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Hormesis depends upon the life-stage and duration of exposure: examples for a pesticide and a nanomaterial.

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Abstract

Tests to assess toxic effects on the reproduction of adult *C. elegans* after 72 h exposure for two chemicals, (3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)), also known as diuron, and silver nanoparticles (Ag NPs) indicated potential, although not significant hormesis. Follow up toxicity tests comparing the potential hormesis concentrations with controls at high replication confirmed that the stimulatory effect was repeatable and also statistically significant within the test. To understand the relevance of the hormesis effects for overall population fitness, full life-cycle toxicity tests were conducted for each chemical. When nematodes were exposed to DCMU over the full life-span, the hormesis effect for reproduction seen in short-term tests was no longer evident. Further at the putative hormesis concentrations, a negative effect of DCMU on time to maturation was also seen. For the Ag NPs, the EC_{50} for effects on reproduction in the life-cycle exposure was substantially lower than in the short-term test, the EC_{50}s estimated by a three parameter log logistic model being 2.9 mg/L and 0.75 mg/L, respectively. This suggests that the level of toxicity for Ag NPs for *C. elegans* reproduction is dependent on the life stage exposed and possibly the duration of the exposure. Further, in the longer duration exposures, hormesis effects on reproduction seen in the short-term exposures were no longer apparent. Instead, all concentrations reduced both overall brood size and life-span. These results for both chemicals suggest that the hormesis observed for a single endpoint in short-term exposure may be the result of a temporary reallocation of resources between traits that are not sustained over the full life-time. Such reallocation is consistent with energy budget theories for organisms subject to toxic stress.

Introduction

Because of the wide relevance to real environmental scenarios, the nature of biological responses to low concentration chemical exposure has developed as a key area of research in toxicology. Within the field, a key area of focus has been on the prevalence of hormesis. This is a well documented low dose phenomenon that is the subject of much debate and controversy (Calabrese, 2008). There are a number of different definitions of hormesis and a range of potentials mechanisms have been proposed (Calabrese, 2005; Calabrese, 2013; Calabrese and Blain, 2011; Cedergreen et al., 2005; Costantini et al., 2010; Gems and Partridge, 2008). Broadly speaking hormesis is defined as a biphasic response with a low dose stimulatory response and a high dose inhibitory response (Calabrese and Baldwin, 2002). The term hormesis has also been applied to a conditioning effect where a low dose exposure leads to improved performance of the organism when exposed to higher doses later in life. In the later respect, hormesis can be linked to observation such as the development of tolerance within generation through adaptive response (Birringer, 2011), potentially as a result of epigenetic effect on gene expression regulation (Vaiserman, 2011).

The study of hormesis is particularly relevant for a range of chemicals, such as pesticides and nanoparticles, for which measurements and modelling generally point to the prevalence of low environmental concentrations (Gottschalk et al., 2013; Hirsch et al., 1999; Johnson et al., 2011; Williams et al., 2012; Carriger et al., 2006; Konstantinou et al., 2006). For pesticides, Cedergreen (2008) has established the potential for hormesis for effects on plant growth that has also been extended in observations made for non-target species (Bonilla-Ramirez et al 2013). These studies suggest that pesticides can elicit hormesis through different mechanisms relevant to different species. Observations of hormesis have also been widely recorded for nanoparticles (Arora et al., 2008; Kawata et al., 2009; Ma et al., 2009; Nations et al., 2011).

This includes positive effects on cell viability of silver (Ag) NPs (Jiao et al. 2014), as well as putative effects on reproduction in *C. elegans* that we observed in previous work (Tyne et al. 2013). Generally, however, these observations of hormesis are based on single observations that in some cases may lack a robust statistical basis for their definition.

A key question in hormesis research has been how observation of low dose stimulation for a single trait in short-term studies are related to effects on multiple traits over a full life time exposure. This question is important because it can determine how hormetic effects may relate to effects at the population level. Jager et al. (2013) highlight the important point that organisms have to obey the conservation laws for mass and energy, with the suggestion there may well be "no such thing as a free lunch". These authors present three explanations for observed hormesis, namely increased acquisition (i.e. increasing the input of energy into the individual), changes in allocation (i.e. rearranging the energy flows over various traits) and medication (i.e. the stressor is an essential element or acts to alleviate disease or infection). Robust assessments of observation of hormesis in relation to these hypotheses are needed to address how hormesis for single traits relate to overall fitness.

To investigate the basis for hormesis, here we conduct an analysis of the phenomena focusing on response of the nematode *Caenorhabditis elegans* exposed to two chemicals, the herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU or diuron) and Ag nanoparticles (Ag NPs), for which our own previous studies have suggested a hormesis response for effects on nematode reproduction (Martin et al. 2009; Tyne et al 2013) The aim of our study was to first establish the reproducibility and statistical validity of potential hormesis in short-term toxicity tests and thereafter to assess the effects at such concentrations for multiple traits within a full life-cycle exposure. Concentration ranges used for our studies were selected to cover

environmentally relevant concentrations for these chemicals. For example, the estimates for the predicted concentration of silver nanoparticles in UK river waters are range between 0.016 ng L^{-1} (Dumont et al., 2015) and 100 ng L^{-1} (Boxhall et al. 2007), while DCMU has been found in marine sediments in the UK at 12 to 13 mg kg^{-1} and at up to 768 ng L^{-1} in the waters of a marina in the UK (Boxhall et al. 2000). Hence, our assessment relates to environmentally relevant exposure conditions.

Materials and Methods

Ag nanoparticles and DCMU

The 3-(3,4-dichlorophenyl)-1,1-dimethylurea was obtained as a pure chemical (98%) from Sigma Aldrich (Poole, UK).The Ag nanoparticles with a reported primary particle size of 8 nm were supplied by Amepox (Warsaw, Poland) as a 1000 mg/L stable dispersion in water. Analysis of the supplied material by transmission electron microscopy and dynamic light scattering confirmed that the material was comprised of primary particles of 3-8nm in diameter and that these particles formed agglomerates in the 100 nm diameter range that were generally well dispersed. Low energy sonication was sufficient to cause the separation of these agglomerates to form a dispersion containing a high number of the primary particles (for a detailed description of the characteristics of these Ag NPs see Tyne et al., 2013).

