able to develop behavioral changes to mechanical and chemical stimuli: they learn to associate vibrations, odors, tastes, changes in temperature and oxygen levels with noxious stimuli or even with food.

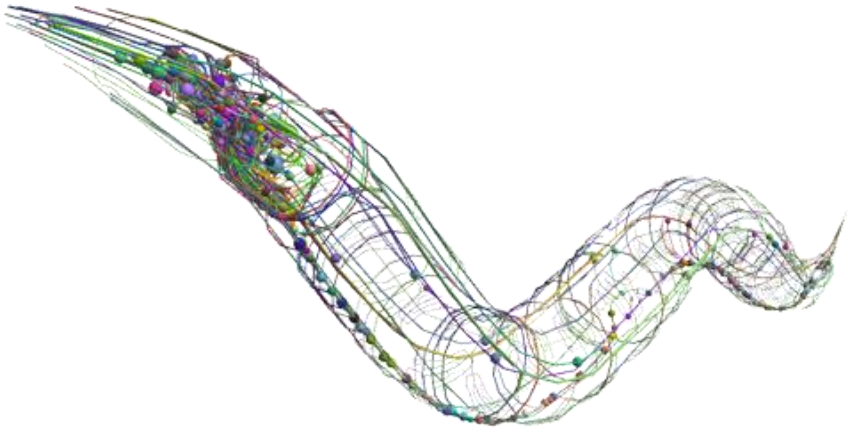

Figure 2. The entirely mapped nervous system of *C. elegans* consists of 302 neurons

(The image is cited from the http://openworm.org/science.html website)

For instance, animals experiencing the combined presence of sodium chlorine and food are later more likely to choose the salt-labeled site on the plate than the control counterpoint. Furthermore, exposure of adult nematodes to a chemoattractant in conjunction with food increases preference for the attractant. The association also works with negative effects: animals experiencing salt and garlic extract together later avoid salt, even if they were previously attracted to it. In addition, conditioning worms with an attractive odor (e.g. diacetyl or benzaldehyde) while being starved, leads to an association between the odor and starvation, thus worms will avoid the otherwise attractive odor. Worms are also able to remember these learned associations and the intensity and

frequency of the associated stimuli determine the length of the induced memory. The altered behavior lasts only for minutes (short-term memory) or for a couple of hours (intermediate-term memory), but repeated associations can increase the length of this changed behavior up to over a day (long-term memory) in a nematode life span scale of 2-3 weeks[22].

However, a similar conditioning of newly hatched worms during the L1 larval stage, in the critical (sensitive) period, forms an imprinted olfactory memory which is retrievable in 5-day adults[23]. Remy and Hobert in 2005 showed that *C. elegans* hatched in the combined presence of food and the chemoattractant benzaldehyde is more attracted to benzaldehyde, which implies the presence of food, even at 5 days of age, and increases the number of laid eggs in the presence of benzaldehyde even without food. Other studies have established that adult wild-type *C. elegans* that transiently pass through the stress-resistant dauer larval stage exhibit distinct gene expression profiles and life history traits, as compared to worms that bypassed this stage[24]. In addition, exposure to a chemoattractant in early life increases attraction to the same olfactory cue in adulthood, and the Alanine tRNA (UGC) plays a central role in this type of regulation of chemoattraction in *C. elegans*[25]. Moreover, recent studies reported that early life exposure to *Pseudomonas aeruginosa* PA14 infection or to the ascr#3 pheromone, respectively, gave rise to imprinted aversive behavior in adult worms[26,27]. These findings suggest that early experiences form a long-lasting neural representation and help the animal to avoid imminent threats of pathogenic attacks, starvation or population overcrowding.

1.4 Cytoprotective stress responses in *C. elegans*

Throughout its life, *C. elegans* encounters a number of adversities both in its natural habitat and under laboratory conditions. To overcome the hardships, besides behavioral avoidance, nematodes possess active physiological defense strategies, especially responding to insults when organismal integrity is injured. In response to various stresses, such as heat, drought, oxidants or toxins, worms induce evolutionarily conserved specific cellular stress responses (Table 1).

Table 1. *C. elegans* stress response genes induced by various stresses (cited from [Rodriguez M, Kammenga J.E, 2013][28] with modifications)

Type of stress	***C. elegans* gene**
heat-shock	*hsf-1*
oxidative	*pink-1, lrk-1, sod-1,-2,-3, gst-4*
heat-shock/oxidative	*daf-16*
nutrient	*daf-18*
hypoxia	*hif-1, vhl-1, egl-9*
osmotic	*gpdh-1, elt-2,-3, osm-12*
oxidative/electrophile/xenobiotic	*skn-1*
mitochondrial	*hsp-6, hsp-60, atfs-1*

Compared to the immediate behavioral response, these cytoprotective transcriptional or translational responses last for hours or even for days. They restore homeostasis by repairing damage and eliminating the cause (such as neutralizing a toxin), thereby provide increased stress tolerance, enhance immunity and promote longevity[28,29]. Accordingly, the function of cell protection is to eliminate harmful agents and repair the damage caused by them[30]. *C. elegans* responds to mitochondrial stress by activating the mitochondrial unfolded protein response (mtUPR) to buffer the mitochondrial folding environment, rewire the metabolic state and promote innate immunity and lifespan extension. The mtUPR is initiated by several modes of mitochondrial stress and activates, among other regulators, the ATFS-1 transcription factor, which upregulates the expression of nuclear genes, such as *hsp-6* encoding a mitochondrial chaperone[31].

In nematodes, as well as in mammals, a number of different molecular processes are responsible for recognizing and overcoming toxic stressors, such as the Nrf2 and FOXO pathways. In nematodes, the Nrf2 orthologue SKN-1 transcription factor is involved in the protection against xenobiotic and oxidative stresses[30]. SKN-1 is also expressed multiple tissues including the intestine and the ASI neuron. Nuclear translocation of SKN-1 is induced by caloric restriction, pathogenic attack and the INS/IGF-1 and TIR-1/PMK-1 pathways, resulting in increased oxidative stress resistance, enhanced immunity and induction of systemic detoxification processes[32,33]. Direct targets of SKN-1 (among

others) are *gst-4* and *gcs-1* genes, encoding for the drug-metabolizing enzyme glutathione S-transferase 4 (GST-4) and the phase II detoxification enzyme gamma-glutamyl cysteine synthetase (GCS-1), respectively[34]. SKN-1 interacts with a number of stress-related pathways and regulators, including DAF-16 and the *C. elegans* heat shock transcription factor ortholog HSF-1, to modulate cytoprotective gene expression patterns[35].

A key oxidative and metabolic stress response regulator in *C. elegans* is the FOXO ortholog DAF-16 transcription factor. It is widely expressed in the germline, in somatic non-neuronal tissues such as the hypodermis, gut and muscle as well as in neurons, such as the ventral nerve cord. In normal conditions, DAF-16 is localized in the cytosol and in response to starvation, desiccation, heat stress or oxidative agents it is activated by nuclear translocation[35]. DAF-16 is responsible for activating genes involved in longevity and resistance to multiple stresses, thereby helping to survive heat shock and to elicit oxidative stress responses[36].

1.5 Connections between cellular and behavioral stress responses in *C. elegans*

A few pieces of experimental evidences show connection between cellular stress responses and neuronal circuitries in *C. elegans*. For instance, the AFD thermosensory neuron in addition to the regulation of thermotaxis, also facilitates the activation of the heat shock transcription factor HSF-1 during heat shock, which augments survival by upregulating the expression of cytoprotective molecular chaperones[37]. In contrast, disruption of essential cellular processes in somatic cells both activate cytoprotective responses and induce an associative aversive behavior which requires the cellular stress activated JNK-like kinase pathway[38].

Findings that neuronal signals promote cytoprotective responses and that cellular stress signals form the basis of learned behavior, indicate mutual regulation of non-neuronal and neuronal responses to the expected danger. Recent studies in *C. elegans* demonstrated that pathogen- and toxin-induced stresses simultaneously trigger cytoprotective responses and aversive behavior[38,39]. This proof suggests that induction of systemic cytoprotective molecular defenses may influence stress-induced aversive behavior and learned behavioral decisions. Other studies have established that food-derived volatile odors, benzaldehyde and diacetyl are attractive to *C. elegans* at low concentrations but repulsive at high concentrations[40,41]. These odors contain both the

chemosensory cue as well as a dual, attractive and aversive property. However, it is unknown whether non-neuronal cellular mechanisms underlie the switch in odor concentration-dependent olfactory preference and thus the change in behavior.

Together with the demonstration of imprinted aversion for pathogen and pheromone exposure, these findings raise several questions. Does the exposure to adversities in early life trigger aversive behavior and cytoprotective responses, respectively? Is imprinted aversion a common consequence of stress experienced in the critical period? If cytoprotective transcriptional responses develop under early life stress, can they be induced by stress-associated sensory cues in adulthood? Are there peripheral cellular processes behind the distinct behaviors given to different concentrations of odors that can influence behavioral decisions? How can these putative mechanisms determine the development of different behavioral responses?

2 Objectives

In the course of my doctoral work, using *C. elegans* as an experimental model, I aimed to:

1.a. Characterize the immediate/early behavioral and cytoprotective responses elicited by early life toxic stress during the critical period.

1.b. Investigate whether an imprinted aversive memory forms and aversive behavior can be retrieved in adulthood by sensory cues that co-occurred with early life stress.

1.c. Examine the imprinting of cytoprotective stress responses and detect whether the expression of cytoprotective genes can be activated by stress-associated sensory cues in adulthood.

2. Elucidate how the adult experience of toxic stress and the different efficiency of cytoprotective defenses influence nematodes in making optimal behavioral decisions when re-encountering stress-associated olfactory cues.

3 Methods

Methods not described in associated co-authored or first-authored publications are detailed in the following section. Methods already published with my authorship[42,43] are excluded from this thesis, following the guidance of the Doctoral School.

Experimental setup belonging to Figure 11.

Imprinting training

3 cm NGM plates were seeded with 50 µl OP50 bacteria in the middle of the plate and left for overnight. Before the experiment, 20 µl 50 µg/ml antimycin or the solvent control, ethanol was overlaid on the bacteria lawn. Then, 15-20 *hsp-6::GFP* transgenic worms were placed on the plates and allowed to lay eggs for 4 hours. Hermaphrodites were removed and plates were incubated at 20°C for 24 hours during the L1 larval stage. After imprinting training worms were washed and placed on 9 cm diameter NGM plates seeded with *Bacillus subtilis* and grown to adulthood. 24 hours prior the examination of reporter expression animals were placed onto new *Bacillus subtilis* seeded plates to provide a constant amount of food. Simultaneously, 10-12 drops of OP50 (or *Bacillus subtilis* bacteria as a control) were placed on the lid of the plates to expose the OP50 odor and prevent direct contact of worm with OP50.

