1  **Electronic supplementary information (ESI)**

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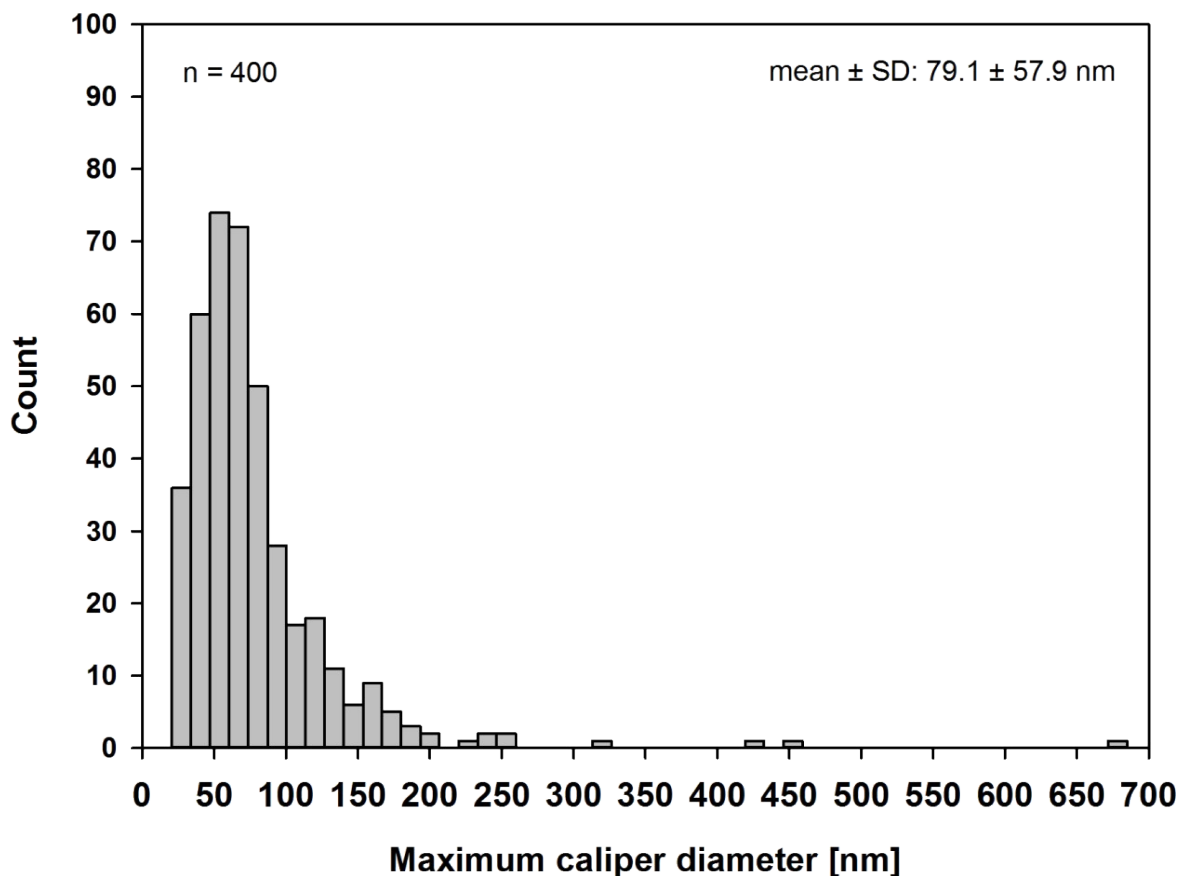


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6  **Figure S1: Primary particle size-frequency distribution based on the maximum caliper diameter of 400**

7  **NPs imaged by electron microscopy.**

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10  **Table S1: Size and shape descriptors of CuO NPs in aqueous dispersion (MQ-water).**

| | Mean | Std. Dev | Std. Error | C.I. of Mean | Max | Min | Median | 25% | 75% |
|---|---|---|---|---|---|---|---|---|---|
| **Maximum calliper diameter (Feret)** | 79.1 | 57.9 | 2.9 | 5.7 | 685.0 | 20.6 | 65.0 | 47.7 | 92.3 |
| **Minimum calliper diameter (MinFeret)** | 51.9 | 25.0 | 1.3 | 2.5 | 156.0 | 14.9 | 46.2 | 34.9 | 62.1 |
| **Aspect ratio (AR)** | 1.6 | 1.2 | 0.1 | 0.1 | 19.7 | 1.0 | 1.3 | 1.2 | 1.6 |