Caenorhabditis elegans culture maintenance and synchronization

Wild type N2 ancestral *C. elegans* obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, MN, USA) were kept as a laboratory culture on NGM agar plates (1.7% bacto-agar, 0.025% bacto-peptone, 50mM $NaCl_2$, 0.005% cholesterol, 1mM $CaCl_2$. 1mM $MgSO_4$, and 25mM KH_2PO_4) seeded with *E. coli* OP50 strain (Brenner 1974). All cultures and tests were conducted at 18°C. This is at the lower end of the standard range used for *C. elegans* studies. This temperature was used to slow reproduction rate and extended brood period to allow daily handling and counting of more test concentrations and/or replicates, notably for the life-cycle test. All toxicity studies were conducted with synchronized worms obtained by transferring 20 young adults to a fresh NGM agar plate for egg laying for 3 hours. The adult worms were then removed and the plates incubated at 18°C to allow eggs to hatch. The resulting juveniles were kept for 72 hours until they were at young adult stage and ready

for use directly in the short-term adult reproduction tests or to be used as a “parental” generation to produce eggs for the life-cycle tests. .

Both the short-term and full life-cycle tests were conducted in Simulated Soil Pore Water (SSPW) without fulvic acid prepared as described by Tyne et al. (2013). This medium consists of a mix of salts designed to provide a low ionic strength solution suitable for the exposure of *C. elegans* to chemicals under conditions that more closely resemble natural soil pore water than does any of the current widely used alternatives such as M9 buffer, K-media or nematode growth medium agar. The greater relevance (e.g. low ionic strength) of SPPW in generating relevant exposure chemistry is particular useful for tests with NPs because it supports a greater NP stability than high ionic strength solutions such as M9 and K-medium (Tyne et al., 2013). As food, the SSPW was always seeded with *E. coli* OP50 at a concentration of 500 Formazin Attenuation Units to provide an excess of the nutritional requirements of the *C. elegans*. Tests were performed in 12 well plates using a 2 mL exposure volume.

For the DCMU study, the test chemical was solubilised in acetone carrier at 9.2 mg/mL and maximum of 2 µL spiked into a 2 mL volume of SSPW to give the test concentration needed for each replicate. Apart from the pure SSPW controls, additional acetone was added to the solvent control and lower concentration treatments to ensure that the total volume of solvent was consistent across all treatments in the experiment. The tests conducted with Ag NPs were prepared in a similar manner to that described in Tyne et al (2013). The Ag NPs were prepared in a stock solution of 500 mg/L and then diluted with SSPW to yield the required concentrations in a total of 2 mL of test solution for each replicate well on the 12 well plates. To start each test, an individual , young adult worm for the short-term test, or individual eggs for the DCMU life-cycle test, was transferred from the synchronous culture agar plates directly to each well

with the appropriately dosed SSPW media. The AgNP lifecycle was started by transferring adult worms to the wells containing appropriately dosed media for 3 hours, during which time sufficient eggs were laid to carry out the trial.

Short-term reproduction test

The DCMU test included seven concentrations in the range 0.82-9.21 mg/L, a solvent control all matched to the 1 µl/mL of acetone required to act as a carrier for the DCMU when making up the top dose, and a separate set of standard controls without acetone. Four replicate wells were used for each tested concentration. To initiate the test, a single age synchronized adult worm was placed in each well and incubated in constant dark at 18°C for 72 hours. At the end of the exposure, 0.75 mL of 1% Bengal red was added to each well to stain the juveniles and eggs, which were then killed by heating to 55°C and counted. The short-term test for DCMU was done to confirm that the hormesis response previously seen in a study on agar (Spurgeon *et. al.*, unpublished data) was also present in liquid media, and the test therefore not repeated, as was done for the Ag NP study.

The Ag NP study used for initial concentration response analysis included a control and seven concentrations of Ag NPs (range 0.0625 – 4 mg Ag/L), with six replicate wells for each treatment. At the test start, an individual young adult worm from the age-synchronized culture was placed in each test well. Worms were then incubated in constant dark at 18°C for 72 hours after which time 0.75 mL of 1% Bengal red was added to each well to stain the juveniles and eggs, which were then killed by heating to 55°C for 30 minutes and counted. As prior data for the concentration response relationship for effects on *C. elegans* reproduction for these Ag NPs was not available, the test was repeated once (i.e. two tests in total) to ensure the reproducibility of any subtle low dose responses. The results are reported as Test 1 and Test 2 in the results.

Confirmation of initially observed hormesis by single dose tests with increased replicates

Data from the short-term reproduction studies was used to identify putative hormesis concentrations that could be brought forward to tests conducted with a degree of replication estimated by power analysis to be sufficient to give an 80% probability to identify a difference as statistically significant. These tests compared 15 replicates of the control and putative hormesis concentration identified short-term exposure assay (0.81 mg/L DCMU, 0.0625 mg/L Ag NP) and were otherwise run following the same dosing and exposure protocol used for the initial toxicity tests. Although only standard controls were used in both the Ag NP and DCMU single dose trials, as no difference in reproduction was found between the standard and carrier controls in the short-term study and solvent controls thus not deemed necessary for this low dose DCMU study running with a lower acetone concentration.

Life-cycle toxicity tests for DCMU and Ag NPs

Full life-cycle toxicity tests for DCMU were conducted comparing 0.81 mg/L DCMU and standard control treatments with twenty replicate wells used for each treatment. To initiate the test, individual eggs were picked from a synchronization plate and placed into the dosed or control wells (1 egg per well) for each replicate. The emergent nematodes were monitored to confirm they were still alive every 24 hours and additionally photographed at 12, 24, 60, 72, 84, 96, 108 120, 132, 144 and 156 hours using a Nikon Coolpix 4500 digital camera to assess growth. Size was measured using Image Pro Express software (Media Cybernetics, Marlow, UK) and volumetric length (cubic root of body volume) calculated (Alda Álvarez et al., 2005). To record temporal development and total brood size, the adult nematode in each well were transferred to new plates with fresh dosed or control media after 96, 144 and 192 hours of exposure. Fresh OP50 was included in both the dosed and control media that was supplied at

every transfer time at a concentration of 500 FAU. This ensured that the nematodes would not be nutrient limited at any point during the test. Eggs and offspring remaining in the test solution after each adult transfer were stained and counted as for the short-term test. The DCMU trial was terminated after 240 hours, when no further reproduction had taken place in the previous period. Hence in this study effects on growth and broodsize, but not life-span, could be assessed.

