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Supplemental Information for:

Divergent brain gene expression profiles between alternative behavioural helper types in a cooperative breeder

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1. Association between egg cleaning and defence

Cleaners were not more likely to defend than non-cleaners (Chi-square test with simulated p-value; X^2 = 2.22, p = 0.45 (Table S1), but cleaners performed more defence behaviours than non-cleaners (Welch two sample t-test; t = 2.79, df = 10.7, p = 0.02, Table S2).

Table S1: Associations of the propensities for egg cleaning and defence.

	cleaners	**non-cleaners**
defence yes	10	8
defence no	0	2

Table S2: Amount of defence behaviours performed by cleaners and non-cleaners during the opportunity.

	cleaners	**non-cleaners**
	1	0
	40	14
	21	7
	56	13
	33	30
	76	4
	2	12
	83	10
	19	5
	26	0
Mean	**35.7**	**9.5**
Standard deviation	**28.3**	**8.8**

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2. Sample sizes throughout the experiment

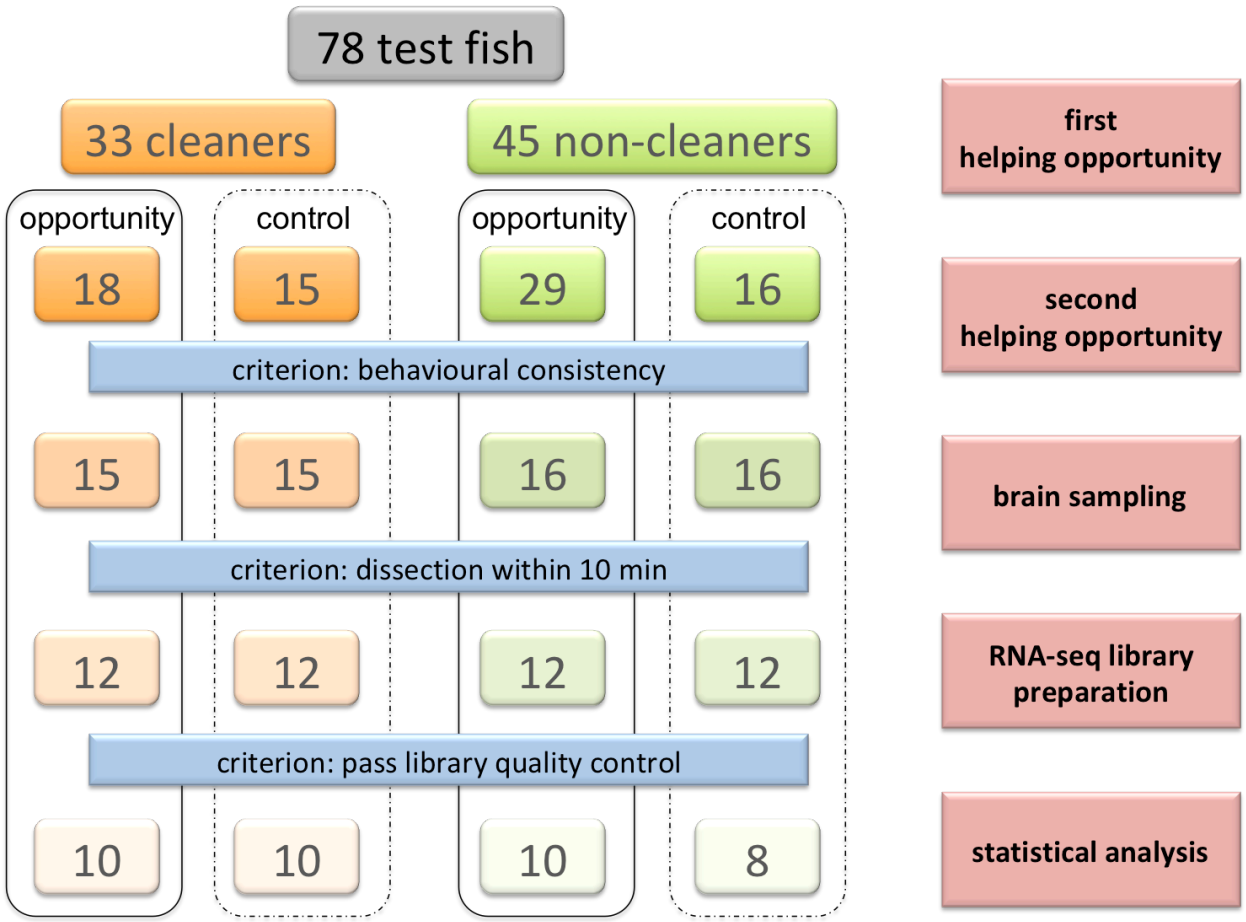

Fig. S1: The initial sample sizes and their reduction through the stages of the experiment. 78 test fish entered the experiment. Subjects were removed from the experiment if they did not show the same egg-cleaning propensity in the second opportunity, when their brain could not be removed within 10 min after sacrificing them, and when the RNA-seq libraries did not fulfill the quality control (see Table S3). Thus, the differential gene expression analysis was based on 38 test fish.

3. Trimmomatic settings

Trimmomatic software v.0.36 (Bolger, Lohse, & Usadel, 2014) was used to remove adapters sequences and low-quality sequences. We set '-phred33' for base quality encoding, using sliding window of 20 base pairs (bp) with a quality threshold of 2, removing sequences at both ends below quality threshold of 2 remove sequences below length of 70 bp (Hebert, 2017).