GFP reporter expression by fluorescence microscopy

Adult worms were placed on a 2% agarose pad and immobilized with 25 mM NaN_3 dissolved in M9 buffer. Images were taken by a Nikon Eclipse E400 microscope with Diagnostic Instruments SPOT model 1.5.0. camera using a GFP fluorescent filter. Images were captured at 10x and 20x magnification. GFP expression levels were evaluated by ImageJ software.

Statistical analysis

Reporter expressions were analyzed by the non-parametric Kruskal-Wallis test using STATISTICA program. Data were expressed as median and first and third quartiles (box) and tenth to 90th percentiles (whiskers) in reporter expression experiments, which displayed non-normal distribution. Statistical levels of significance are as follows: *$p<0.05$; **$p<0.01$; ***$p<0.001$.

4 Results

The text of the two published articles is the most expedient presentation of the results, thus this chapter is based on the literal text of the results section of our articles entitled *A cellular defense memory imprinted by early life toxic stress*[42] and *Toxic stress-specific cytoprotective responses regulate learned behavioral decisions in C. elegans*[43] with minor changes.

4.1. Early life stress

In the critical period, the detrimental effect (stress) induced by the simultaneous presence of various toxins damaging essential cellular functions are employed in the presence of OP50 food bacteria, in which case the toxic effect and the organoleptic properties of the food (smell, taste) is simultaneously experienced by the animals. If these memories (associations between the smell of a particular food and the toxic effect) are imprinted, then in adulthood the appearance of the OP50 smell will trigger a behavioral response to stress in the absence of the toxin. We employed a *C. elegans* nematode model for early life stress by exposing worms to toxic compounds, such as antimycin A and paraquat during the L1 stage and investigated behavioral and cytoprotective responses during development and in adulthood.

The first step of our work was the selection of stress effects in the L1 age, the setting of a behavioral test method and the characterization of early behavior. Then we mapped the adaptive molecular responses and examined the imprinting of responses to sensory cues associated with toxic stress that compromised the integrity of the individual.

4.1.1 Early life toxic stress induces food aversion behavior

Adult *C. elegans* exhibits an aversive behavioral response to pathogen-derived sensory cues after an earlier infection that occurred either in adulthood or in the L1 larval stage[44, 26]. Likewise, both adults and L3-L4 larvae cease feeding and leave the toxin-contaminated bacterial lawn[38]. We tested whether L1 larvae are able to exhibit aversive behavior in response to toxic stresses by exposing them to a combination of *E. coli* OP50 food source overlaid by antimycin (AM) or paraquat (PQ). AM is a bacterial toxin, an

inhibitor of complex III of the mitochondrial electron transport chain, while PQ is a synthetic herbicide, a reactive oxygen species (ROS) generator[45,46]. Although their chemical structures and mechanisms of action are different, both toxins cause severe damage and compromise mitochondrial energy production. Worms were hatched on *E. coli* OP50 supplemented with the respective toxins and their food leaving behavior was monitored after 24 hours, at the end of the critical period of *C. elegans* (Fig. 3a).

We observed that naive L1 larvae remained on the lawn, whereas exposure to either AM or PQ, respectively, induced robust food leaving behavior (Fig. 3b and c). In addition, both toxin exposures resulted in slight growth retardation (cf. the size of nematodes in Fig. 3b and c), consistent with the developmental effects of the toxic stress. Nevertheless, L1 larvae are already able to sense toxicity, make a behavioral decision and avoid the otherwise nutritious bacterial lawn. Moreover, dose response curves (Fig. 3d and e) demonstrate that the decision making is proportional to the extent of toxic stress, indicating an ability to carry out an adaptive behavioral response.

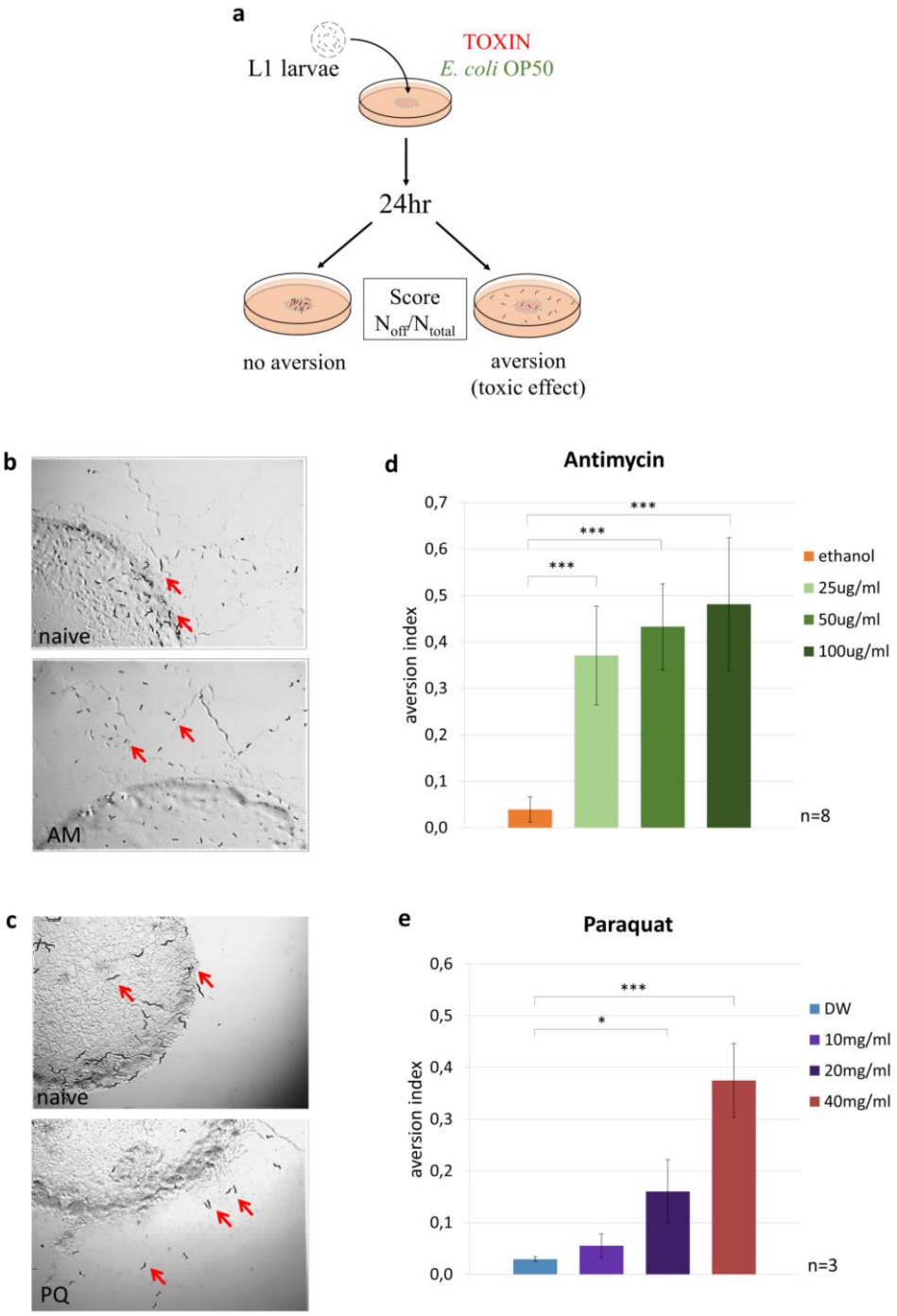

Figure 3. Early life toxic stresses trigger food avoidance behavior

(**a**) Schematic of early life toxin exposure and food aversion assay. Representative images of the effect of antimycin A (AM) (**b**) or paraquat (PQ) (**c**) exposure on food aversion phenomena after 24 hr. Red arrows are pointing to the L1 larvae. Quantification of food leaving behaviors in response to AM (**d**) or PQ (**e**). n = number of independent assays. p values were generated by one-way ANOVA followed by Tukey's HSD post-hoc correction. *p<0.05, **p<0.01, ***p < 0.001.

4.1.2 Early life toxic stresses stimulate specific cytoprotective responses

Exposure to toxic stresses triggers a highly conserved array of cytoprotective molecular stress responses that promote organismal survival[47]. Therefore, we employed several GFP reporters to monitor the activation of stress-responsive transcriptional programs. Mitochondrial dysfunction leads to the induction of the mitochondrial unfolded protein response including *hsp-6*, the mitochondrial paralog of nematode *hsp-70*[48] and ortholog of mammalian *grp*-75[49]. Indeed, both AM and PQ exposure enhanced the fluorescence of the mitochondrial stress marker *hsp-6::GFP* in transgenic L1 larvae (Fig. 4a,d and b,e).