The life-cycle toxicity tests for the Ag NPs were based on a more elaborate design than for DMCU, which considered responses at four exposure concentrations including the putative hormetic concentration of 0.0625 mg/L as well as three higher concentrations (0.7, 2.1 and 6.5 mg/L) that were found to result in reduced reproduction in a short-term adult test. The latter were included as positive controls for Ag NP toxicity. To ensure enough juveniles were available for each dose four adults worms were used to seed each well containing 2 mL of control or dosed medium. These adults were removed after 3 hours and the plates then incubated at 18°C for 48 hours. Individual emerging juveniles that hatched from the eggs laid by these worms were then used to set-up 18 replicate wells containing one worm each for the control, putative hormesis and 3 putative higher exposure concentrations used. After 60, 84, 108, 132, 156, 228, 252, 276, 300, 324, 384, and 408 hours of exposure, the nematodes were visual checked for survival and to assess the onset and end of egg laying (n.b. any male nematodes unintentionally included were excluded from assessment). After 60, 120, 168, 228, 276, 324, 360 and 408 hours of exposure, the adult nematodes were transferred to wells in new plates containing fresh medium at the relevant Ag NP dose. Fresh food as *E. coli* OP50 at 500 FAU was included at all transfers to avoid nutritional limitation. Juveniles and eggs remaining following adult removal were stained, killed and counted as above. The visual monitoring of

the nematodes was more frequent during the early phase of the trial to enable screening for any instances of the internal hatching juveniles and mortality due to handling.

Data analysis

Measurement of reproduction (combined counts of eggs and juveniles) over the 72 hr exposure period used for the short-term exposure in the concentration response trial were initially tested for normality using the Kolmogorov-Smirnov test and then analyses using a fixed parameter one way analysis of variance (ANOVA) in Minitab 16 followed by Tukey's pair-wise comparisons to ascertain if differences between treatments were statistically significant. Additionally, the data was also fitted using a logistic 3 parameter model fitting in SigmaPlot10 (Systat Software Inc.(SSI), San Jose, California) to allow parameters that described the maximum level of reproduction, model slope parameter and test EC_{50} value to be estimated. For the test comparing the single concentration exposures to controls, two sample one-tailed and two-tailed t-tests were performed in Minitab 16 to assess if differences in reproduction between treatments were statistically significant. In the life-cycle test, time series measurements of body size were both analyzed through a series of paired t-tests in Minitab, and through fitting to a Gompertz model generated by the SSgompGrowth.1 function in R. The curve values for the Gompertz regressions were estimated by a weighted nonlinear least squares method fitted to the equation: $fx = ae^{-bc^{x}}$, where: a is the upper asymptote, b sets the displacement on the x axis and c is the gradient function (c is equivalent to e^{-c} in the standard form of the Gompertz equation) The differences in lifespan for the Ag NP lifecyle test were compared by using a fixed parameter one way analysis of variance (ANOVA) followed by a series of two-tailed t-tests in Minitab 16 to assess if differences in the lifespan between different treatments were statistically significant. .

Results

Short-term reproduction test

The concentration response relationships for all short-term reproduction tests conducted with DCMU and the Ag NP are shown in Figs 1 and Fig 2 a/b respectively. The results suggest a biphasic response relationship for both chemicals. For DCMU the average value of reproductive output of 240 at the lowest exposure concentration of 0.81 mg/L) was higher than the control value of 214 (and was also higher at 1.22 and 1.82 mg/L DCMU, with reproductive outputs of 235 and 226 respectively). Similarly, the Ag NPs reproductive output at 0.0625 mg/L of 89 was higher than the control value of 75 in the first test. With the second test confirming the observation with an average of 117 offspring at 0.0625mg/L compared to 99 in controls. Typical for observations of hormesis, the differences between the higher rates of reproduction at the lowest concentrations compared to those of the controls were not statistically significant. In fact for DCMU, the significant effect of this chemical (ANOVA, $F(7,28) = 4.41$, $p = 0.002$) on reproduction was not associated with difference between the control and any of the DCMU concentrations used, but rather significant differences in reproduction between the lowest DCMU treatment and the two highest concentrations (T values of -3.841 and -4.063; adjusted p values 0.013 and 0.007 for differences between reproduction at 0.81 and those at 6.14 and 9.2 mg/L DCMU respectively). For Ag NPs, the significant effects seen in both tests (ANOVA $F(7,41) = 6.12$, $p<0.001$ and $F(7,41) = 15.25$, $p<0.001$) were related to reduction in reproduction at the highest test concentrations, compared to the controls as well as the 0.0625 mg/L treatments (Ag NPs $t = -3.169$, adjusted p values = 0.017 for Test 1 control vs 2 mg/L Ag NP and T values – 4.342 and -5.653 with adjusted p values = 0.002 and <0.001 for control vs 2 and 4 mg/L respectively in Test 2).

Reproduction in the single dose trials

These highly replicated comparisons of control against the putative hormesis concentrations of respectively 0.81 mg/L DMCU and 0.0625 mg/L Ag NPs, were designed using power analysis of the effect size and variance information from the short-term tests to determine the replication needed to identify a significant effect in 80% of cases. The required number of replicates was 13. Hence all single dose tests were all conducted to at least this replication (15 replicates used). In the DMCU single dose trial, the difference in mean number of offspring in control wells of 202.5 ± 22 (SD) compared to 226.8 ± 18 (SD) at 0.81 mg/L DCMU was significant (T = -3.3, P = 0.003, DF = 27). For the Ag NPs, the mean control reproduction of 125.2 ±17.3 (SD), was also significantly lower that the mean of 142.1 ± 15 (SD) offspring at 0.0625 mg/L (T = -2.86, P = 0.008, DF = 27). These tests, therefore, provided confirmation of statistically significant hormesis at these exposure concentrations for both DCMU and Ag NPs. Box plots drawn from these results are provided as supplementary files (supplementary Figs. 1 and 2).

Life-cycle toxicity tests for DCMU and Ag NPs

The continuous monitoring in the life-cycle test allowed assessment of cumulative offspring production, full broodsizes and patterns of growth and/or life-span. In the DMCU test, mean full broodsize in the control of 190 ± 35 (SD) was significantly (T = 4.95, P < 0.001) higher than the 148 ± 23 (SD) observed for the 0.81 mg/L treatment indicating a toxic effect of the low DCMU concentration over the full life-cycle as opposed to a stimulation. Body size measurements made were fitted using the three parameter Gompertz model based on the measurements of body size for each made at each timepoint (Fig.3). Two data points from the control measurements, one at 108 hours and one at 144 hours were outliers and were excluded from the analysis. Models could be fitted to both data sets (control: $fx = 2390e^{-4.1*0.968^{x}}$; 0.81 mg/L: $fx = 2150e^{-4.66*0.963^{x}}$). Initially growth patterns for the control and DCMU

exposed nematodes were almost identical, however after 72 hours, the growth rate of the DCMU exposed nematodes reduced resulting in a smaller final body size (control 25800 ± 2400 μM^3, 0.81 mg/L DCMU 23000 ± 2100 μM^3) ($t = 2.75$ and $p = 0.013$). This pattern was confirmed by a series of paired t-tests at each time point which showed no significant differences between the treatments until 120 hours, after which time body size in DCMU treated worms was significantly ($p<0.05$) lower than controls.