4. Quality control criteria for libraries

All RNA-seq libraries had to fulfill the following criteria to enter the final analysis for differential gene expression: i) a number of reads >10M, ii) a proportion of duplicates <80%, iii) an average per-base quality (Phred score) ≥35, and iv) balanced sequence content (proportion of A, T, C and T must not vary importantly from one position to the next). This led to the exclusion of 10 samples (see Table S3).

Table S3: Quality control of samples grouped by experimental condition and helper phenotype for RNA extracts (RIN – RNA Integrity Number) and RNA-seq libraries. Diagnostics below the threshold (see text above) are highlighted in red, removed samples are highlighted in gray.

sample	condition	phenotype	RIN	total reads	% duplicates	per-base quality	sequence content
1I6b	control	cleaner	NA	29857326	62	36	good
1X9b	control	cleaner	9,3	21362334	53	37	good
4G1b	control	cleaner	9,7	19208554	57	36	good
4P8b	control	cleaner	9,6	30866178	56	36	good
5U1c	control	cleaner	9,1	17282729	50	37	ok
5V7c	control	cleaner	9,8	19844389	59	36	good
6J7g	control	cleaner	9,7	32406684	58	36	good
6Ü7b	control	cleaner	9,9	21301108	51	37	good
7X9c	control	cleaner	9,5	35521062	68	36	good
8P4e	control	cleaner	9,7	23347931	53	36	good
8V8e	control	cleaner	9,7	5277152	94	35	bad
3Q1b	control	cleaner	NA	11704954	80	35	bad
3A3c	control	non-cleaner	10,0	20465876	52	36	good
3Q9c	control	non-cleaner	8,7	25160465	56	36	good
4K3d	control	non-cleaner	9,5	26388400	54	36	good
5Ü7b	control	non-cleaner	9,3	22927709	57	36	good
6J9f	control	non-cleaner	9,8	23246291	55	36	ok
6V3c	control	non-cleaner	9,9	36305924	64	36	good
8V8d	control	non-cleaner	9,8	21664611	52	36	good
9A9d	control	non-cleaner	9,8	18594990	49	37	good
4M7b	control	non-cleaner	9,8	512706	87	28	very bad
9F6b	control	non-cleaner	9,5	884513	96	33	very bad
1O1b	control	non-cleaner	10,0	15239373	97	31	bad
6J7i	control	non-cleaner	NA	1174304	82	35	bad
1I6e	opportunity	cleaner	9,7	26397069	59	37	good