**Table S2. Overview of experiments carried out to determine CuO NP dissolution under different experimental conditions.**

| Original solution/dispersion (1 mg Cu mL$^{-1}$) | Further dilution in MQ before loading onto Amicon tubes | Incubation time | Ultra-filtration | Information obtained |
|---|---|---|---|---|
| **Experiment n° 1** | | | | |
| CuCl$_2$ inMQ | No | 0.5 h | No | Dissolution of CuO NPs in MQ water |
| CuCl$_2$ in MQ | No | 0.5 h | Yes | |
| CuO NPs in MQ, sonic. | No | 0.5 h in ice/water bath[a] | Yes | |
| CuO NPs in MQ, sonic. | No | 0.5 h in ice/water bath[a] | Yes | |
| | | | | Effect of sonication on CuO NP dissolution |
| **Experiment n° 2** | | | | |
| CuCl$_2$ in MQ | 1:20 (50 µg mL$^{-1}$) [b] | 0.5 h | No | Dissolution of CuO NPs in MQ with time |
| CuCl$_2$ in MQ | 1:20 (50 µg mL$^{-1}$) [b] | 0.5 h | Yes | |
| CuO NPs in MQ, sonic. | 1:20 (50 µg mL$^{-1}$) [b] | 0.5 h in ice/water bath[a] | Yes | |
| CuO NPs in MQ, sonic. | 1:20 (50 µg mL$^{-1}$) [b] | 0.5 h in ice/water bath + 7.5 h [c]at RT on rotary susp. mixer | Yes | |
| CuO NPs in MQ, sonic. | 1:20 (µg mL$^{-1}$) [b] in SGF (pH=4) | 0.5 h in ice/water bath+ 7.5 h[c] at RT on rotary susp. mixer | Yes | Dissolution of CuO NP during gastric residence time |

[a] =time and conditions used for preparation and sonication
[b] = corresponds to Cu concentration in food packages (50 µg Cu g$^{-1}$ ww food)
[c] = estimated gastric half-life in stickleback [1]
RT = room temperature, SGF = simulated gastric fluid (2.0 g NaCl L$^{-1}$ MQ, 0.0001 N HCl, pH 4).

**Table S3. Information about stickleback primers and corresponding qPCR assays.**

| Gene | Transcript ID | Sequence (5'->3') of forward (fw) and reverse (rv) primers | | Conc. [nM] | Temp. [°C] | Eff. [%] | Source |
|---|---|---|---|---|---|---|---|
| 18S rRNA (*18S*) | see reference | fw | GACTCCGGTCCTATTTTGTGG | 500 | 60 | 88.6 | [2] |
| | | rv | GCTAGTTGGCATCGTTTATGG | 500 | | | |
| β-Actin (*β-act*) | see reference | fw | CTGTCTTTCCCTCCATCGTC | 500 | 60 | 103.4 | [3] |
| | | rv | CTCTTGCTCTGGGCTTCATC | 500 | | | |
| Ubiquitin (*ubq*) | see reference | fw | AGACGGGCATAGCACTTGC | 500 | 60 | 93.4 | [2] |
| | | rv | CAGGACAAGGAAGGCATCC | 500 | | | |
| Tight junction protein 1a (*zo-1*) | ENSGACT00000020584.1 | fw | CTCTCTTAGGAGGCCCACCA | 250 | 62 | 98.6 | Designed |
| | | rv | TCTCCCCGTGTTTTCTACGC | 250 | | | |
| High affinity copper transporter (*ctr1*) | ENSGACT00000020453.1 | fw | TCAACGTCCGCTACAACTCC | 500 | 60 | 103.0 | Designed |
| | | rv | ACCTGGACGATGTGCAACAG | 500 | | | |
| Metallothionein-A (*mta*) | see reference | fw | CCCCTGCTGCCCGACTG | 500 | 60 | 102.4 | [4] |
| | | rv | TGTTCAAACTGCCGCCATCTC | 500 | | | |
| Glutamate-cystein-ligase, catalytic subunit (*gcl*) | ENSGACT00000008490.1 | fw | CGTGTTGAAATGGGGCGATG | 250 | 60 | 104.8 | Designed |
| | | rv | TCCAAAGGGTGGGGTGATTG | 250 | | | |
| Gluathione reductase (*gr*) | ENSGACT00000023248.1 | fw | GCTGCAAAACTCTGGTGTGG | 500 | 60 | 98.0 | Designed |
| | | rv | CATTTCCAAACCCATGGCGG | 500 | | | |
| Glutathione peroxidase (*gpx*) | see reference | fw | ATCAGGAGAACTGCAAGAATGAAG | 100 | 60 | 110.3 | [5](modifed) |
| | | rv | GTTCACCTTCTCAAGGAGCTG | 100 | | | |
| Superoxide dismutase-1 (*sod-1*) | ENSGACT00000027267.1 | fw | AGCAGGAGAGCGATAAAGCG | 250 | 60 | 102.5 | Designed |
| | | rv | TCATCATTAGGGCCTGCGTG | 250 | | | |
| Catalase (*cat*) | see reference | fw | ACCAAGGTTTGGTCCCACAAAG | 500 | 60 | 102.4 | [5](modified) |
| | | rv | TGCTCCACCTCTGCAAAGTAG | 500 | | | |