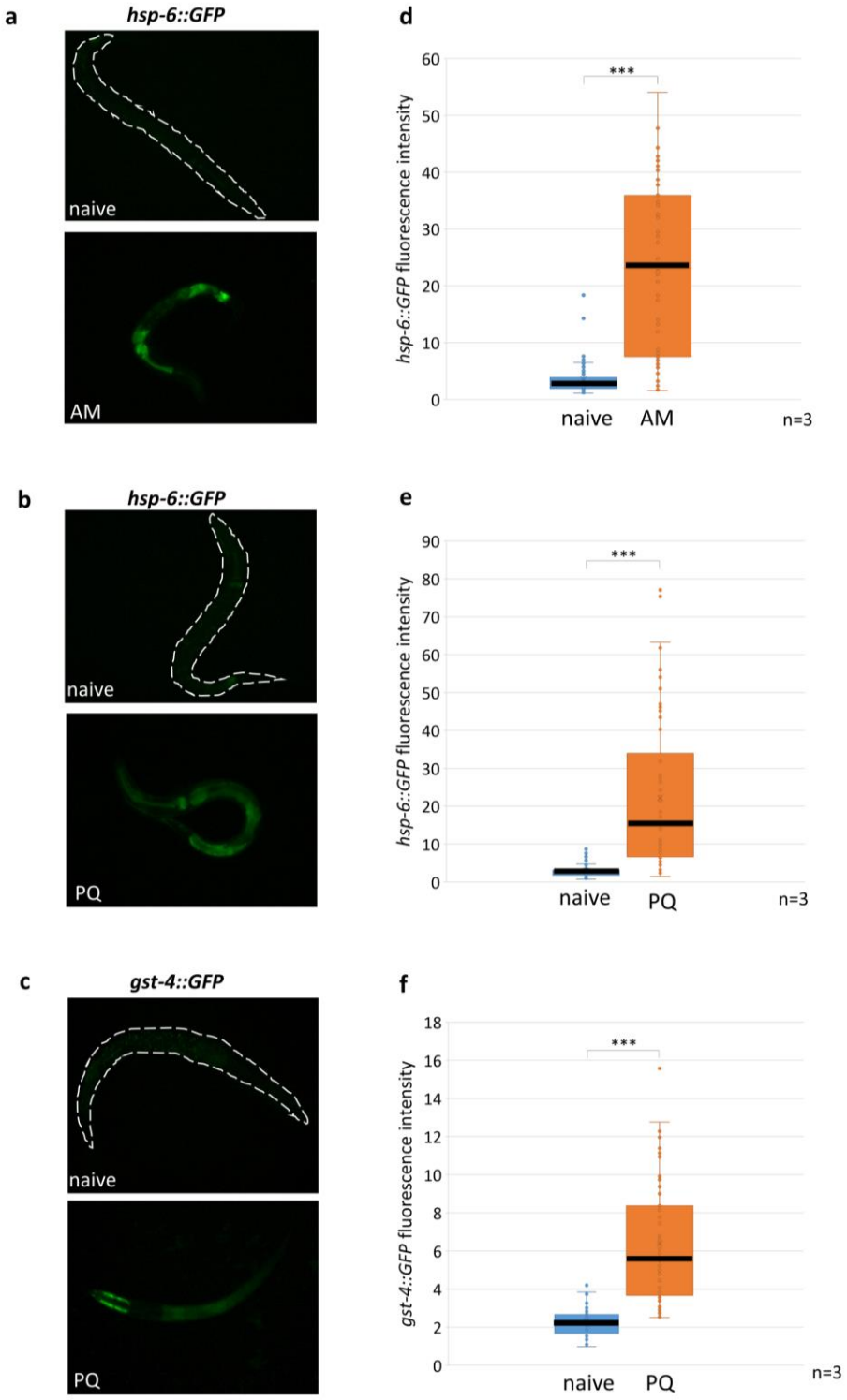

Figure 4. Toxin exposure at the first larval stage induces specific stress and detoxification responses

Epifluorescent microscopic images (**a-c**) representing, and quantification of (**d-f**), the effect of AM exposure on *hsp-6::GFP* (**a**, **d**) and PQ exposure on *hsp-6::GFP*

(**b**, **e**) and *gst-4::GFP* (**c**, **f**) reporter expression. GFP expression was imaged and quantified immediately after training. Boxes represent median and first and third quartiles and whiskers represent tenth to 90th percentiles. n = number of independent assays. p values were generated by the non-parametric Kolmogorov-Smirnov test. *p<0.05, **p<0.01, ***p < 0.001.

Stresses might also upregulate drug detoxification responses that neutralize and help excrete toxic chemicals. Assaying two enzymes of glutathione metabolism we found that PQ, but not AM, induced the phase II glutathione S-transferase *gst-4::GFP* (Fig. 4c and f and Fig. 5a), whereas none of them affected the expression of the glutamate cysteine ligase *gcs-1::GFP* (Fig. 5b).

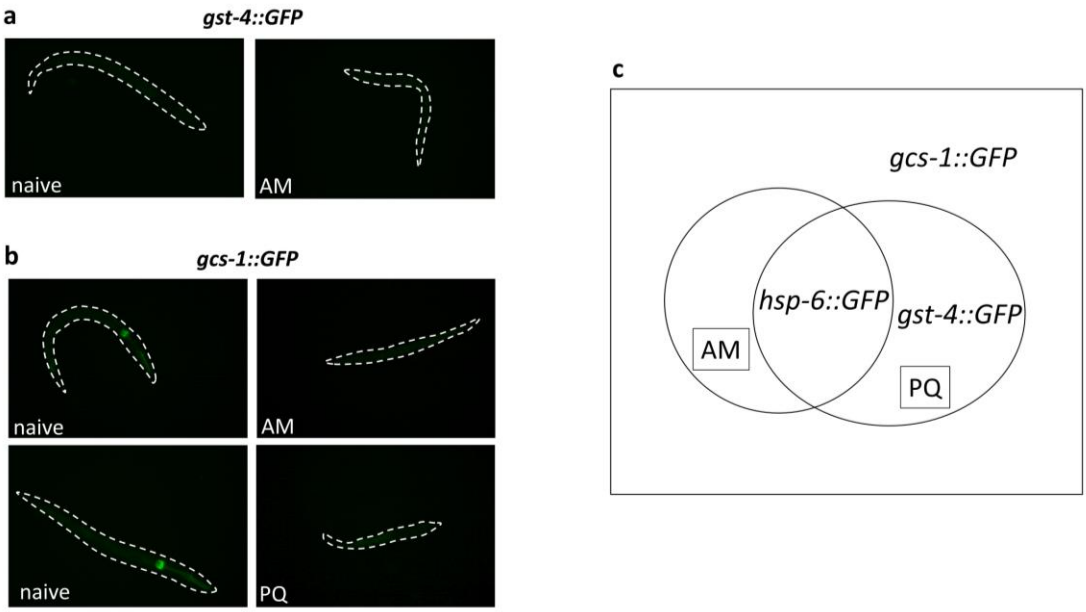

Figure 5. Specificity of toxin induced stress and detoxification responses

AM does not induce *gst-4::GFP* (**a**), whereas both AM and PQ appear to slightly inhibit the baseline expression of *gcs-1::GFP* (**b**), reporters in L1 larvae. To confirm the lack of activation, both AM (100 µg/ml) and PQ (40 mg/ml) was employed at the highest concentration that induced aversive behavior. (**c**) Venn diagram showing the AM and PQ induced genes in L1 larvae.

Hence, early life stress induced by AM and PQ respectively, gives rise to specific and overlapping cytoprotective responses in L1 larvae (Fig. 5c).

4.1.3 Lack of early life stress associated aversive memory in adulthood

By the first day of adulthood, toxin-treated worms caught up with mock-treated controls, compensating for the developmental delay caused by early life AM and PQ exposure. Next, we asked whether the bacterial sensory cues experienced during the toxic insult early in life could evoke an avoidant behavior in adults. To this end, naive and toxin exposed L1 larvae were washed after 24 hours, transferred to the non-pathogenic *Bacillus subtilis* NRS 231 (BS) strain and grown till adulthood. We employed BS for several reasons. Although *C. elegans* has a very delicate chemosensory system, the sensory cues of the Gram positive BS strain are largely different from those of OP50 allowing a clear distinction between them. Moreover, BS has similar nutritive value, as worms raised on BS appear as healthy as those raised on OP50. Finally, BS is equally attractive compared to OP50 (see choice of naive worms on Fig. 6b and c). We tested adult worms in a classic olfactory food choice assay, placing them in the middle of a plate containing OP50 and BS spots at the opposite sides and allowing them to explore for an hour (Fig. 6a). In this experiment, due to the short incubation time, animals choose between the two diets based on olfactory stimuli. We observed no significant difference between the choice of OP50 and BS, which suggest that, AM and PQ treatment at an early age did not affect adult food preference (Fig. 6b and c).

To ensure that these results were not due to any specificity involving the BS strain, toxin exposed worms were grown to adulthood in non-pathogenic *Pseudomonas fluorescens* NCTC 10038 (PF) bacteria. Similarly, we observed no difference between the food preference of AM and PQ treated, compared to naive worms (Fig. 6d and e).

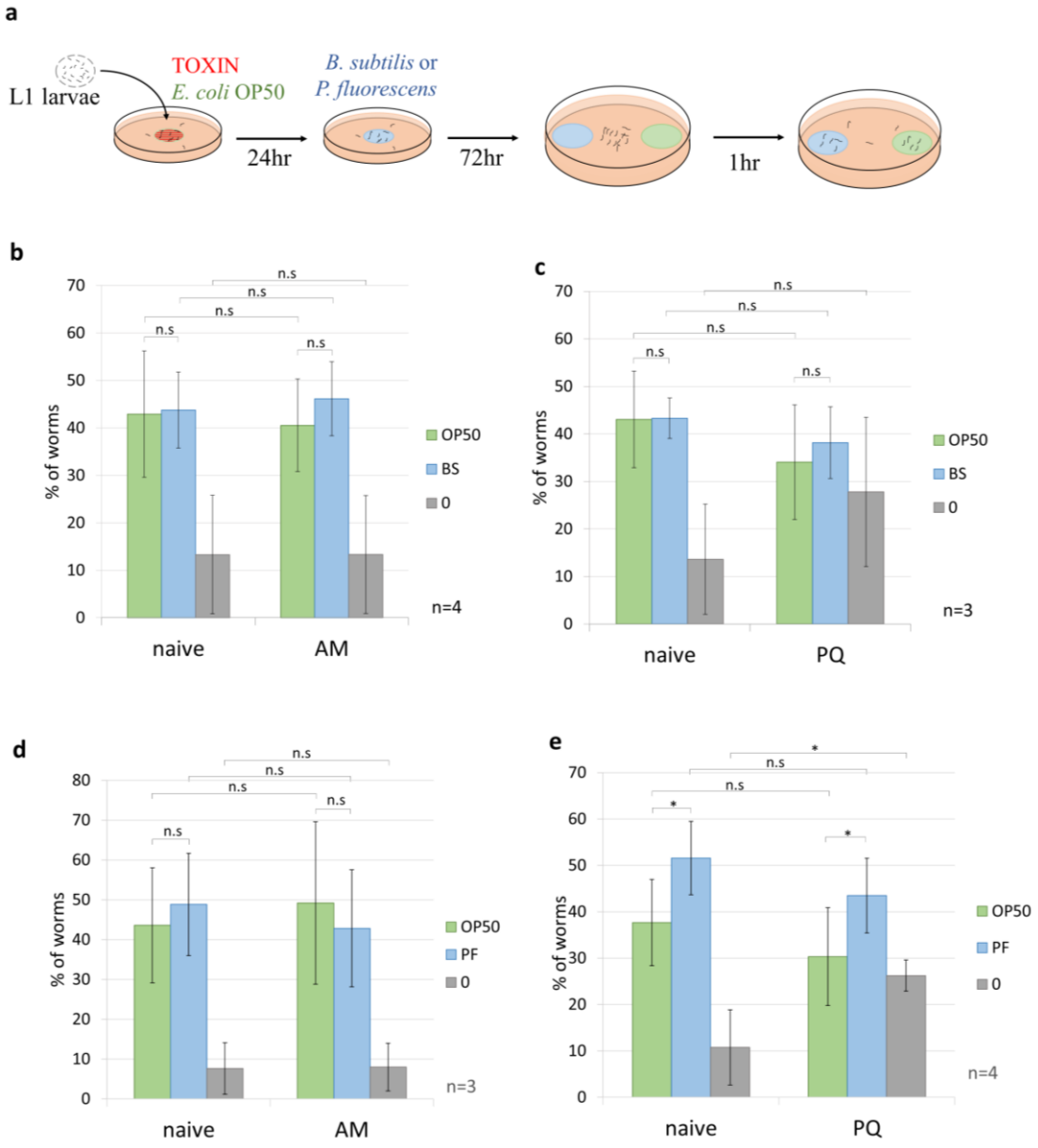

Figure 6. Adult worms do not avoid food olfactory cues experienced during early life toxic stress