In the Ag NP exposure, the mean brood size of control nematodes was 182 compared to 168, 98 and 5 juveniles at 0.0625, 0.7 and 2.1 mg/L of Ag NPs respectively (shown in supplementary material Fig. 3). No eggs were laid by nematodes exposed to 6.5 mg/L. Mean brood size was, thus, higher in the controls than in all Ag NP exposed worms including the putative hormesis concentration. Patterns of offspring production (Fig. 4) indicated peak rate in unexposed nematodes between Days 5-6. Observation indicated this was apparently delayed to between Days 6-7 at 0.0625 mg/L Ag NP and even further delayed to Days 7-9 for 0.7 mg/L. Final egg production was at Day 9 for all concentrations except at 0.0625 mg/L Ag NP where a single nematode continued egg laying until Day 12, producing 43 eggs between Day 9-12. Overall these differences in reproduction patterns (time to first egg, time of maximum egg production, brood period length) were marginal compared with the large differences seen in overall reproduction.

For life-span, there was a significant reduction (ANOVA, $F = 64.48$, $P > 0.001$) with increasing Ag NP concentration (Fig. 5). This shortening was significant (Tukey, $p < 0.05$) compared to lifespan of controls (18 days ± 3.808) at 2.1 (6 days ±0.577) and 6.5 mg/L, (2 days ± 0.0) but not (Tukey, $p > 0.05$) at 0.0625 mg/L (19 days ± 3.347) and 0.7 mg/L (15.857 days ± 4.22).This

data, therefore, points to a concentration related effect on Ag NPs in overall life-span at higher exposure concentrations.

Discussion

The past 25 years have begun to see the progressive clean up of industrial discharges and effluents, and a move towards less persistent and more specifically targeted pesticides. These advances in the management of the most severe pollutant effects, at least under some jurisdictions, have shifted the focus of environmental policy makers to more pernicious and complicated ecotoxicological problems, such as mixture toxicity, chemical/environment interactions, secondary effects and sub-group sensitivities (Daam and Van den Brink, 2007; Holmstrup et al., 2010; Hooper et al., 2005; Kille et al., 2013; Nota et al., 2011; Van Gestel et al., 2010). Regarding the nature of the dose-response curves, evidence has been put forward to suggest that the widely applied S-shaped logistic model, concentration-response curves may not be appropriate in all cases. Instead some, or many, response relationships may be characterised by low-dose stimulation and high-dose inhibition (toxicity), and more accurately represented by a J- or U-shaped curve. Such patterns are identified as the classical hormesis response (Calabrese and Baldwin, 2003b; Costantini et al., 2010).

To date low dose stimulation has been reported to occur in a wide range of species for metals/ metalloids (e.g. Cd, As, Hg) (Calabrese and Blain, 2004; Helmcke et al., 2010), dioxins (Rozman et al., 2005), pesticides (Cedergreen, 2008) and therapeutic agents (Calabrese and Baldwin, 2003a). The repeated occurrence of low-dose stimulation has led to suggestions that the hormesis model could even replace the logistic as the default in toxicology (Calabrese, 2008; Calabrese et al., 2006). This could have profound implications for chemical management, as regulation using hormesis (over traditional logistic models) could both alter permissible levels (Calabrese and Cook, 2005) and also providing a public perception of risk that “a little stress might be good for you” or more eloquently “what doesn’t kill you makes you stronger”

Although frequently observed, the overall impact of hormesis responses for single endpoint parameters on populations is not always obvious because response profiles can vary dependent on a range of factors. These include the stressor type; endpoint; test medium and life stage used and studied. As just one example, the toxicity of zinc chloride for on *Caenorhabditis elegans* movement shows a biphasic response pattern in unbuffered K media, however, in a buffered media no evidence for hormesis was found (Ma et al., 2009). Because of such differences, analysis of the impacts of hormesis for individuals and populations and its consideration in ecological risk assessment needs a more holistic context that considers this range of variation. Of particular importance is the relationship between hormesis for individual life-cycle parameters and response profiles for other traits. As emphasised by Jager et al. (2013) organisms have to obey the conservation laws for mass and energy and hence trade-off between traits can be expected to be the norm, with implications for the relationship between hormesis effects and fitness.

In this study, the initial short-term toxicity for two chemicals (DCMU and Ag NPs) for *C. elegans* reproduction showed a typical hormesis profile at concentrations within the environmentally relevant ranges of both chemicals. A highly replicated analysis of single dose control and putative hormesis concentration confirmed the statistical significance of this hormetic low dose stimulation. While such response profiles could be viewed as clear examples in support of hormesis, further investigation including measurement of responses across multiple traits and following extended exposure across the full *C. elegans* life-cycle indicated that this biphasic response pattern is not conserved for fitness at the population level when the exposure period is extended. Hence, exposure to the DMCU and Ag NP concentrations that caused observable hormesis in the short-term and single dose tests resulted in overall negative

effects on brood size, growth and survival in the life-cycle tests. The lifecycle tests for DCMU and Ag NP employed different methodologies with different outcomes in terms of the scope and type of data obtained as a result. There are advantages and disadvantages to both of the approaches. The advantage of the Ag NP approach is that, as well as time to death being recorded, a range of doses were used and the same shift in terms of greater impact of toxicity (lower reproduction, reduced lifespan etc) could be observed across the dose range. The DCMU approach, with its focus on the single, putative hormesis dose allows for the collection measurements of growth with far greater ease. The simple inclusion of lifespan data by extending the length of the lifecycle test would provide a much clearer analysis of the putative hormesis dose response than that obtained by the Ag NP approach, however for a study of how full dose response curve is modulated by the lifestage and length that an insult is present the Ag NP approach is more informative.

The disconnect between results from the short-term and life-cycle tests highlights issues with regards to the definition and application of the hormesis term. Most definitions of hormesis are centred on the notion of a stimulatory effect of a toxicant at a low (sub-inhibitory) dose (Calabrese and Baldwin, 1997; Calabrese and Baldwin, 2002; Chapman, 2002; Southam and Ehrlich, 1943). The length of time to which organisms are subject to the low dose does not form part of these definitions. Further, the precise stage of the organism lifecycle at which exposure occurs is not explicitly considered. Presence of hormesis in the short-term test which used adult worms, but not the life-cycle test initiated with early stage juveniles, suggests test exposure duration, or life-stage, or both may be critical factor in determining the overall nature of the concentration response relationship.