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**Table S4. Cu amounts ingested and egested by each fish of the different treatment groups.** The fish marked with an asterisk (*) rejected or consumed only part of the test diet during the first two feedings. See footnote (a) for information on statistically significant differences.

| Fish ID | Treatment | Total amount of Cu administered throughout experiment [µg] (nominal) | Daily administered amount of Cu [µg] (nominal) | Total amount of Cu measured in pooled faeces [µg] | Cu concentration in faeces [µg/g faeces dw] |
|---|---|---|---|---|---|
| 7 | Control | 0 | 0 | 0.04 | 0.18 |
| 26 | Control | 0 | 0 | 0.06 | 0.09 |
| 51 | Control | 0 | 0 | 0.06 | 0.34 |
| | **Mean (n=4)** | | | **0.05** | **0.2** |
| | SD (n=4) | | | ±0.01 | ±0.1 |
| 28 | $CuCl_2$ | 16.94 | 2.42 | 1.57 | 2.39 |
| 47 | $CuCl_2$ | 17.08 | 2.44 | 0.19 | 0.40 |
| 49 | $CuCl_2$ | 16.38 | 2.34 | 0.35 | 0.51 |
| *11 | $CuCl_2$ | *13.44 | 2.24 | 2.64 | *9.42 |
| | **Mean (n=4)** | | | **1.19** | **3.18** |
| | SD (n=4) | | | ±0.90 | ±3.69 |
| | *Mean (n=3) | | | 0.71 | 1.10 |
| | *SD (n=3) | | | ±0.62 | ±0.91 |
| 2 | CuO NP | 25.34 | 3.62 | 4.94 | 6.10 |
| 9 | CuO NP | 22.12 | 3.16 | 4.27 | 8.89 |
| 18 | CuO NP | 15.4 | 2.2 | N.A. | N.A. |
| 19 | CuO NP | 22.26 | 3.18 | 4.16 | 4.57 |
| 53 | CuO NP | 14.7 | 2.1 | 6.41 | 18.98 |
| | **Mean (n=4)** | | | **4.94** | **9.64** |
| | SD (n=4) | | | ±0.99 | ±5.62 |