(**a**) Schematic representation of the experimental setup of the imprinting paradigm using an olfactory food choice test. Quantification of olfactory food choice of adult worms (grown on *Bacillus subtilis*) between OP50 and *Bacillus subtilis* after early life exposure to AM **(b)** or PQ **(c)** in the presence of OP50. Food choice of adult animals (grown on *Pseudomonas fluorescens*) between OP50 and *Pseudomonas fluorescens* upon exposure to AM **(d)** and PQ **(e)** in the presence of OP50 in early life. Worms were placed in the middle of the plate in the olfactory food choice

assay. Choice was quantified by scoring worms on *E. coli* OP50, *Bacillus subtilis* (BS) or on the empty agar surface (0). Choice was quantified after 1 hour by scoring worms on *E. coli* OP50, *Bacillus subtilis* (BS) or *Pseudomonas fluorescens* (PF) lawn or on the empty agar surface (0). n = number of independent assays. p values were generated by two-way ANOVA followed by Fisher's LSD post-hoc correction. n.s, not significant, *p<0.05, **p<0.01, ***p < 0.001.

After that, we wanted to make sure that the lack of difference in adult food preference between the toxin-treated and control groups is not due to the methodology used. In the above experimental conditions, the choice was based on predominantly olfactory cues. We also used a mixed population of worms that showed toxin-induced aversion or remained on the lawn. We hypothesized that worms that previously avoided the lawn as larvae might have a higher tendency to avoid it as adults, hence avoidant and non-avoidant larvae were separately grown into adulthood. Also, we aimed to better mimic the original sensory experience and to eliminate the negative impact of hunger on the choice in case animals might leave the OP50 lawn. Therefore, we developed a novel, food leaving-food choice assay. Here, worms were hatched on OP50 bacteria in the presence of AM or PQ. After 24 hours avoidant (OFF) and non-avoidant (ON) L1 populations were separated and animals were grown on *Bacillus subtilis* similarly to the above experimental setup. In this case, adult worms were placed on plates containing both OP50 and *Bacillus subtilis* bacteria at the opposite ends, with the difference that in this experiment we placed the animals on the OP50 bacterial spot. After allowing the worms for 20 hours to explore, we counted the animals remaining on the OP50 bacterial spot, leaving OP50 bacteria and choosing *Bacillus subtilis* bacteria (Fig. 7a). In this experiment, due to the longer incubation time of 20 hours, animals decided which food to choose based on not only olfactory but also gustatory stimuli, similar to the early toxin-induced food aversion test. In this assay, naive worms displayed a much greater preference for OP50 compared to BS probably because they were more likely to remain on the food source available (Fig. 7b and c).

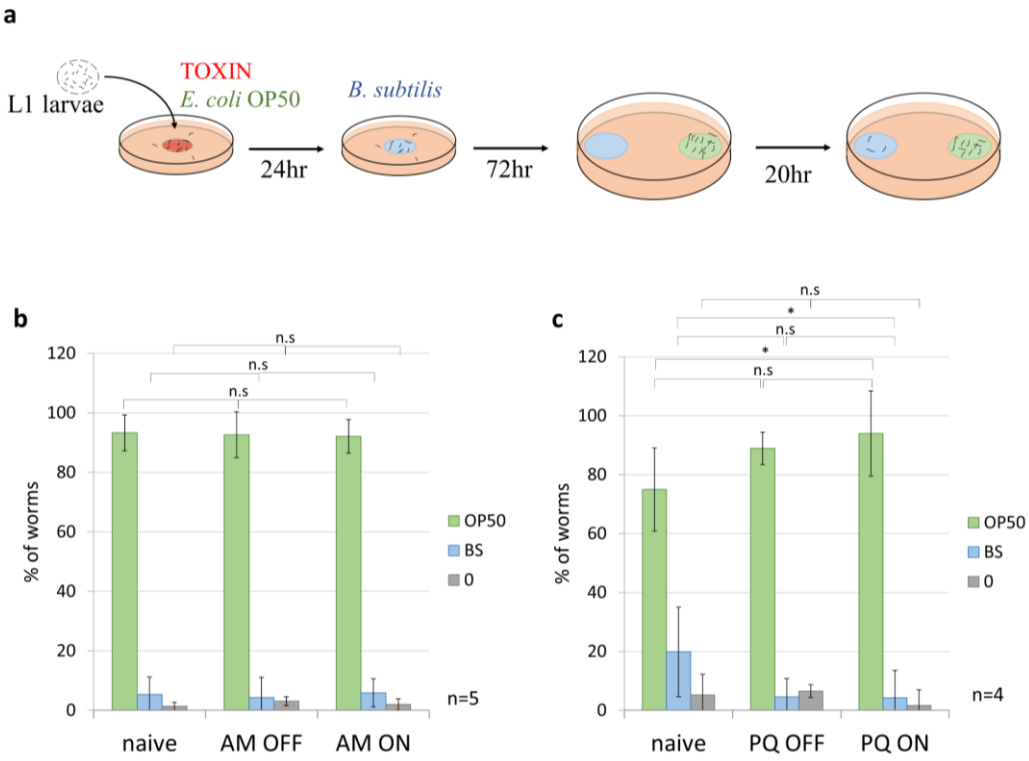

Figure 7. Food preference of adult worms is not affected by food sensory cues associated with toxic stress in early life

(a) Schematic of the experimental setup of the imprinting paradigm using a food leaving-food choice assay. Quantification of adult food leaving-food choice after early life exposure to AM (**b**) or PQ (**c**), respectively, in toxin-avoidant (OFF) or non-avoidant (ON) groups of worms. Worms were placed on the *E.coli* OP50 lawn in the food leaving-food choice assay. Choice was quantified after 20 hours by scoring worms on *E. coli* OP50, *Bacillus subtilis* (BS) or on the empty agar surface (0). n = number of independent assays. p values were generated by two-way ANOVA followed by Fisher's LSD post-hoc correction. n.s, not significant, *p<0.05, **p<0.01, ***p < 0.001.

Importantly, neither worms that avoided (OFF), nor those that remained on (ON) the toxic lawn during the critical period, altered their preference as adults towards OP50 sensory cues compared to each other or naive nematodes (Fig. 7b and c). Thus, early life stresses induced by AM and PQ do not appear to imprint an associative aversive behavior.

4.1.4 Early life stress associated sensory cues revive an imprinted, stress-specific molecular defense memory in adulthood

Next, we were interested whether a re-exposure of adult worms to the sensory cues co-occurring with the respective early life stresses would induce the corresponding cytoprotective responses. Therefore, we examined the *hsp-6::GFP* and *gst-4::GFP* reporter expressions of control and toxin-treated adult animals after re-exposure to OP50 bacteria. In this experiment, after the first 24 hours of toxin treatment, worms were grown to adulthood on *Bacillus subtilis* as described above, and then animals were placed onto OP50 or *Bacillus subtilis* as a control. Reporter expressions were examined 24 hours later (Fig. 8).

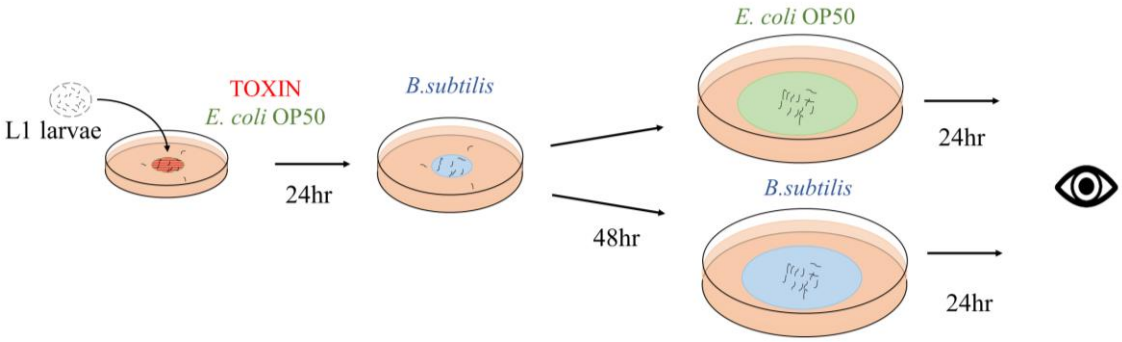

Figure 8. Schematic of the experimental setup for retrieving early life cytoprotective memory in adult worms.

hsp-6::*GFP* fluorescence was comparable in naive and AM treated worms on BS lawn as well as in naive worms on OP50 showing that reporter expression of adults was neither affected by a previous AM exposure nor by the re-exposure to OP50 alone. Strikingly, the re-encounter with OP50 of adult worms exposed to AM in the L1 stage induced a marked *hsp-6::GFP* expression predominantly in the tail region (Fig. 9a and d). Although *hsp-6::GFP* fluorescence remained higher in PQ treated worms maintained on *Bacillus subtilis*, OP50 sensory cues significantly stimulated it throughout the whole body (Fig. 9b and e). Likewise, a combination of early life PQ exposure and a re-encounter with OP50 significantly increased *gst-4*::*GFP* expression, especially in the proximal head-pharynx region (Fig. 9c and f).