Table S3 continued

sample	condition	phenotype	RIN	total reads	% duplicates	per-base quality	sequence content
1X9a	opportunity	cleaner	NA	16988848	70	36	good
3Q1c	opportunity	cleaner	10,0	20285452	51	37	good
4P8c	opportunity	cleaner	9,7	25149072	59	37	good
5U1a	opportunity	cleaner	9,6	23762752	55	36	good
6V9b	opportunity	cleaner	7,1	17040636	65	35	good
7R6e	opportunity	cleaner	9,7	17728033	48	36	good
7X9b	opportunity	cleaner	9,8	20196519	51	36	good
8V6b	opportunity	cleaner	9,5	18828265	52	37	good
9V2d	opportunity	cleaner	9,4	19404757	50	37	good
6Ü7c	opportunity	cleaner	9,8	3316930	87	35	bad
5V7d	opportunity	cleaner	9,8	16084735	98	32	bad
1O1a	opportunity	non-cleaner	9,9	20446246	50	36	good
2S3a	opportunity	non-cleaner	9,1	21359521	64	36	ok
3Q9h	opportunity	non-cleaner	9,8	23077228	61	37	good
4K3b	opportunity	non-cleaner	9,8	28037022	57	37	good
4M7a	opportunity	non-cleaner	9,3	24905698	55	36	good
5Ü7d	opportunity	non-cleaner	9,8	28731210	56	37	good
6J7c	opportunity	non-cleaner	9,6	28923836	57	36	good
6J9a	opportunity	non-cleaner	9,7	19014450	55	37	good
9A9c	opportunity	non-cleaner	9,3	21447518	54	36	good
9F6d	opportunity	non-cleaner	7,0	23051743	54	36	good
8V8c	opportunity	non-cleaner	9,7	719673	94	36	ok
6V3e	opportunity	non-cleaner	9,5	20133658	79	33	bad

5. Aligning reads to reference vs de novo transcriptome assembly

We chose to align raw reads onto the published Nile tilapia (*Oreochromis niloticus*) genome instead of a de novo transcriptome assembly from the reads sequenced in this experiment. In the following, we list the reasons for this decision. The raw data generated in this study does not apply to this approach because they are short and single-end. Performing a de novo transcriptome assembly with short single-end reads using data from multiple individuals typically results in a highly fragmented and repetitive reference (Conesa et al., 2016), which becomes problematic when analyzing gene expression levels. In this context, we expect that the de novo transcriptome will be characterized by numerous very similar contigs due to allele splitting, i.e. sufficiently different alleles will be split into independent contigs by the assembler, thus increasing the number of redundant sequences in the final assembly and creating an abundance of unusable multi-mapping reads. This would ultimately bias gene expression levels. Accurate transcript reconstruction from short and unpaired reads is extremely difficult, not

recommended and generally considered suboptimal (Engström et al., 2013). Complete reconstruction of a transcriptome with the type of raw data that we have is a challenging problem that will result in hundreds of thousands of contigs accounting for fragmented transcripts. This will result in a final read-count matrix with hundreds of thousands of potential transcripts with very few counts for each transcript in each individual, a scenario in which the detection of differentially expressed genes becomes almost impossible, or at least very unlikely (too many comparisons and not enough per-individual counts to have sufficient statistical power). Previous work on RNAseq experiments evaluating different designs and strategies showed that mapping short reads on a well annotated genome of a divergent sister clade (especially in the context of non paired-end reads) outperformed de novo assembly in the identification of differentially expressed genes (Vijay, Poelstra, Künstner, & Wolf, 2013). In addition, due to the high content of gene duplicates in the genomes of cichlids in general, assembling a de novo transcriptome with short unpaired reads seems like an impossible task.