26 a Statistical comparison between treatment groups taking into account Cu concentrations in faeces of all listed fish (Control:
27 n=3, $CuCl_2$: n=4, and CuO NP: n=4) indicated significant differences for CuO NP vs. control (Kruskal-Wallis One Way
28 ANOVA on Ranks followed by multiple comparison using Dunn's Method). Excluding fish 11 and 53, which had a
29 considerably higher Cu concentration in the faeces than the rest of the fish of their treatment group, statistical comparison
30 indicated significant differences for CuO NP vs. control (p=0.05) and CuO NP vs. $CuCl_2$ (p=0.07), but no significant
31 differences for $CuCl_2$ vs. control (One Way ANOVA followed by multiple comparison using Holm-Sidak method).¨
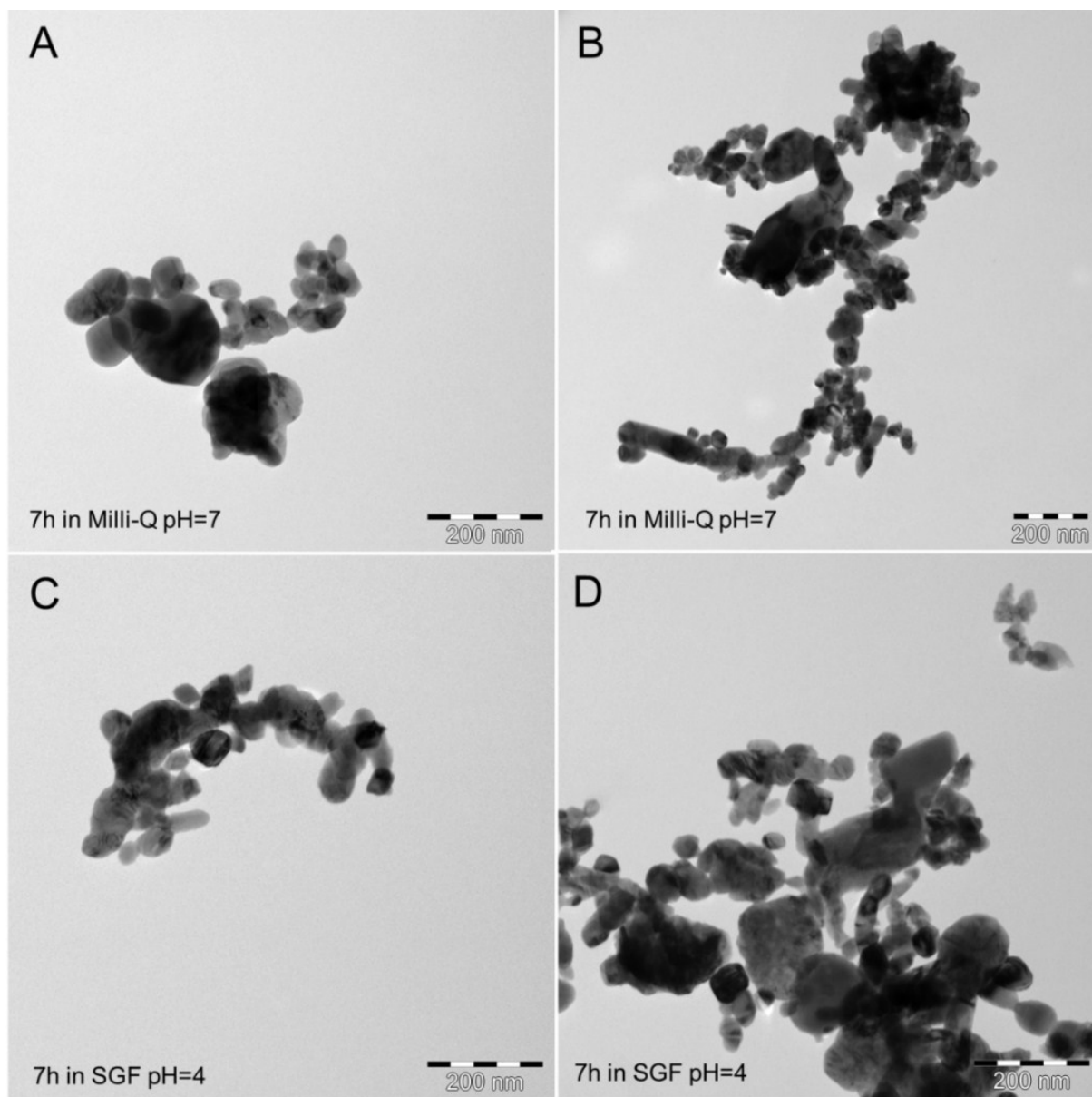
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**Figure S2:** Photograph exemplarily showing the appearance of the faeces that were daily collected from each fish.

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A
7h in Milli-Q pH=7
200 nm

B
7h in Milli-Q pH=7
200 nm

C
7h in SGF pH=4
200 nm

D
7h in SGF pH=4
200 nm

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**Figure S3: Effect of prolonged incubation time and pH on CuO NP morphology.** A and B show TEM images of CuO NPs incubated for 7h in MQ water (pH=7). C and D show CuO NPs incubated for 7h in simulated gastric fluid (SGF) (pH=4). Scale bars in all images correspond to 200 nm. Image A, C and D were taken at 100k x magnification. Image B was taken at 63k x magnification.