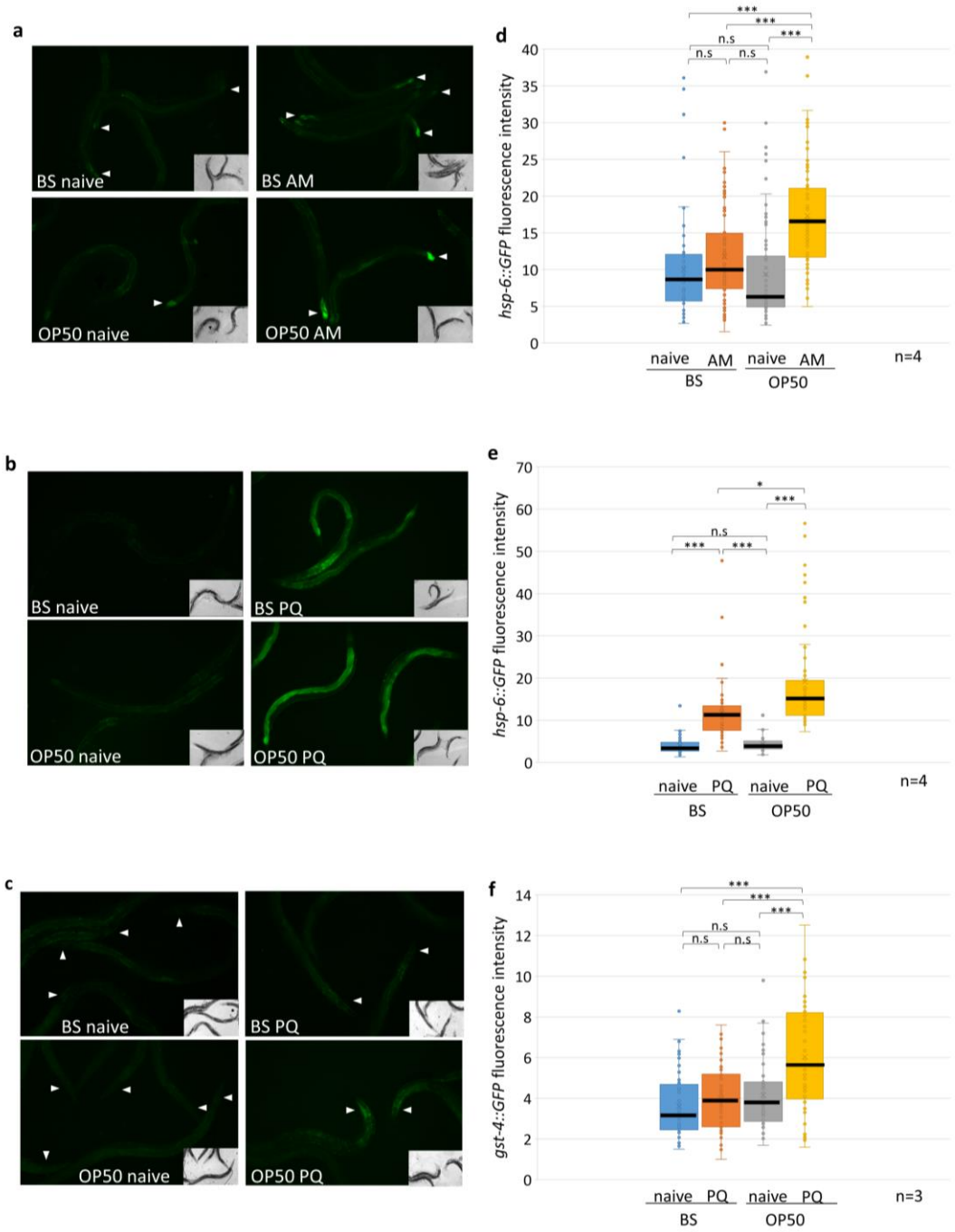

Figure 9. Reactivation of cytoprotective responses in adult worms by early life stress associated sensory cues

Epifluorescent microscopic images (**a**, **b**, **c**) representing, and quantification of (**d**, **e**, **f**), the effect of re-encountering *E. coli* OP50 on the expression of *hsp-6::GFP* in AM treated (**a**, **d**), as well as *hsp-6::GFP* (**b**, **e**) and *gst-4::GFP* (**c**, **f**), in PQ

treated adult nematodes, respectively. Boxes represent median and first and third quartiles and whiskers represent tenth to 90th percentiles. n = number of independent assays. Statistics: p values were obtained by the non-parametric Kruskal-Wallis test. n.s, not significant, *p<0.05, **p<0.01, ***p < 0.001.

One of the criteria of imprinting is that memories are imprinted in the critical (sensitive) period, the L1 stage in *C. elegans*. Therefore, we examined and showed that AM and PQ treatments in the L2 developmental stage do not result in elevated *hsp-6::GFP* and *gst-4*::*GFP* expressions in adult worms by OP50 re-exposure (Fig. 10a and c). In contrast, in control experiments, as already described, when AM or PQ treatment was applied during the L1 larval stage, re-exposure of adult worms to OP50 increased the expression of *hsp-6::GFP* and *gst-4::GFP* reporters (Fig. 10b and d). We note, that worms treated in L2 larval stage show higher reporter expressions either on BS or on OP50 lawn compared to worms treated in L1 stage. These residual expressions are probably due to the one-day less resting time after toxin treatment, hence the turnover of toxin-induced reporter GFP levels was not complete.

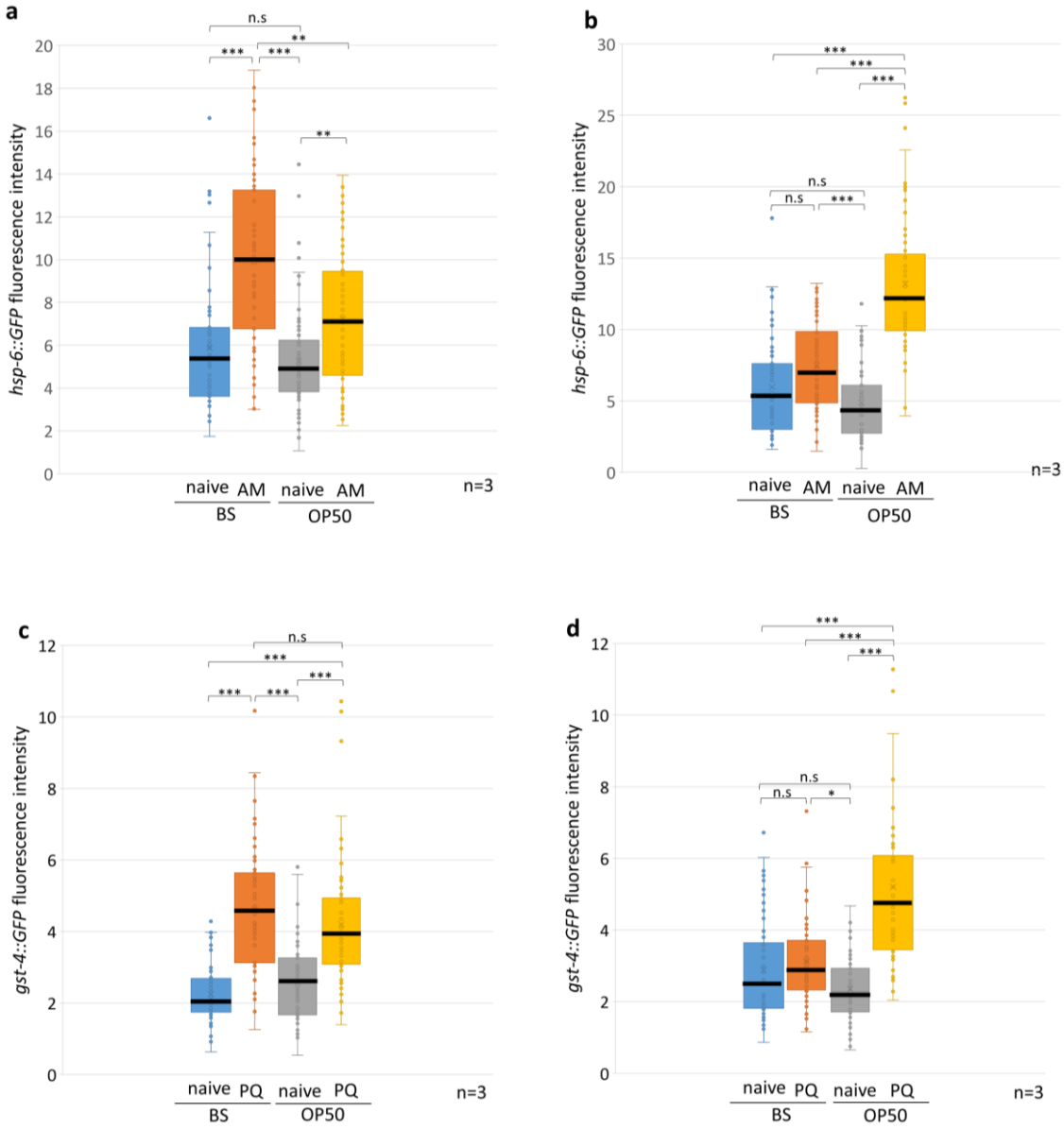

Figure 10. Imprinting of early life stress induced cytoprotective memory occurs in the L1 stage

Exposure to OP50 in animals treated with AM or PQ in the L2 larval stage does not increase *hsp-6::GFP* **(a)** or *gst-4::GFP* **(c)** expression in adulthood. While in control worms treated in L1 stage, re-encountering *E. coli* OP50 increases the expression of both reporters **(b, d)**. Boxes represent median and first and third quartiles and whiskers represent tenth to 90th percentiles. n = number of

independent assays. p values were generated by the non-parametric Kruskal-Wallis test. n.s, not significant, *<0.05, **p<0.01, ***p < 0.001.

These findings demonstrate that sensory cues experienced during toxin exposure, during but not after the critical period, re-engage stress-specific cytoprotective molecular responses in adult nematodes.

Finally, we investigated whether stress-associated olfactory cues, i.e. the bacterial odors *per se* are sufficient to retrieve the imprinted cytoprotective response. Therefore, adult worms were exposed only to the odor of OP50 during memory recall. Our results show that in worms treated with AM in the presence of OP50 in L1 larval stage, OP50 odor exposure also induces *hsp-6::GFP* expression in adulthood (Fig. 11a and b).