Considering all of these elements combined together, we chose to use a very well constructed and annotated genome generated with long PacBio sequencing reads (Conte, Gammerdinger, Bartie, Penman, & Kocher, 2017), thus ensuring that we have a proper gene annotation to work with, as well as complete and non redundant gene sequences on which to align our short reads. *O. niloticus* and *N. pulcher* are two closely related species separated by ~15-25 My of evolution (Genner et al., 2007). *O. niloticus* represents the closest and most complete and well annotated genome that can be used in the context of this study and the high quality of this reference genome accounts for more in the “mapping success” than the divergence time between the two species (Henning & Meyer, 2014). Furthermore, in a study on adaptive radiation in African cichlid fish (Brawand et al., 2014) the authors note in the supplemental information that they used the *O. niloticus* genome as a proxy for other cichlids because they did not detect any major genomic rearrangements between them. Using cytogenetic mapping, (Mazzuchelli, Kocher, Yang, & Martins, 2012) concluded that there is strong chromosomal conservation among nine cichlid genomes. Further evidence for high synteny and coding sequence

conservation comes from a study on coding sequences of isotocin and vasotocin in African cichlids (O'Connor, Marsh-Rollo, Ghio, Balshine, & Aubin-Horth, 2015).

Nonetheless, we decided to perform a genome-guided de novo transcriptome assembly to try to evaluate the type of reference sequences that we could produce with our raw sequencing data, as compared to the Nile tilapia reference genome. Using the Trinity pipeline, we generated the following transcriptome, and compared it to the Nile tilapia gene set (Table S4).

Table S4: Assembly metrics of *N. pulcher* transcriptome using the Trinity pipeline compared to the Nile tilapia reference genome (Conte, Gammerdinger, Bartie, Penman, & Kocher, 2017).

	N. pulcher	***O. niloticus***
Total number of contigs assembled	132 804	~50 000
Total transcriptome length	65 Mb	168 Mb
Max contig length	8 489	92 220
Min contig length	251	92
Median contig length	363	2612
N50	511	4278

As expected, we obtained a shorter and much more fragmented transcriptome assembly with our data, as compared to the current Nile tilapia reference genome. We tried to reduce potential redundancy created by split alleles or gene isoforms, using CD-HIT-EST (http://weizhongli-lab.org/cd-hit/) on the assembled contigs. This program clusters very similar sequences into longer consensus sequences. We were able to reduce the number of contigs to 110,781 (instead of 132,804) based on a relaxed 90% similarity threshold and on an 85% alignment coverage, but it still represents a high number of transcripts to annotate and work with. The annotation part would also be very problematic and would potentially generate inaccuracy in the identification of transcripts, and thus biased measures of gene expression. These results further support our initial approach of read mapping on a closely related species, in this case *O. niloticus*.

6. Code used for differential gene expression analysis in DESeq2

a) creating DESeq2 objects:

Create DESeq2 object with interaction. 'help' refers to helper type, 'cond' to condition

```
deseqInt <- DESeqDataSetFromMatrix(countData = dataRNAseq, colData = colData,
design = ~ help*cond, tidy = FALSE)
deseqInt$help <- relevel(deseqInt$help, ref = "NH") # reorder factor levels
so that nonhelp (NH) is reference
deseqInt$cond <- relevel(deseqInt$cond, ref = "C") # reorder factor levels so
that control (C) is reference
```

Create DESeq2 object without interaction. 'group' is concatenated label of condition – C(ontrol) and T(est) with helper type HH(elper) and N(on)H(elper). 4 groups: CHH, CNH, THH, TNH.

```
deseq <- DESeqDataSetFromMatrix(countData = dataRNAseq, colData = colData,
design = ~ group, tidy = FALSE)
deseq$group <- relevel(deseq$group, ref = "CNH") # reorder factor levels so
that control nonhelp (CNH) is reference
```

b) Pre-filtering:

Before DEG analysis, remove genes that have no value for the comparison due to their low expression levels to reduce computation time.

```
dds <- deseq[rowSums(counts(deseq)) >1,]
```

c) DEG analysis:

```
dds <- DESeq(dds, test = "Wald", fitType = "parametric")
```

d) Contrasts:

interaction model:

i) Interaction

```
resultsInter <- results(dds.Int, name="helpHH.testT")
```

ii) Is gene expression in nonhelpers and helpers different in control?

```
resultsCNHvsCHH <- results(dds.Int, contrast = c("help", "HH","NH"))
```

iii) How do helpers react to cooperation opportunity?

```
resultsCHHvsTHH <- results(dds.Int, list(c("cond_T_vs_C", "helpHH.condT")))
```

iv) How do nonhelpers react to cooperation opportunity?

```
resultsCNHvsTNH <- results(dds.Int, contrast = c("cond", "T", "C"))
```

v) Is gene expression in nonhelpers and helpers different in the opportunity?

```
resultsTNHvsTHH <- results(dds.Int, list(c("help_HH_vs_NH", "helpHH.condT")))
```

without interaction:

i) Is gene expression in nonhelpers and helpers different in control?

```
resultsCNHvsCHH <- results(dds, contrast = c("group", "CHH","CNH"))
```

ii) How do helpers react to cooperation opportunity?

```
resultsCHHvsTHH <- results(dds, contrast = c("group", "THH", "CHH"))
```

iii) How do nonhelpers react to cooperation opportunity?

```
resultsCNHvsTNH <- results(dds, contrast = c("group", "TNH","CNH"))
```

iv) Is gene expression in nonhelpers and helpers different in the opportunity?

```
resTHHvsTNH <- results(ddsN, contrast = c("group", "THH", "TNH"))
```

For each contrast, the resulting gene lists were ordered according to the FDR-corrected p-value, for instance:

```
resultsOrdCNHvsCHH <- resultsCNHvsCHH[order(resultsCNHvsCHH$padj),]
```

7. Filtering before differential gene expression analysis

In order to increase confidence in our results, we attempted to increase statistical power of the differential gene expression analysis by applying more stringent filtering to the read counts. The default approach recommended in the DESeq2 online manual (https://www.bioconductor.org/packages/devel/bioc/vignettes/DESeq2/inst/doc/DESeq2.html #pre-filtering) is to pre-filter all genes that have equal to or less than 1 read in total (across all samples) and thus have no value for the comparison due to their low expression levels (see 5b). This mainly serves the purpose of reducing computation time, whereas the actual filtering based on Cook's distance is performed within the DESeq2 function. However, since this filtering method does not consider any biological information (e.g. number of test fish per group that express a certain gene) we tried out a range of filters to explore whether they lead to the exclusion of more genes but, most importantly, also to an increase of DEGs. To do so, we defined 3 different criteria for genes to exclude from the final dataset: in a specific test condition – helper phenotype combination (i.e. cleaners in control, cleaners in test, non-cleaners in control and non-cleaners in test): a) ½, b) 2/3 and c) all individuals had to express the gene. Genes not fulfilling the criterion were removed from the DESeqDataSet object and hence from all contrasts (Table S5). This led to a removal of 10288 genes in the original analysis and 17397 for a), 18976 for b) and 21817 for c). The differential gene expression analysis was then conducted on the filtered gene lists and Table S5 lists the number of differentially expressed genes in the different filtering scenarios for FDR < 0.1 and FDR < 0.05. Even though the filters led to the removal of more genes and smaller datasets, the amount of DEGs stayed more or less the same as in the original analysis, regardless of the stringency of the filter.