Classic trade-off, such as that between reproduction and life-span reflect the competing requirement of the relevant processes for overall metabolic resources (Barnes and Partridge, 2003; Forbes, 2000; Kooijman et al., 1999; Kooijman, 1993).The extension of lifespan has been linked with a number of traits including reduction in fecundity (Hercus et. al., 2003), delayed onset of re production, reduction in growth and reduced resistance to oxidative stress (Saul et al., 2013). The increase in reproduction has been linked to a reduction in growth as well as other more complex traits (Weltje et al., 2005). Recognising this, Jager et al (2013) used the principles of Dynamic Energy Budget theory to identify three categories of explanations for hormesis that obey mass and energy conservation laws. These were related to

1. An increase in energy acquisition either due to increase in feeding rate or an increase in the efficiency of energy assimilation.

2. Changes in allocation of energy resources which would lead to an improvement in the performance of one trait at the expense of another.

3. Medication due to, either the toxicant impacting on pathogens of the study organism or the toxiciant acting as a required supplement at low doses.

In all cases, these mechanisms imply that the organism was living under sub-optimal conditions that were the cause of sub-optimal trait performance prior to the application of the hormetic substance.

Assessment against these three potential causes for the two chemicals in this study identify some possible causes that may explain why hormesis was observed in short-term reproduction tests, but not the full life-cycle exposures. For DMCU, the mode of action in plants related to effects on electron transport chain between photosystems. Transcriptomic studies of the effects of a herbicide with a related mode of action (atrazine) on *C. elegans* have identified that this

chemical primarily targets mitochondrial function including electron transport (Swain et al., 2010). Because the effects of DCMU can also be expected to impact the mitochondria function, it seems unlikely that an increase in energy acquisition will underpin hormesis. Further this chemical is not known to have an antimicrobial function or to provide an essential nutrient. Hence for this chemical, a change in energy allocation resulting in a trade-off that favours reproduction against other traits such as somatic growth or maintenance to support extended life-span are likely to underpin the hormesis seen.

For Ag NPs, the primary use of the material in product application has so far been as an antimicrobial compound. Thus, for this material there is the potential that the level of exposure seen may have a knock down effect against microorganisms present on the nematode surface or its gut microbiome. However, while this explanation may initially appear plausible, given that the nematodes were reared under laboratory conditions for multiple generations with a single strain of *E. coli* (OP50) as a food source, it ultimately seems unconvincing to relate hormesis to recovered pathology. Studies of trace metal (e.g. cadmium) toxicity for nematodes most often report a suppression of energy assimilation (Alda Álvarez et al., 2006a; Alda Álvarez et al., 2006b; Swain et al., 2010), a finding supported by evidence that cadmium can suppress feeding rate (Jones and Candido, 1999). Since suppression in assimilation is linked to negative impact on all traits including reproduction, this change would not result in a hormesis response. At low concentrations that do affect acquisition, trade-offs between traits may occur (Alda Álvarez et al., 2005). This suggests that again, as for DCMU, trade-offs between traits are the most likely mechanisms responsible for the hormesis observed in the short-term Ag NP exposure.

While the hypothesis that a reallocation of resources as the mechanism for the observed increase in reproduction at low doses of DCMU and Ag NP appears reasonable it is by no means proven. A clear analysis of the response of multiple traits at low doses would, potentially provide evidence for a change in resource allocation. In this study multiple traits were examined as part of the lifecycle studies, however the hormetic response was not apparent in the lifecycle studies and no inference as to a mechanism for the hormesis in the short term trials can be inferred from them. The choice of traits to examine during a short term trial are more limited than those available for a study which extends across the full lifecycle, however multiple traits can still be studied (e.g. head thrashes and metabolic rate) and could provide a useful platform for future work into the hormetic responses observed in this study.

The majority of examples of hormesis that have been reported in the literature to date have been based on observation of single traits made on the basis of short-term toxicity tests. The results presented here, indicated that such observations may not always be observed after a life-time exposure. This demonstrates that life-cycle tests can be valuable tools by which to investigate the validity of hormesis observations in short-term tests. For *C. elegans*, life-cycle exposures are relatively straight forward given the comparatively short life-cycle. Clearly life-cycle exposures are not always feasible for organisms with a longer lifespan. In such cases it may a series of parallel trials carried out using cohorts at different life stages may be able to provide some indication of the variation in response patterns resulting from exposure in relation to the age and life stage of the exposed organism (Spurgeon et al., 2003). Inclusion of such data in life-table analyses have the potential to integrate these effects to provide a valuable overview of the potential fitness consequences of low concentration exposure (Forbes et al., 2011) and the role of possible mechanisms (Jager et al., 2013).

Acknowledgments

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Legends to Figures

Figure 1. Histogram showing the mean reproductive output (± SD, n = 4) of individual adult *C. elegans* exposed for 72 hours to a range of DCMU concentrations (mg/L) in simulated soil pore water. Letters indicate groupings according to Tukey's test at 0.05 family error rate.

Figure 2. Histograms showing the mean reproductive output (± SD, n = 6) of individual adult *C. elegans* exposed for 72 hours to a range of Ag NPs concentrations (mg/L) in simulated soil pore water for two independent experiments panel a) and b), respectively. Letters indicate groupings according to Tukey's test at 0.05 family error rate.

Figure 3. Body volumetric length (µm^3) and fitted Gompertz growth curves for individual *C. elegans* (n = 20) exposed over the full life-cycle to unspiked ("+" symbol, solid line) and 0.81 mg/L DCMU ("◊" symbol, dashed line) in simulated soil pore water.

Figure 4. Cumulative reproduction patterns for *C. elegans* (n = 18) exposed over the full life-cycle to a range of Ag-NP (mg/L) concentrations in simulated soil pore water (control: "◇" symbol/solid line; 0.0625 mg/L: "☐" symbol/dash dot line; 0.7 mg/L: "△" symbol/dash line; 2.1 mg/L "○" symbol/dotted line).The mean reproductive output for each treatment is indicated by the solid symbols.

Figure 5. Box plot showing the mean(⊕), median (–) and 2nd to 3rd quartile (the boxed region) with mean connect line for the lifespan of *C. elegans* exposed over the full life-cycle to a range of Ag-NP (mg/L) concentrations in simulated soil pore water. The whiskers indicate the full extent of the data range (Q1 – Q4). Outliers are indicated by asterix.