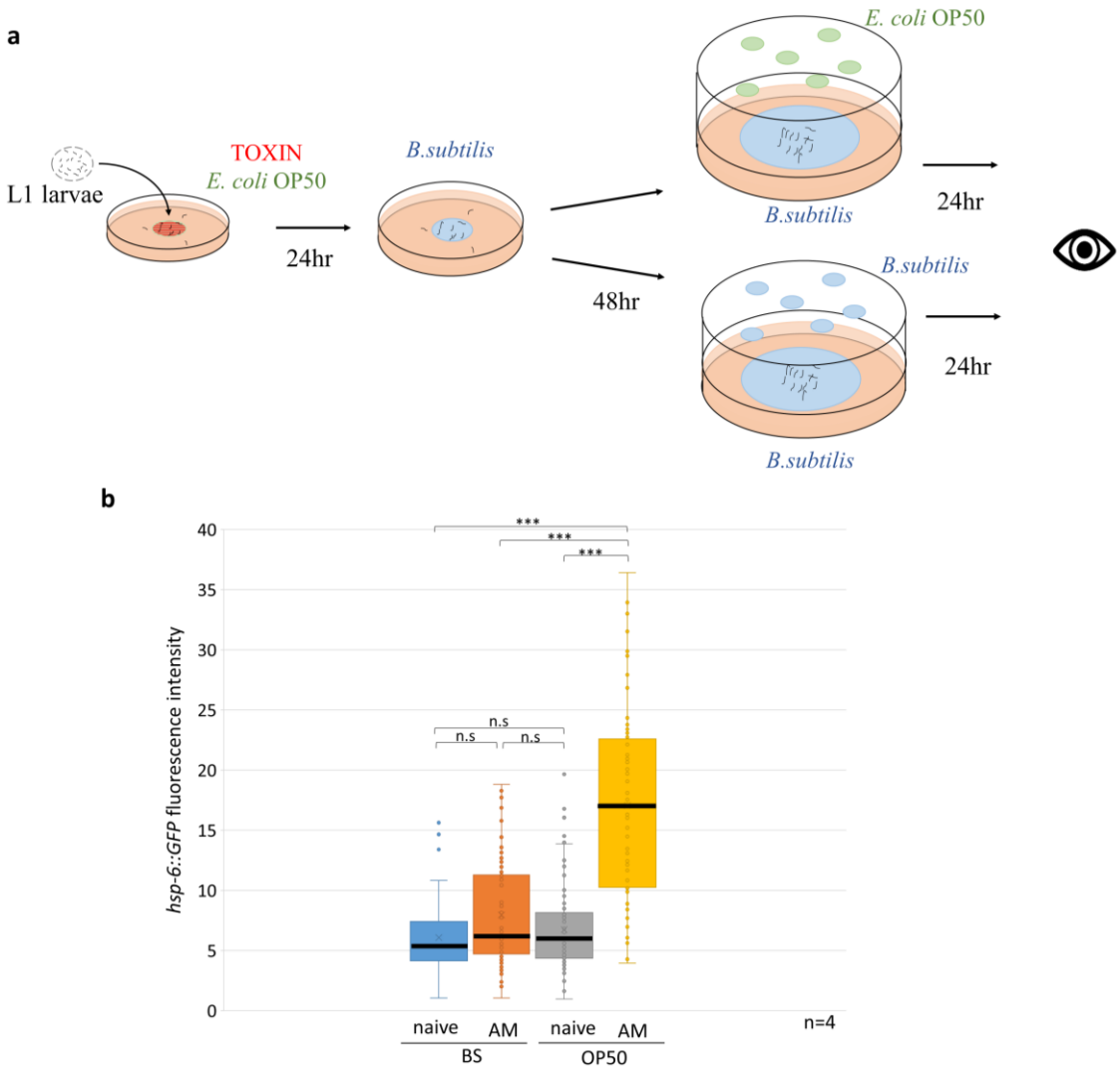

Figure 11. Revival of cytoprotective responses in adult worms by the odor of OP50 associated with stress in early life

(**a**) Schematic of the experimental setup for recalling early life cytoprotective memory in adult worms by only the odor of OP50 bacteria. Quantification of the effect of re-encountering *E. coli* OP50 odor on the expression of *hsp-6::GFP* in AM treated adult worms (**b**). Boxes represent median and first and third quartiles and whiskers represent tenth to 90th percentiles. n = number of independent assays. Statistics: p values were obtained by the non-parametric Kruskal-Wallis test. n.s, not significant, *p<0.05, **p<0.01, ***p < 0.001.

This finding shows that the induction of cytoprotective responses neither requires a direct contact of *C. elegans* with bacteria eliciting a potential non-neuronal metabolic effect nor the involvement of tactile or gustatory cues in neural memory formation. The evidence on the involvement of olfactory cues specifically identifies this phenomenon as olfactory imprinting. Thus, early life stress gives rise to an imprinted cellular defense memory mediated by the nervous system.

4.2 Efficient or deficient cellular defenses generate relevant learned behaviors to stress-associated olfactory cues

In addition to the involvement of cytoprotective responses in imprinting, we also investigated in adult worms the role of cytoprotective responses in shaping learned behavioral decisions. *C. elegans* develops aversive behavior towards initially attractive pathogenic or toxic bacteria through a process called avoidance learning, based on associations between the internal experience of stress and the co-occurring sensory cues[38,50,39]. According to the results of my colleague Gábor Hajdú, in *C. elegans* the undiluted odorants benzaldehyde and diacetyl (refer to *concentratus* BA and *concentratus* DA, ccBA and ccDA) both induce a concentration- and time-dependent development of food aversion which is a consequence of their toxic effects. In contrast to the aversive effect of these undiluted odorants, *C. elegans* is attracted to diluted, 1% concentrations of BA and DA as both volatiles in low concentration implicate bacterial food[43]. This dual property makes these odorants a suitable tool to study the link between cytoprotective stress responses and behavior. Hajdú found that ccBA and ccDA exposures caused

paralysis and also death in a dose- and time-dependent manner. He observed that a preconditioning exposure with ccBA induced specific cytoprotective responses. These cytoprotective responses reduced aversion to a subsequent high-dose odor re-exposure and inhibition of them restored aversion. On the other hand, in case of DA, effective cytoprotection did not appear to develop and preconditioning with ccDA enhanced aversion to a subsequent ccDA exposure. Accordingly, his results suggested that the efficiency of cellular stress defenses determined the behavioral response to a toxic effect.

Based on these results, we aimed to find out how the previous experiences of odor toxicity and different efficiency of physiological protection influenced nematodes in decision making when they re-encountered the olfactory cues that were previously present during toxic stress. To explore this, we investigated worms' behavior in three different behavioral assays (chemotaxis, food aversion and odor choice) towards attractive, 1% concentrations of BA and DA odors after preconditioning with undiluted, toxic concentrations of BA and DA, respectively (Fig. 12a). We observed that ccDA-preconditioned worms showed a significantly reduced chemotaxis to 1% DA compared to naive worms (Fig. 12b). In food aversion assays, more than 60% of the worms chose to leave the bacterial lawn in the presence of ccDA. Importantly, after ccDA preconditioning approximately half of this faction vacated the food source containing the DA olfactory cue (Fig. 12c). In an odor choice assay, we examined how prior stress affects worms' decision if they are offered to choose between 1% DA and the also attractive 1% BA. After preconditioning with ccDA the odor preference of worms shifted almost completely towards BA, thus confirming that 1% DA become aversive (Fig. 12d). Potential mechanisms reducing DA preference might include olfactory adaptation (decreased signal processing in the chemosensory neuron) or the non-associative learning habituation (reduced behavioral response to the irrelevant or familiar sensory cue). Adaptation to olfactory cues usually occurs during starvation however, in our experiments the presence of food during preconditioning inhibits habituation and greatly represses adaptation[51]. Even if worms are able to adapt to DA, a comparison of the extensive food avoidance and the decrease in chemotaxis suggest that the observed behavioral changes are primarily caused by the learned avoidance of the expected threat.

In contrast, in chemotaxis tests where worms could choose between BA and vehicle, ccBA-preconditioned animals maintained their chemotaxis and were equally attracted to

1% BA as their naive counterparts (Fig. 12e), indicating that ccBA-preconditioning did not cause adaptation to BA. Furthermore, although 60% of the worms left the bacterial lawn in the presence of ccBA, after ccBA-preconditioning almost all the worms remained on the lawn in the presence of 1% BA (Fig. 12f). However, it is remarkable that in the odor choice assay ccBA-preconditioned worms showed an increased preference of DA compared to BA (Fig. 12g). Altogether these results indicate that worms exhibit selective avoidance towards the diluted odorant experienced during toxic stress, demonstrating the development of learned avoidance of the specific olfactory cue. In line with this, positive learning indices show that ccDA- and ccBA-preconditioned worms learn to avoid both stress-related odors, but they show different degrees of avoidance consistent of the history of experienced stress (Fig. 12h). The strong lawn avoidance of ccDA-preconditioned worms in the presence of DA suggests an anticipation of stress represented by the DA cue that is dominant over the nutritive, attractive qualities of food. In case of BA, the lower learning index, the maintenance of chemotaxis and the remaining of worms on the bacterial lawn suggest a resiliency in learned behavioral decisions. These results are in accordance with the formation of avoidant or tolerant learned behaviors, which appear to depend on the previous internal experience of stress, originated from efficient or deficient cytoprotection.