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Table S5: Number of DEGs for each of the contrasts and two different levels of FDR for the original pre-filter suggested in the DESeq2 manual, for a filter based on a) 50%, b) 2/3 and c) 100% of individuals in each condition-phenotype combination. The number of genes that the DESeq2 inbuilt filter removed is given below for each contrast (# of genes removed).

contrast	**FDR**	**original**	**a)**	**b)**	**c)**
control	< 0.1	1	2	2	1
non-cleaners vs cleaners	< 0.05	1	1	2	1
# genes removed		2	0	0	0
non-cleaners	< 0.1	15	12	12	12
control vs opportunity	< 0.05	12	15	15	15
# genes removed		10912	4077	2263	0
cleaners	< 0.1	24	24	21	13
control vs opportunity	< 0.05	10	13	11	9
# genes removed		4912	1223	0	0
opportunity	< 0.1	0	0	0	0
non-cleaners vs cleaners	< 0.05	0	0	0	0
# genes removed		0	0	0	0
interaction	< 0.1	0	0	0	0
	< 0.05	0	1	1	0
# genes removed		2	0	0	0

Table S5 clearly demonstrates that applying stricter filtering to the lists of genes fed into the DEG analysis does not yield a higher number of DEGs. To the contrary, it seems that filtering that is too strict (2/3 or 100% of individuals have to express a particular gene) leads to the removal of genes that were identified as differentially expressed in some contrasts in the original analysis.

8. Permutation tests

In order to check whether the number of differentially expressed genes (DEGs) in our experiment could have arisen by chance alone we conducted a permutation test. In this test we permuted the assignment of each individual to a specific experimental group/cleaner phenotype combination and carried out differential expression analysis in DESeq2 as specified for the real data in the Methods section of the main text. We repeated this process 2,000 times and compared the resulting null distribution of the number of DEGs obtained to the number of DEGs in the analysis with the real dataset. Fig. S2 shows the location of the results of the differential gene expression analyses with the original data in relation to these null distributions for the overall analysis (Panel A) as well as for single pair-wise comparisons (Panels B to E). It can be seen from these results that in cases where we find more than one DEGs in the real data (A sum over all pairwise comparisons, C cleaners control vs opportunity and D non-cleaners control vs opportunity), most of the null distributions yield a lower number of DEGs, i.e. lie to the left of the real value. This indicates that the number of DEGs is unlikely to have arisen by chance. In the comparison that resulted in only one (B control cleaners vs non-cleaners) or zero DEG (opportunity cleaners vs non-cleaners), this effect is certainly less pronounced because the distributions are bounded by zero. Hence, whereas this method is suitable to support the results of differential gene expression analysis when the number of DEGs lies in the tens, it is less suitable when the number of DEGs is even lower than that because there is much less opportunity for random results being smaller or equal to the real number of DEGs.