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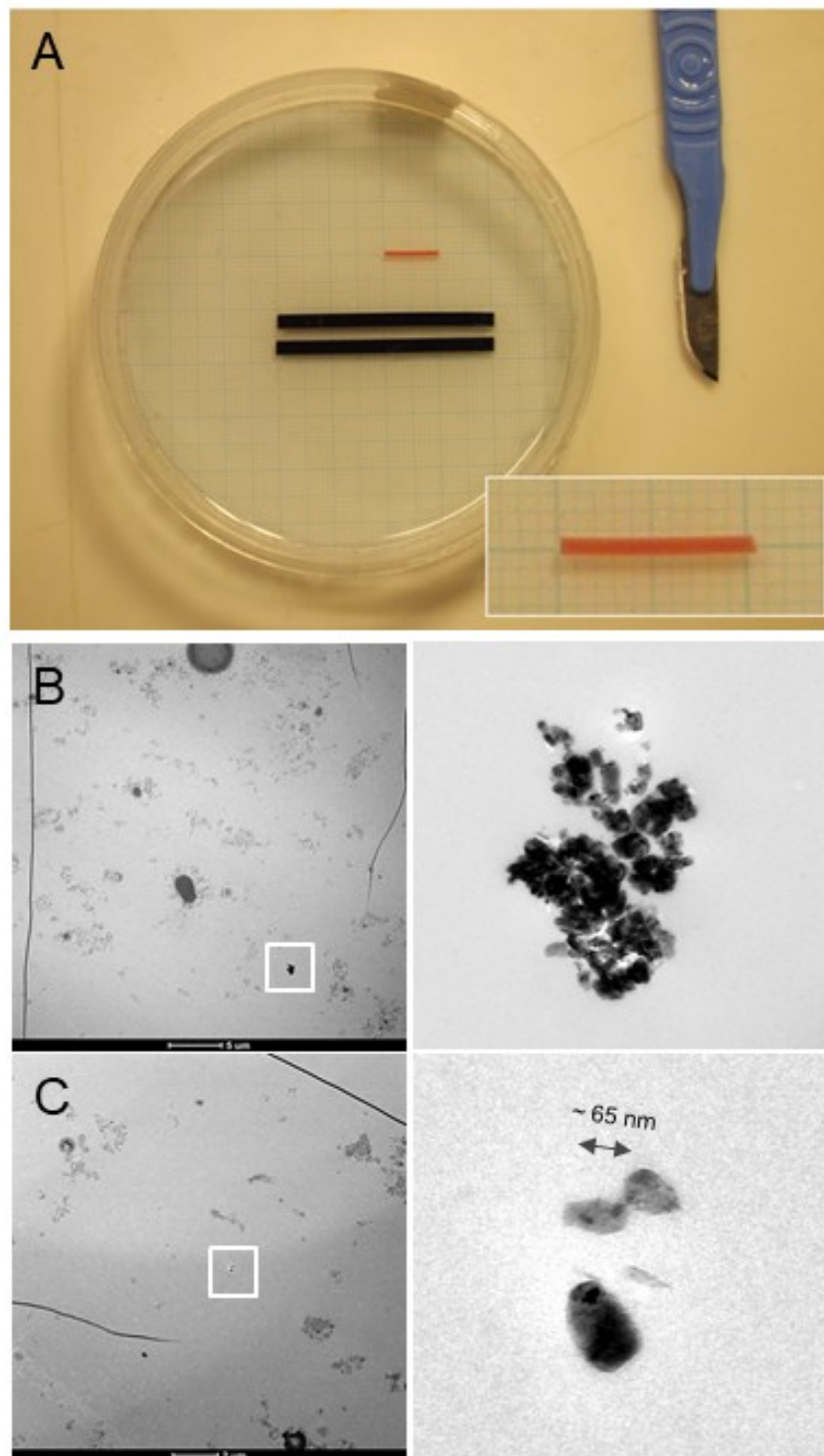
**Figure S4: Food package development and characterisation.** A. Photograph of a worm-shaped food package of pre-defined diameter and adjustable length, which was prepared from *t.tubifex* homogenate and used for accurate and reproducible dietary Cu/CuO NP exposure of three-spined sticklebacks. B and C. TEM images of CuO NPs inside the food packages. The boxed-in areas are shown at higher magnification in the images on the right. Scale bars in B and C correspond to 5 and 2 μm, respectively.