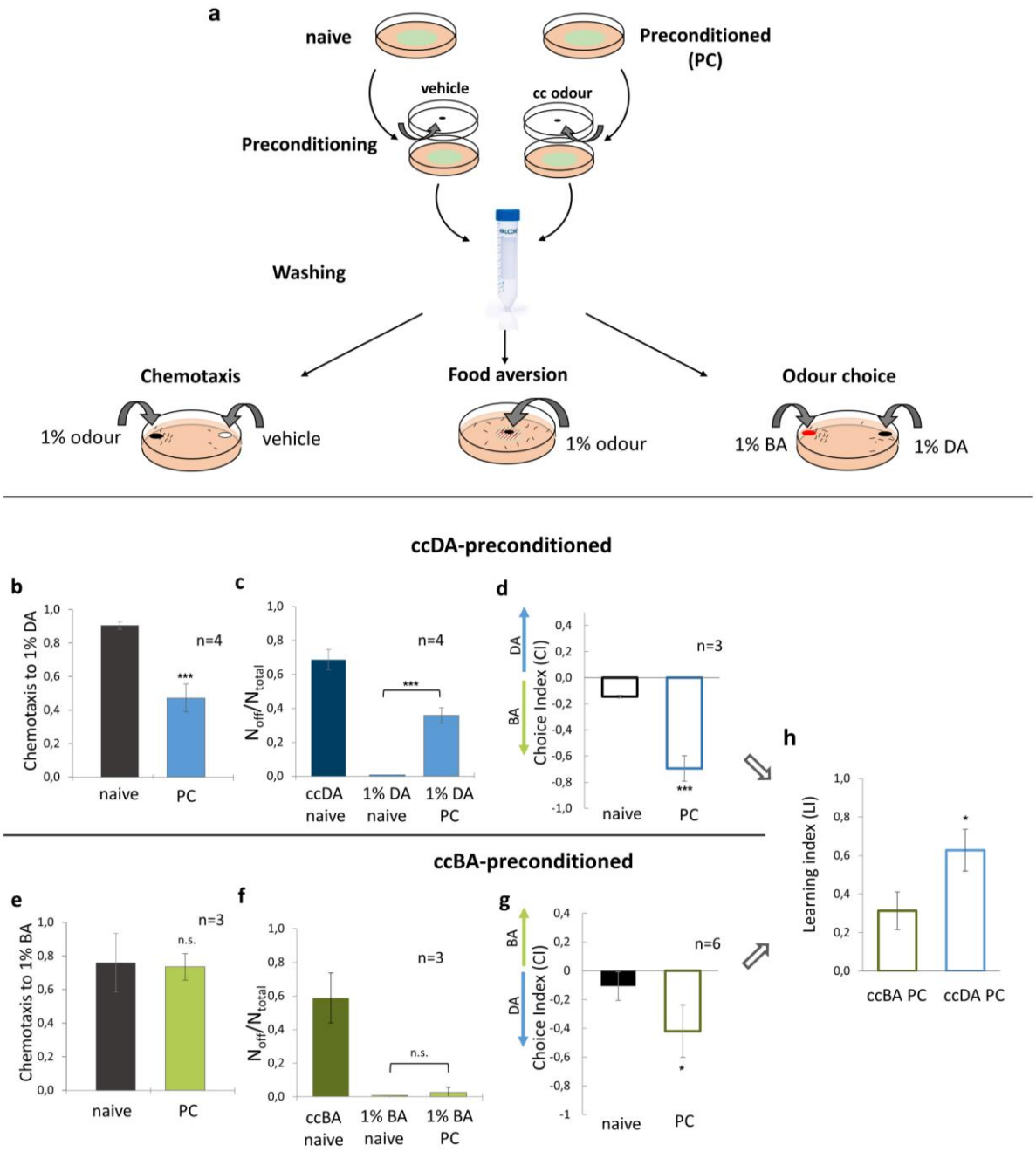

Figure 12. Avoidant and tolerant learned behaviors elicited by stress-associated olfactory cues

(**a**) Experimental setup for the toxic odor preconditioning-induced learning paradigm. Animals were exposed to a hanging drop of undiluted odor (preconditioned, PC) or vehicle (naive), washed and assayed for chemotaxis, food aversion and odor preference using diluted, 1% odors. Effect of ccDA-preconditioning on chemotaxis to 1% DA **(b)**, on lawn avoidance in the presence

of 1% DA **(c**) and on odor choice between 1% DA and 1% BA (**d**). Choice indices were calculated as CI = (worms on DA – worms on BA) / (worms on DA + worms on BA). Effect of ccBA-preconditioning on chemotaxis to 1% BA (**e**), on lawn avoidance in the presence of 1% BA (**f**) and on odor choice between 1% BA and 1% DA (**g**). Choice indices were calculated as CI = (worms on BA – worms on DA) / (worms on BA + worms on DA). **(h**) Learning indices from panels e and i, calculated as LI = CI (naive) – CI (preconditoned). Error bars represent mean ± SEM. n = number of independent experiments. p values were obtained by one-way ANOVA (for chemotaxis and food leaving assays), and by two-way ANOVA (for odor choice assays) with Fisher's LSD post hoc test. n.s.: not significant; *p < 0.05; **p < 0.01; ***p < 0.001.

5 Discussion

Associative learning provides attachment to essential qualities and fundamental conditions, in addition ensures the avoidance of adversities, dangers and noxious stimuli which all serve survival for individuals. The recognition and effective coping with re-emerging (anticipated) stresses originating either from adulthood or from the critical perinatal period is dependent on intricate, potentially different associative learning mechanisms and memory formation. In our research work, using *C. elegans* as a model, we investigated the involvement of physiological defenses, i.e. cytoprotective stress responses in learned systemic, organismal adaptation. As a result, I observed that the efficiency of cytoprotective responses at the time of stress regulates learned behavioral decisions in adulthood. On another time scale, we demonstrated that in response to perinatal stresses animals are able to learn, and form a persistent memory of a transcriptional cytoprotective response. This novel phenomenon we refer to as „imprinted cellular defense memory".

We have established a novel experimental paradigm of early life stress and imprinting by exposing newly hatched *C. elegans* to toxic chemicals. We have shown that early life exposure to toxic stresses induces both locomotory avoidance of the toxin-containing food as well as toxin-specific cytoprotective responses in L1 larvae. This experience, however, does not form a persistent aversive behavioral memory, but imprints a cytoprotective memory that is reactivated by re-encounter of adults with toxin-associated bacterial sensory cues (Fig. 13).

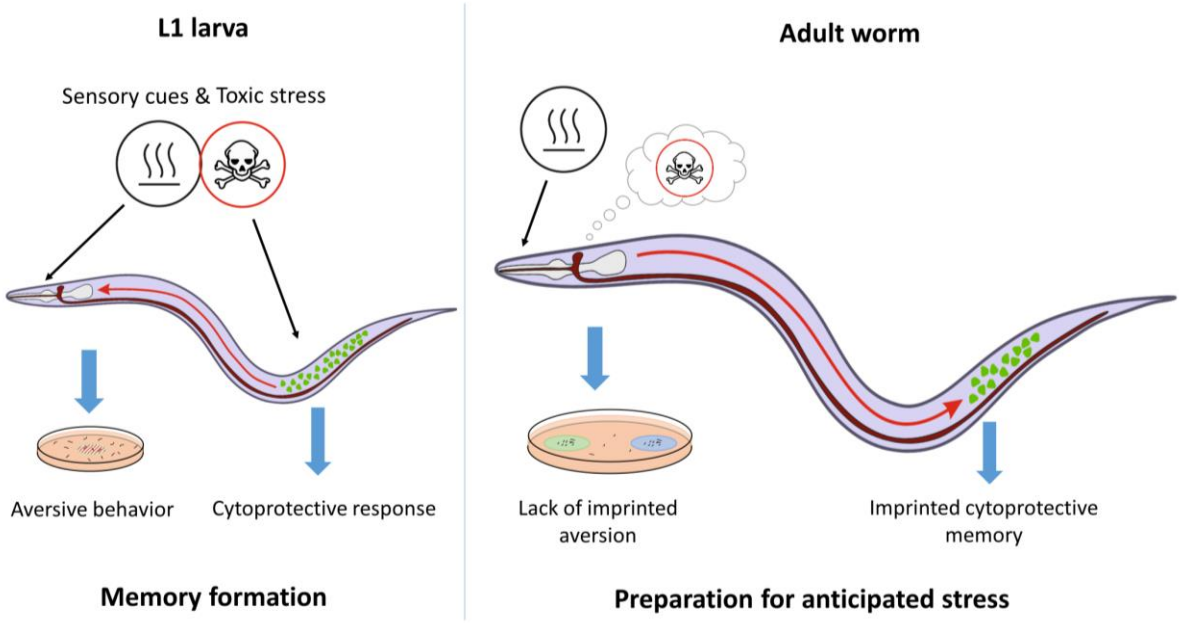

Figure 13. Model of the imprinted cellular defense memory

Exposure of L1 larvae to toxic stresses during the critical period results in tissue damage and toxin-specific systemic cytoprotective responses (green dots). Somatic cells transmit the stress-responsive signals to the nervous system through unknown pathways (red arrow pointing from body to head) which are integrated with the experience of toxicity and the co-occurring sensory cues, giving rise to toxin-induced aversive behavior and an associative memory formation. Re-encounter of the adult worm with the previously experienced sensory cues retrieves the stored stressful memory, which alerts the somatic cells through unknown pathways (red arrow pointing from head to body). Memory retrieval does not evoke aversion, but reactivates the previously induced cytoprotective responses (green dots). The induction of imprinted cellular defenses might be an attempt to help the worm prepare for impending threats.

C. elegans, in its natural habitat, has to deal with dangerous chemicals, which can be of natural origin, such as toxins released by microorganisms or toxic substances from pollution. Previous studies have shown that animals exposed to toxic compounds during their development respond to toxic stress with specific stress responses[52,53] or both behavioral and cellular stress responses[54,55] in L4 larval stage or in adulthood,

respectively. Our results show that worms treated at a very early age, immediately after hatching, with toxic doses of antimycin (AM) or paraquat (PQ) in L1 larval stage are already advanced enough to effectively protect themselves against toxic stress effects at both the cellular and the behavioral levels (Fig. 3 and 4). These results indicate that *C. elegans* employs a combined defense strategy to overcome adversities during early development. Our results also demonstrate and independently confirm earlier work that L1 larvae are capable of neural integration and formation of enduring associative memories.

Previous studies show that behavioral aversion can be imprinted in *C. elegans*. *Pseudomonas aeruginosa* infection[26] or ascr#3 pheromone exposure[27] in L1 larvae results in the development of avoidance for these cues in adulthood, suggesting that these negative experiences during the sensitive period form long-term aversive memories. In our experiments, despite the explicit aversive behavior of L1 larvae, adult nematodes did not avoid sensory signals of OP50 that co-occurred with the toxic stress (Fig. 3, 6 and 7). These experiments were performed with two unrelated toxins and two unrelated bacterial strains, and all results show the lack of adult aversion in classical olfactory food choice assays (Fig. 6). Nematodes did not show aversive behavior even after the separation of avoidant and non-avoidant populations of L1 larvae and the application of a novel test method that mimicked the original conditions and eliminated the negative effect of hunger on choice (Fig. 7). Toxin-treated worms developed into normal, healthy adults after early life exposure and they did not show any differences in their appearance or locomotion during food choice assays, which may have prevented the manifestation of the aversive behavior. Although the reason of the lack of adult aversive behavior is not clear, our results suggest that in *C. elegans* imprinted aversion is not necessarily a consequence of adverse conditions experienced during the sensitive period. However, imprinting of other stress-induced behavioral changes may be conceivable, which requires further investigations.

In contrast to the lack of aversion, adult worms encountering sensory cues experienced during toxin exposure in early life activate toxin-specific stress and detoxification genes (Fig. 9). Both AM and PQ are potent toxins and cause severe damage[45,46], therefore a dysregulated state of the organism can cause random activation of toxin-inducible genes. However, toxin-induced putative metabolic or homeostatic

changes are unlikely due to the down-regulation of toxin-specific cytoprotective responses, normal health status and normal behavior of the worms. Since sensory cues are able to activate these cellular stress responses even in the absence of toxic stress, this suggests a regulated neuronal process that evokes systemic protective responses based on previous experiences. Although the identification and understanding of the signaling pathways and neuronal circuits require further studies, our findings suggest an associative learning mechanism and are consistent with two recent studies which demonstrated the systemic activation of intracellular HSF-1- and DAF-16-dependent stress responses, respectively, only by PA14- or starvation-associated olfactory cues[56,57]. In addition, cellular stress signals through a JNK-like pathway or *hsf-1* are also essential for aversive behavior to occur under stressful conditions[38,56]. Taken together, these studies expand our knowledge and reveal that, in parallel with the visually well-perceptible behavior, learned responses exist also at the molecular level and cause systemic and highly influential changes that, among others, affect behavior.