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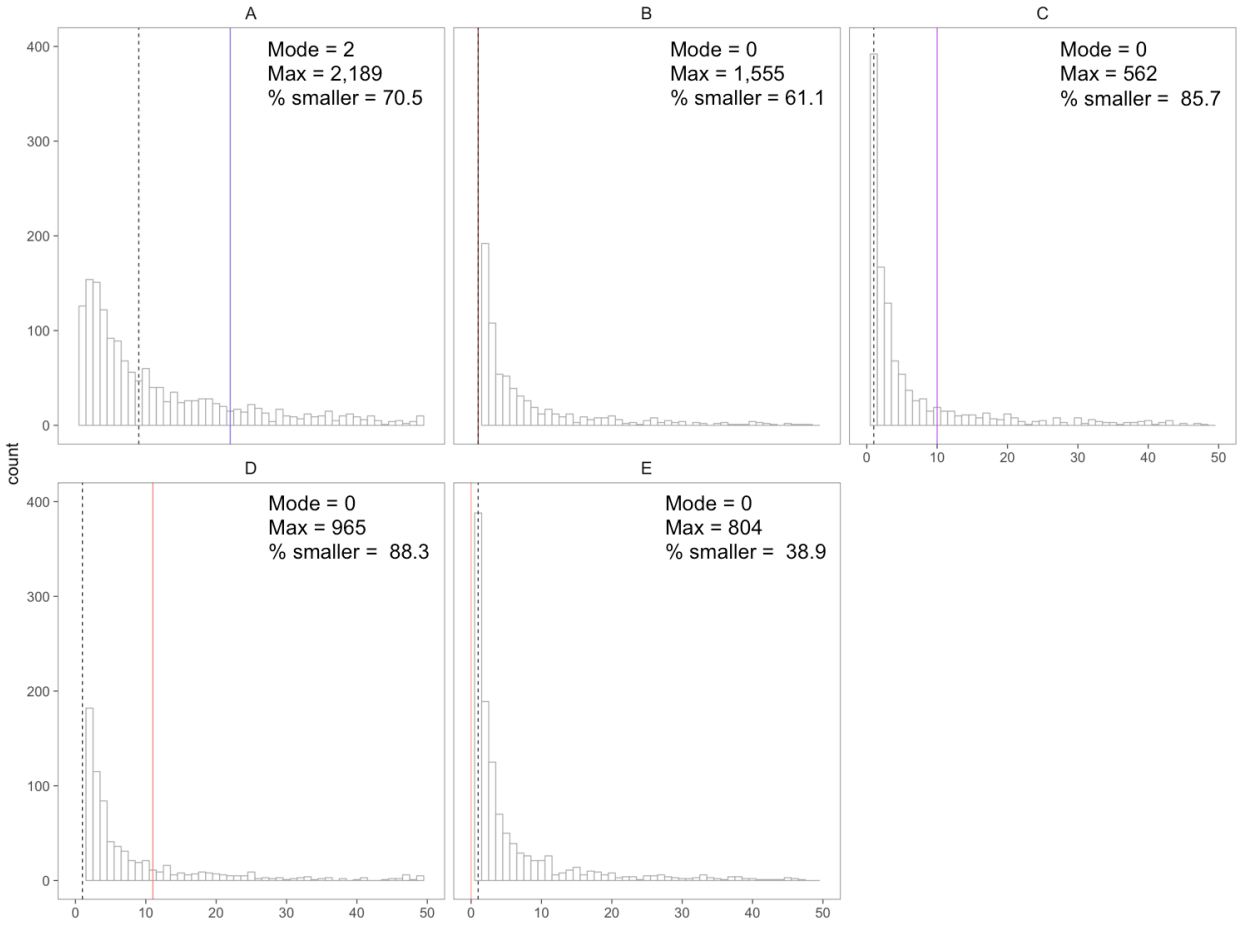

Fig. S2: Histograms of null distributions of differential gene expression analyses created by 2,000 random permutations of experimental group (control or opportunity) and cleaner type (cleaners or non-cleaners) combination. Dashed vertical lines denote the medians of the null distributions, and colored vertical lines the number of differentially expressed genes obtained with the real data. Panel A shows the sum of differentially expressed genes across all pairwise comparisons. Panel B shows non-cleaners vs cleaners in control, panel C cleaners in control vs opportunity, panel D non-cleaners in control vs opportunity and panel E non-cleaners vs cleaners in opportunity. For reasons of visibility the x-axis is cut off at a count of 50, even though the distributions have long tails (maximum indicated by 'max' in the in box in the upper right corner of each panel). The modes of the distributions, which is the most frequent number of genes identified as differentially expressed, as well as the percentage of the null distribution that yield lower or equal numbers of differentially expressed genes than the real data ('% smaller') are also given in the box.

9. Correlations of behaviours and gene expression

The number of reads of genes identified as differentially expressed did not correlate with the amounts egg-cleaning or with defence behaviours. This indicates that the most informative part of the variation is indeed expressed in the dichotomy of cleaners vs. non-cleaners rather than in a quantitative measure of these behaviours.