Fig 1

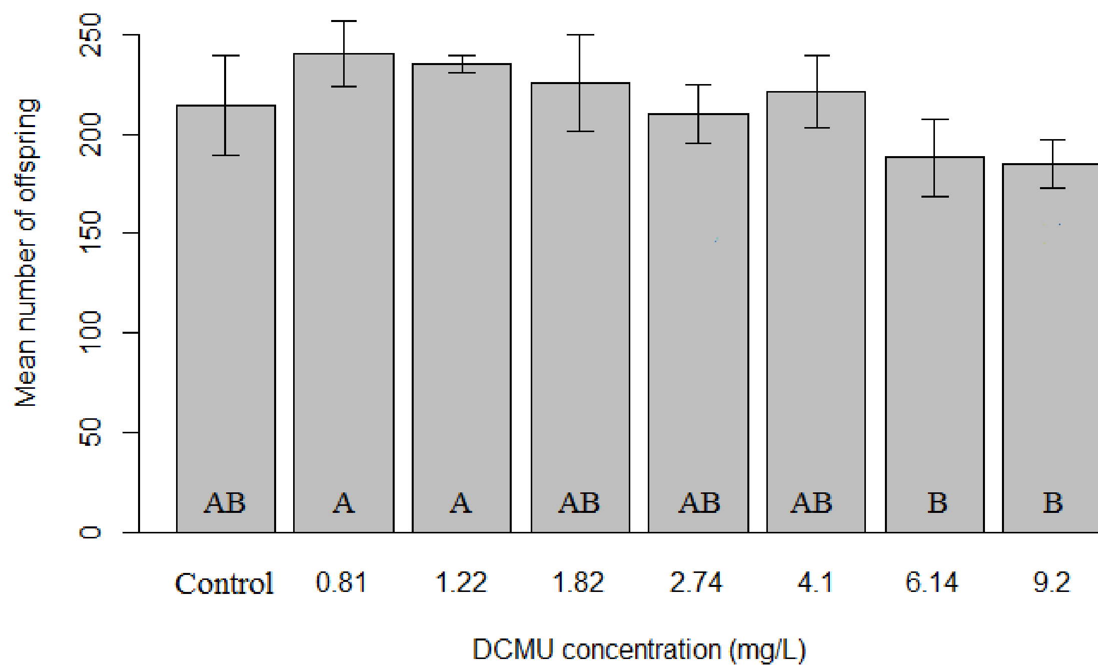
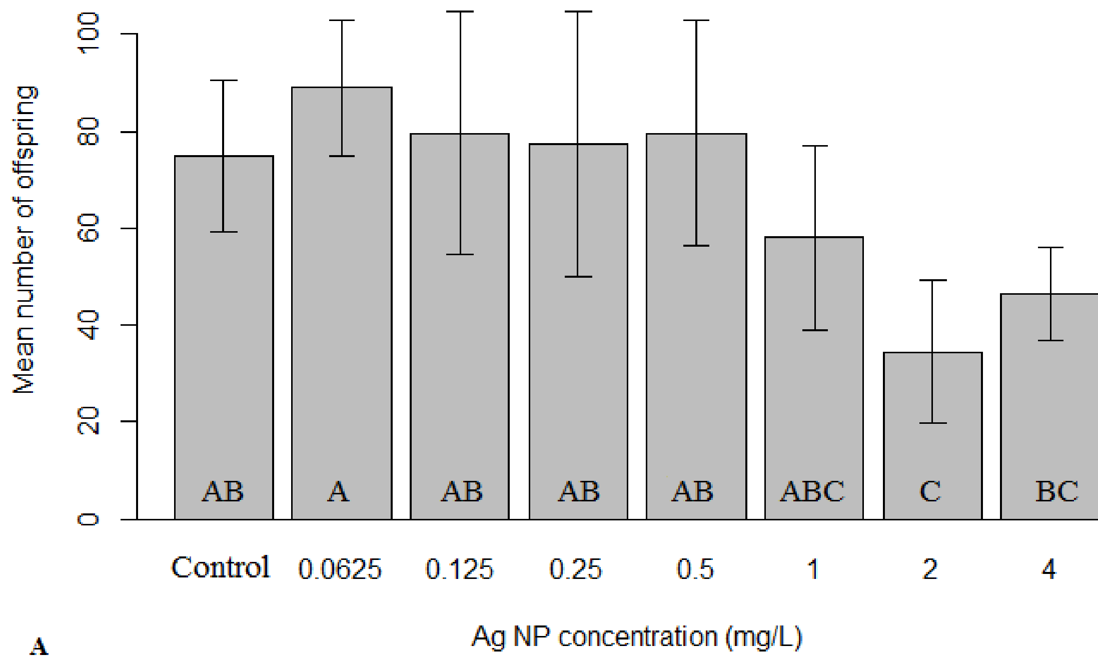
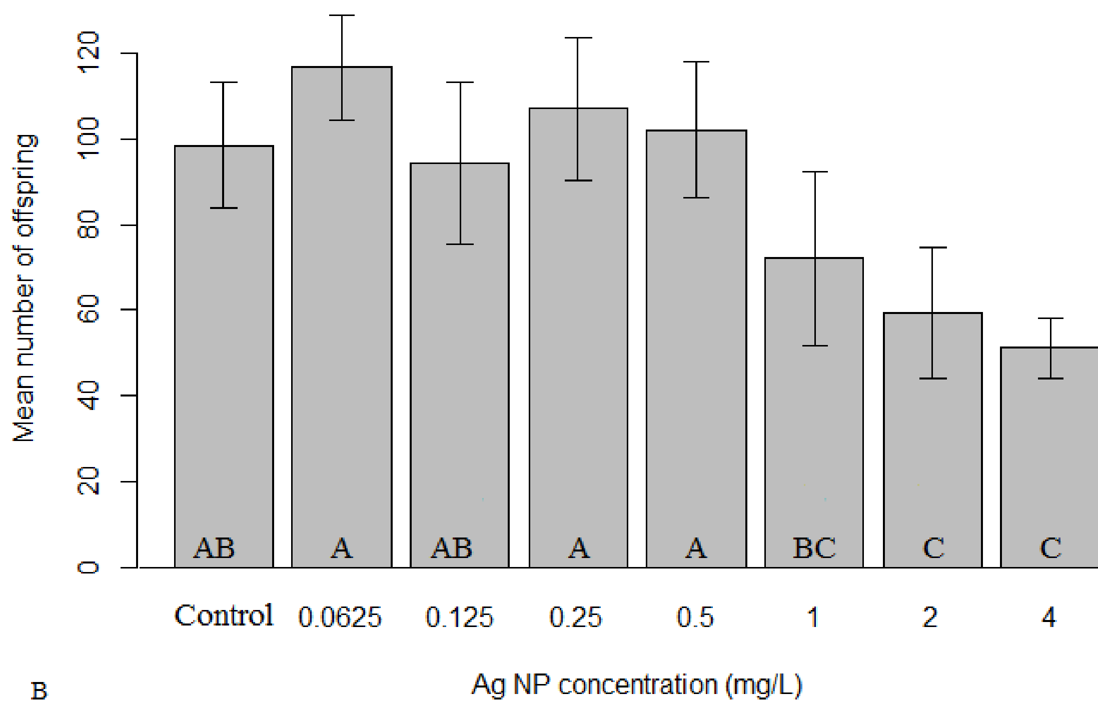