Previous studies on associative learning and imprinting, with the exception of the two studies[56,57] mentioned above, have mostly examined behavioral outcomes[21]. We observed a learned associative cytoprotective memory which is formed only in the L1 stage and can be evoked by toxin-associated sensory signals in adult worms (Fig. 9 and 10). By integrating the critical period, the specific sensory component and the persistent cellular response, we identified a thus far unknown, novel form of olfactory imprinting. To distinguish from the behavioral imprinting and to clarify its location at the cellular level, we refer to it as "imprinted cellular defense memory", the model of which is shown in Figure 13. Interestingly, a specific odor exposure associated with 24-hour starvation induces DAF-16 nuclear translocation that is enduring beyond 48 hours, thereby also being able to form a long-term memory[57]. However, in that case the associative cytoprotective memory was not imprinted in the L1 stage, indicating that early life stresses do not necessarily lead to imprinted cellular defenses[57], consistent with their selective effects on aversive behavior[26,27]. Further studies are needed to determine whether other stresses, such as pathogen attack or population crowding, induce imprinted cellular protection, and what other, hitherto unknown factors and which conditions conduce to the development of different forms of imprinted memories.

In connection with the imprinting results, we have investigated the link between toxin-induced cellular defense responses and learned behavioral avoidance in adult nematodes. In these experiments we also studied the neuronal associations between the internal experience of stress induced by toxicity and a co-occuring chemosensory cue. We applied toxic concentrations of undiluted odorants, benzaldehyde (ccBA) and diacetyl (ccDA) and low concentrations of the same odorants, which are naturally attractive to *C. elegans* and characterized their learned behavioral decisions combining three different experimental paradigms. Our results have shown that adult nematodes preconditioned with toxic ccDA are unable to cope with toxicity at the cellular level, thus these animals respond to a subsequent 1% DA exposure by avoidance. In contrast, ccBA preconditioning induces cytoprotective responses that prepare animals for a subsequent stress, resulting that worms develop tolerant behavior upon 1% BA (Fig. 12).

We have demonstrated that the induction of tolerant or avoidant behaviors by BA and DA olfactory cues after preconditioning showed that the presence or absence of the appropriate cytoprotective responses during stress is a critical regulator of future behavioral "fight-or-flight" decisions in response to the expected stress. These results suggest that internal experiences under stress, more specifically the efficiency of the cellular protective responses, greatly influence the outcome of learned behavior upon encounter with the olfactory cues. The increased preference of DA over BA after ccBA preconditioning suggests that worms perceive BA as a putative stress factor, presumably through their prior experiences of toxic stress and induced defense processes that associated with the BA odor. Such a representation allows individuals to reflect and decide whether resources for self-defense are needed or not, to obtain food.

Summarizing and investigating our results on both time scales, examining the connection between toxic stress-induced avoidant behavior at the organismal level and the induction of molecular protective responses at the cellular level, we observe that the experience of effective physiological protection helps to prepare individuals for a subsequent, anticipated stress situation through associative learning.

In case of imprinting, toxicity and the olfactory cues of OP50 food bacteria are associated at an early age, during the sensitive period. Importantly, in parallel, cytoprotective responses are also induced by the toxic stress, which are thus become part of the association as well with the addition of the internal experiences of stress. This

complex experience composed of these three main constituents can be evoked in adulthood merely by the odor of OP50. Re-encountering with the odor of OP50 elicits the cellular stress responses *per se* in the absence of toxic effects. Interestingly, in our experimental conditions it did not appear to induce aversive behavior, indicating that the individual is already prepared for an impending stress at the cellular level and when required (i.e. when toxic effects reappear) it will be able to react quickly and robustly to an already experienced stress situation.

We have also studied the effects of cytoprotective responses on behavioral decisions on a shorter time scale examining adult worms, and we have obtained similar results. Preconditioning with the toxic concentration of BA induces effective protective cellular stress responses in non-neuronal cells that are also able to prepare the individual for an expected, possibly re-emerging threat. Thus, when adult worms re-encounter with low concentration of BA, i.e. the stress-associated olfactory cue, which is naturally attractive to them, they show tolerant behavior instead of avoidance. While in case of DA, odor-preconditioning with toxic concentration do not result in the induction of adequate, effective defense mechanisms at the cellular level. Therefore, when animals are subsequently exposed to the same sensory cue, they respond to the already experienced stress at the behavioral level and they must actively avoid the source of stress which might even be fatal. Therefore, we propose that associative learning enabled worms to alter the neural representation of DA to react even to the otherwise attractive 1% DA by avoidant behavior.

Our studies imply that in *C. elegans* the ability or inability to counteract toxic stress with cellular defense mechanisms regulate behavior under stress and influence learned behavioral decisions upon re-encountering with the stress-related olfactory cues. This regulatory link between cellular defense responses and behavioral responses is present both in terms of associative learning in adulthood. Moreover, such a link might be present in imprinting during the sensitive perinatal period.

The foundations of stress responses and learning are conserved between worms and humans. Therefore, the induction and imprinting of similar cytoprotective responses during the sensitive period as well as in adulthood is plausible in higher organisms. Consistent with this idea, several pieces of experimental evidence suggest such a mechanism[10,11,12], although the association between stress and a chemosensory cue have

not yet been demonstrated. Therefore, this hypothesis needs further experimental confirmation. Similarly, the regulatory link between cellular stress responses and behavioral responses revealed by our work might also exist in mammals and humans. In case of imprinting, persistent behaviors and somatic changes imprinted by early life exposure to predators or violence appear to play a role in the increased resilience of both animals[58] and humans[59] in a predatory or violent environment. Resilience is typically defined as the capacity to recover from adversities, difficult life events. Among animals, this mutual regulation between physiological and behavioral stress responses and associative aversive learning is considered to be adaptive, by preparing animals for a potential stress situation. Besides, our work suggests, on the one hand, that memories of past stresses associated with deficient cellular defenses may condition to avoidance. At the same time, our results also imply that basically independent, otherwise neutral sensory stimuli can be associated with stress and upon encounter, can elicit stress responses.

What might be potential and highly putative implications of our findings for human health? In humans, an increasing number of maladaptive, stress-related physiological and emotional responses are recognized as elements of various mental, emotional[8,9,60] and somatic diseases, including chronic pain syndromes[13,14,15], phobias, panic attacks and eating disorders. In both the adaptive as well as the maladaptive cases, among other yet unknown effectors, the acute or sustained activation of the hypothalamic–pituitary–adrenal (HPA) axis plays a causative role[61,62,63,64]. It remains to be seen whether imprinted or learned cellular defense memories of prior stressful physical experiences govern behaviors and somatic conditions in response to sensory cues in vertebrates.

6 Conclusion

In my doctoral work, I investigated the connection between toxin-induced cytoprotective stress and behavioral responses and associative learning during early development and in adulthood.

The most important new results of my doctoral work are the following:

1. We found that in response to toxic stress L1 larvae exhibit both physiological and behavioral defenses.
2. We identified a hitherto unknown, novel form of olfactory imprinting by showing an imprinted transcriptional cytoprotective response we named "imprinted cellular defense memory". This memory develops only in the critical (sensitive) period and can be evoked by toxin-associated sensory cues in adulthood.
3. We did not find early life toxic stress associated aversive memory in adulthood, suggesting that in *C. elegans* early life stresses do not necessarily cause imprinted associative aversive behavior.
4. We demonstrated that in adult *C. elegans* the efficient or deficient cytoprotection during toxic stress regulates learned behavioral decisions upon re-encountering with the stress-associated olfactory cues.

7 Summary

The nematode *C. elegans* is a simple organism that, despite its simplicity, is characterized by complex behavior, associative learning, long- and short-term memory. The existence of physiological and behavioral defenses together with learning enables nematodes to adequately respond and remember to environmental stress ensuring survival and fitness. However, few studies have examined the involvement of intracellular molecular stress responses in learned adaptation to stress. It was previously discovered that a special form of associative learning, called imprinting already exist in *C. elegans*. Imprinting forms especially persistent memories and develops lasting patterns of behavior. Previous studies on associative learning and imprinting have mostly examined behavioral outcomes. However, whether intracellular transcriptional stress responses might be imprinted is unknown.

The aim of my doctoral dissertation was to better understand the involvement of cytoprotective responses in learned defenses against toxic stress in early life and in adulthood. We established that in newly born L1 larvae, early life exposure to antimycin A or paraquat toxins simultaneously stimulates the expression of the *hsp-6* and *gst-4* cytoprotective stress reporter genes, and behavioral avoidance of the toxin-containing bacterial lawn. In adulthood, we did not find imprinted aversive behavior towards stress-associated bacterial sensory cues. In contrast, the mere re-encounter with the stress-associated olfactory cues reactivated the transcription of previously induced cytoprotective genes. Thereby, we identified a hitherto unknown form of olfactory imprinting, referred to as “imprinted cellular defense memory”.

Using a toxic stress paradigm of undiluted odorants in adulthood, we demonstrated that adult worms learn the previous experience of odor toxicity together with the different efficiency of cytoprotective responses. The lack of apparent cytoprotection induced avoidant behavior, while the efficient cytoprotection elicited flexible behavioral responses. This, in turn, led to the expression of learned behavioral decisions upon re-encountering with the stress-related olfactory cues.

Our findings reveal an important connection between physiological and behavioral defenses. Specifically, they identify a hitherto unrecognized role of cytoprotective stress responses in non-neuronal cells in the regulation and expression of learned defenses and adaptive strategies to anticipated stress.

8 Independent work

Imprinting assay

- Establishing an early life stress paradigm in *C. elegans* and assaying appropriate toxins and stress reporters.
- Setting up and testing behavioral aversion in response to toxic stress in L1 larvae.
- Measuring GFP reporter expressions by fluorescent microscopy and evaluation of the GFP fluorescence intensity by ImageJ softwer in L1 larvae and in adult worms.
- Assaying toxin-treated worms' behavior in olfactory food-choice tests.
- Developing a novel, food leaving-food choice assay to examine food choice of adult worms more accurately.
- Statistical analyses of all imprinting data using STATISTICA program

Adult learned avoidance assays

- Performing chemotaxis, food leaving and odor choice assays
- Statistical analyses of chemotaxis, food leaving and odor choice data using IBM SPSS Statistics and STATISTICA programs

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10 Bibliography of the candidate's publication

Publications directly related to this thesis

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Publication not directly related to this thesis

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