49 **Table S5: Pearson Product Moment Correlation matrix for variables measured in stickleback intestine.**
50 The cells in the first, second and third row show the correlation coefficient (Pearson's r), p-value and number
51 of samples (n) of each variable pair. Note: Pairs of variables with positive correlation coefficients and $p<0.05$
52 tend to increase together. Pairs with negative correlation coefficients and $p<0.05$, one variable tends to
53 decrease while the other increases. For pairs with $p> 0.05$, there is no significant relationship between the two
54 variables. Statistically significant correlations are indicated with asterisks (* $p<0.05$ and ** $p<0.01$).

| | ΔCq zo-1 | ΔCq ctr1 | ΔCqgcl | ΔCq gr | ΔCqmta | ΔCq sod1 | ΔCq cat | ΔCqgpx |
|---|---|---|---|---|---|---|---|---|
| **[Cu] int.** | -0.718 | -0.0732 | 0.0806 | 0.308 | -0.851 | 0.198 | 0.256 | -0.279 |
| | 0.0195* | 0.841 | 0.825 | 0.387 | 0.00179** | 0.583 | 0.475 | 0.435 |
| | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| **ΔCq zo-1** | | -0.0573 | -0.431 | -0.177 | 0.57 | -0.282 | -0.59 | 0.131 |
| | | 0.867 | 0.186 | 0.624 | 0.0671 | 0.401 | 0.0562 | 0.701 |
| | | 11 | 11 | 10 | 11 | 11 | 11 | 11 |
| **ΔCq ctr1** | | | -0.139 | 0.638 | 0.251 | -0.227 | 0.347 | -0.18 |
| | | | 0.683 | 0.0472* | 0.456 | 0.502 | 0.296 | 0.596 |
| | | | 11 | 10 | 11 | 11 | 11 | 11 |
| **ΔCqgcl** | | | | 0.061 | -0.339 | 0.00499 | 0.355 | 0.135 |
| | | | | 0.867 | 0.308 | 0.988 | 0.284 | 0.692 |
| | | | | 10 | 11 | 11 | 11 | 11 |
| **ΔCq gr** | | | | | -0.106 | -0.016 | 0.248 | -0.11 |
| | | | | | 0.772 | 0.965 | 0.489 | 0.763 |
| | | | | | 10 | 10 | 10 | 10 |
| **ΔCqmta** | | | | | | 0.178 | 0.125 | -0.155 |
| | | | | | | 0.602 | 0.714 | 0.649 |
| | | | | | | 11 | 11 | 11 |
| **ΔCq sod1** | | | | | | | 0.378 | -0.429 |
| | | | | | | | 0.251 | 0.188 |
| | | | | | | | 11 | 11 |
| **ΔCq cat** | | | | | | | | -0.497 |
| | | | | | | | | 0.12 |
| | | | | | | | | 11 |

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## RT-qPCR results obtained in stickleback liver

Seven days dietary exposure to $CuCl_2$ resulted in elevated expression levels of target genes that are involved in cellular uptake, transport and storage of Cu as well as in glutathione-dependent oxidative stress defence (Table S6). *Ctr1*, *mta*, *gcl*, *gr* and *gpx* were ~1.2, ~1.2, ~1.4, ~1.4 and ~1.5-fold increased with respect to the control (=fish receiving non-contaminated food packages), respectively. In statistical terms, the increase in mRNA expression levels was however not significant ($p > 0.05$; One Way ANOVA, Kruskal-Wallis One Way ANOVA on Ranks and t-test). For *sod-1* no differences were observed compared to the control. For *cat* the $CuCl_2$-treatment seemed to have caused a slight decrease in mRNA expression levels. In liver of CuO NP-exposed fish no differences in the expression of the selected target genes were observed compared to control fish Table S6. Only *gcl* and *gpx* were found to be slightly higher expressed at the mRNA level (1.1-fold and 1.2-fold, respectively). For *cat* the CuO NP-treatment seemed to have caused a slight decrease in mRNA expression levels (0.7-fold). Interestingly, *ctr1* expression levels were strongly correlated with *gr* and *gpx* mRNA mRNA expression levels (Pearson's r=~0.7 and p=~0.015). Furthermore, there was a moderate, albeit statistically not significant correlation between *ctr1* and *gcl* transcript levels (Table S 7). This implicitly suggests that there was a relationship between hepatic Cu uptake and induction of the glutathione system in the liver of sticklebacks. However, representation of the corresponding data in form of a scatter plot showed that there was no treatment-specific clustering of the individuals, which had higher hepatic expression levels of the above stated genes (not shown).