Fig 2 a and b



A



B

Fig 3

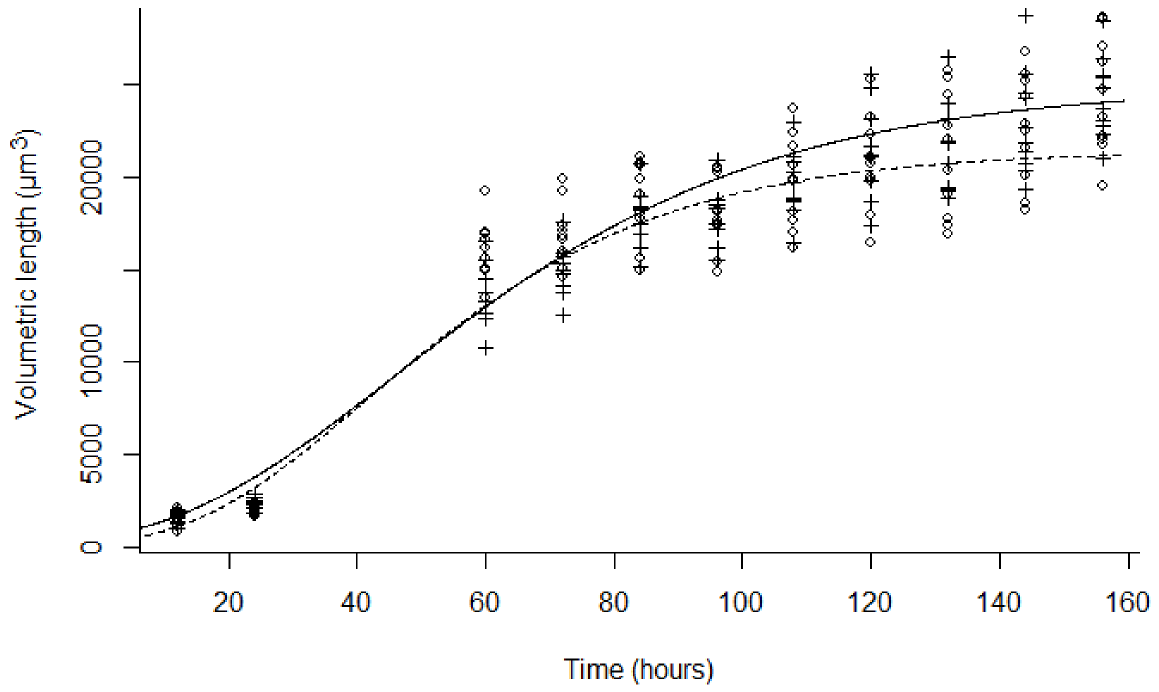


Fig 4

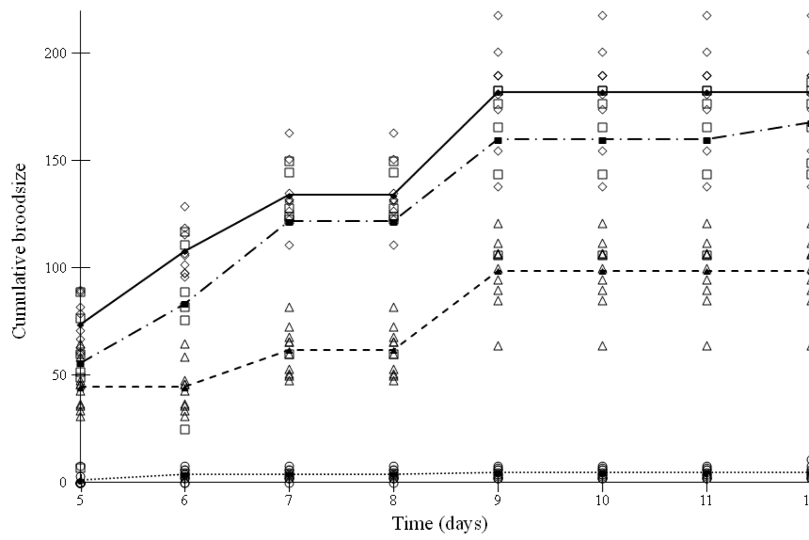
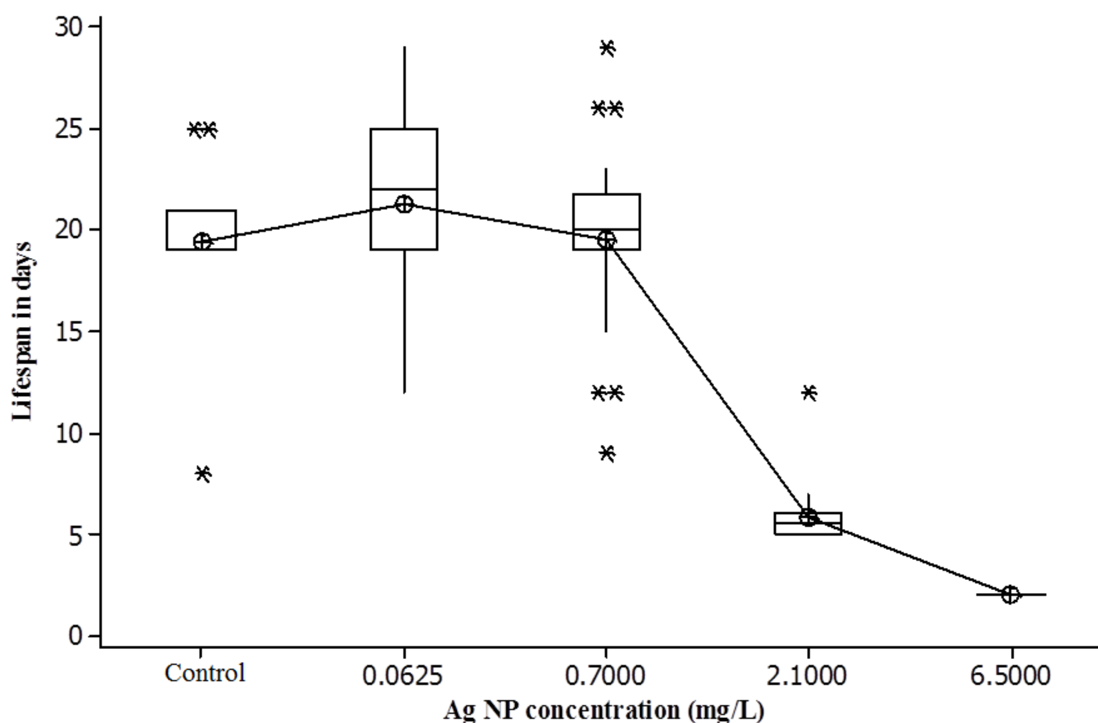


Fig 5



Supplementary material.

Legends to supplementary figures

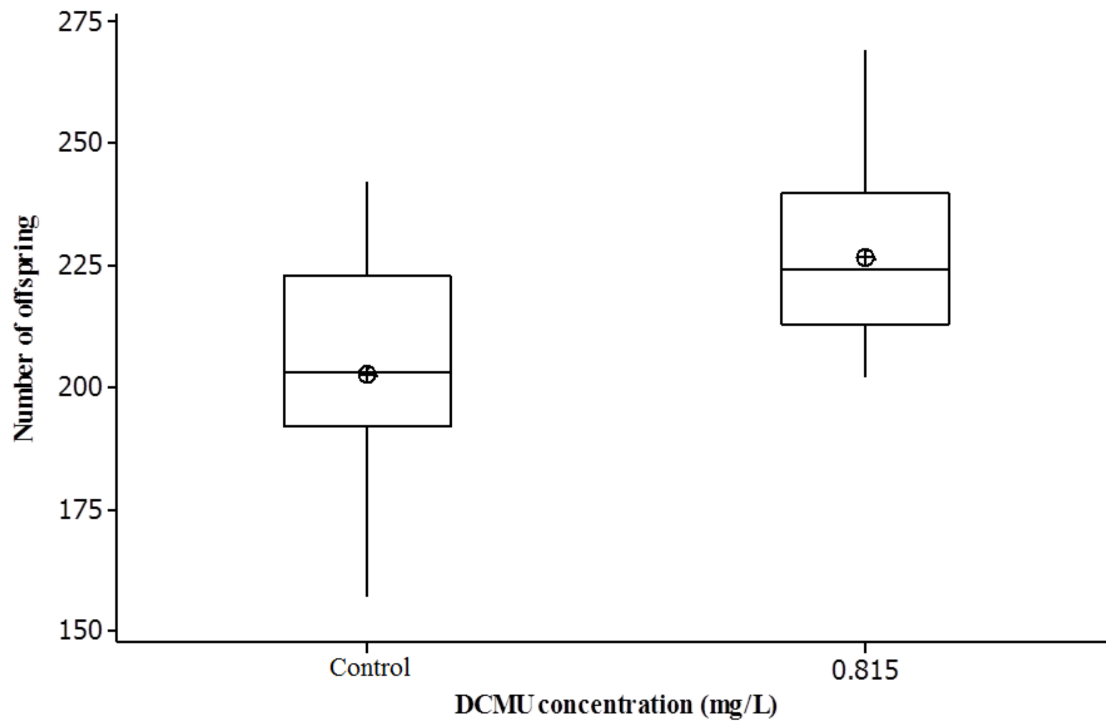
Supplementary Figure 1. Box plot showing the mean(⊕), median (–) and 2nd to 3rd quartile (the boxed region) for the reproductive output of *C. elegans* exposed over 72 hours to undosed simulated soil pore water and simulated soil pore water dosed at 0.815 mg/L of DCMU. The whiskers indicate the full extent of the data range (Q1 – Q4).

Supplementary Figure 2. Box plot showing the mean(⊕), median (–) and 2nd to 3rd quartile (the boxed region) for the reproductive output of *C. elegans* exposed over 72 hours to undosed simulated soil pore water and simulated soil pore water dosed at 0.0625 mg/L Ag NP. The whiskers indicate the full extent of the data range (Q1 – Q4).

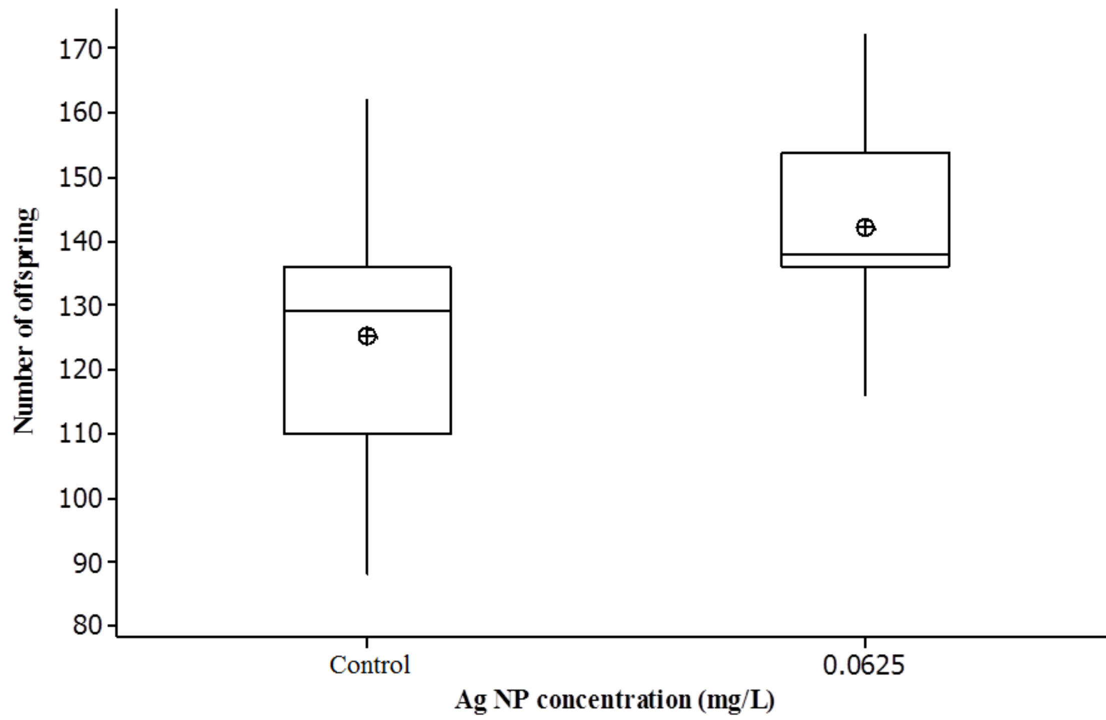
Supplementary Figure 3. Box plot showing the mean(⊕), median (–) and 2nd to 3rd quartile (the boxed region) for total brood size of *C. elegans* exposed over the full life-cycle to a range of Ag-NP

(mg/L) concentrations in simulated soil pore water. The whiskers indicate the full extent of the data range (Q1 – Q4).

Supplementary Fig 1



Supplementary Fig 2



Supplementary Fig 3