**Table S6: Relative mRNA expression levels of selected target genes in liver of three-spined stickleback upon seven days of dietary exposure to $CuCl_2$ and CuO NPs.** Values in bold represent the mean fold change (FC) with respect to the negative control (cntrl). Values in square brackets indicate the deviations from the mean FC in both directions, which were calculated from the SD of $\Delta\Delta C_q$ values (n ≥ 3). No statistically significant differences were observed between treatment groups.

| Target gene | Fold change | | |
|---|---|---|---|
| | Cntrl | $CuCl_2$ | CuO NP |
| *mta* | **1.00** [-0.09 / +0.10] | **0.70** [-0.17 / +0.23] | **0.67** [-0.25 / +0.40] |
| *ctr1* | **1.00** [-0.37 / +0.60] | **1.19** [-0.43 / +0.67] | **0.92** [-0.36 / +0.60] |
| *gcl* | **1.00** [-0.49 / +0.97] | **1.35** [-0.27 / +0.34] | **1.13** [-0.31 / +0.43] |
| *gr* | **1.00** [-0.41 / +0.69] | **1.38** [-0.49 / +0.76] | **1.02** [-0.26 / +0.35] |
| *gpx* | **1.00** [-0.23 / +0.31] | **1.49** [-0.74 / +1.48] | **1.22** [-0.29 / +0.37] |
| *sod-1* | **1.00** [-0.54 / +1.20] | **0.95** [-0.49 / +1.00] | **1.07** [-0.36 / +0.54] |
| *cat* | **1.00** [-0.55 / +1.24] | **0.77** [-0.17 / +0.21] | **0.68** [-0.27 / +0.46] |

88 **Table S7 : Pearson Product Moment Correlation matrix for variables measured in stickleback liver.** The
89 cells in the first, second and third row show the correlation coefficient (Pearson's r), p-value and number of
90 samples (n) of each variable pair. Note: Pairs of variables with positive correlation coefficients and $p < 0.05$
91 tend to increase together. Pairs with negative correlation coefficients and $p < 0.05$, one variable tends to
92 decrease while the other increases. For pairs with $p > 0.05$, there is no significant relationship between the
93 two variables. Statistically significant correlations are indicated with asterisks (* $p < 0.05$ and ** $p < 0.01$).

| | ΔdCq ctr1 | ΔCqgcl | ΔCq gr | ΔCqmta | ΔCq sod-1 | ΔCq cat | ΔCqgpx |
|---|---|---|---|---|---|---|---|
| **[Cu] liver** | -0.0411 | 0.013 | 0.0184 | -0.342 | 0.248 | 0.379 | -0.0249 |
| | 0.91 | 0.972 | 0.96 | 0.333 | 0.489 | 0.28 | 0.945 |
| | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| **ΔCq ctr1** | | 0.392 | 0.7 | -0.183 | 0.62 | -0.0103 | 0.711 |
| | | 0.233 | 0.0164* | 0.591 | 0.042 | 0.976 | 0.0141* |
| | | 11 | 11 | 11 | 11 | 11 | 11 |
| **ΔCqgcl** | | | 0.633* | 0.316 | 0.592 | 0.553 | 0.498 |
| | | | 0.0364 | 0.343 | 0.0549 | 0.0778 | 0.119 |
| | | | 11 | 11 | 11 | 11 | 11 |
| **ΔCq gr** | | | | -0.202 | 0.797 | -0.00108 | 0.778 |
| | | | | 0.551 | 0.00332** | 0.997 | 0.00483** |
| | | | | 11 | 11 | 11 | 11 |
| **ΔCqmta** | | | | | -0.0081 | 0.583 | -0.043 |
| | | | | | 0.981 | 0.0595 | 0.9 |
| | | | | | 11 | 11 | 11 |
| **ΔCq sod-1** | | | | | | 0.183 | 0.788 |
| | | | | | | 0.59 | 0.00395** |
| | | | | | | 11 | 11 |
| **ΔCq cat** | | | | | | | -0.108 |
| | | | | | | | 0.751 |
| | | | | | | | 11 |

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