Table S6: Correlations of gene expression (of DEGs) and amounts of cleaning / defence behaviours during the opportunity.

	egg cleaning		**defence**	
gene name	**rho**	**p-value**	**rho**	**p-value**
irx2	0.09	0.696	0.04	0.867
c-fos	0.05	0.843	0.39	0.086
H38	-0.04	0.879	0.14	0.563
mb9.15	0.36	0.119	0.28	0.240
csrnp1b	-0.09	0.715	0.02	0.940
epsti1	0.39	0.091	-0.37	0.111
rsad2	-0.06	0.803	-0.22	0.362
ido2	-0.12	0.613	0.18	0.460
neurod1	0.25	0.295	-0.23	0.331
dach1	-0.26	0.259	-0.15	0.527
npas4	0.19	0.433	0.22	0.362
npasdc4b	0.28	0.230	-0.01	0.960
npasdc4b-like	-0.10	0.662	-0.07	0.765
plk2	0.17	0.479	0.06	0.791
egr1	0.22	0.360	-0.12	0.629
egr2	0.22	0.341	0.26	0.264
ier2	0.13	0.595	0.08	0.722
uncharacterized (XR_002063607.1)	0.03	0.884	-0.31	0.178

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10. Comparison of gene sets in cleaners and non-cleaners

Table S7: Genes that were up-regulated in non-cleaners during the opportunity showed similar regulation in cleaners (A). Numbers in parentheses denote fold-change in the original comparison (see Table 2). Genes that were differentially regulated in cleaners during the opportunity mainly showed no expression differences in non-cleaners (B). Numbers in parentheses denote fold-change in the original comparison (see Table 1). The full lists of genes (i.e. including those with FDR > 0.05) are given in Table S9.

	GenBankID	abbr	mean	FC	lfcSE	stat	pvalue	padj
A	XM_003444618.4	npas4	2495.747	1.3 (1.4)	0.096	4.037	5.42E-05	0.098
	XM_019367106.1	nps4b	33.575	1.4 (1.6)	0.119	3.873	0.0001	0.160
	XM_003452282.4	egr-1	1512.105	1.3 (1.4)	0.099	3.333	0.0009	0.346
	XM_005452100.3	plk2	2954.527	1.2 (1.4)	0.096	3.152	0.002	0.424
	XM_019367107.1	nps4l	27.634	1.3 (1.5)	0.124	2.770	0.006	0.625
	XM_003454061.4	egr-2	37.455	1.3 (1.5)	0.126	2.607	0.009	0.652
	XM_003452801.4	ier2	23.450	1.2 (1.5)	0.126	1.906	0.057	0.847
	XR_002063607.1	unch	13.765	1.0 (1.3)	0.085	0.217	0.828	0.998
B	XM_003453237.3	rsad2	6.846	1.0 (1.3)	0.084	0.797	0.425	1
	XM_005450549.3	irx2	9.136	-1.0 (-1.4)	0.083	-0.105	0.917	1
	XM_005474264.3	dach1	205.732	-1.1 (-1.4)	0.107	-0.837	0.403	1
	XM_003439202.4	csrnp1b	800.369	1.2 (1.3)	0.087	2.809	0.005	1
	XM_005457455.3	ido2	4.8345	1.0 (1.4)	0.116	0.605	0.545	1
	XM_003451558.4	neurod1	75.538	-1.0 (-1.3)	0.089	-0.703	0.482	1
	XM_005475227.3	epsti1	13.615	-1.1 (1.4)	0.095	-1.071	0.284	1

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11. Additional tables

Table S8: Complete gene lists from DESeq2 analysis for differential gene expression including phenotype-by-condition interaction. The lists include Entrez gene ID, annotation, mean expression across groups, fold change, log2 fold change, SE of log2 fold change, Wald statistic, p-value and FDR-adjusted p-value. Each gene list is presented on a separate sheet: **A** interaction, **B** control non-cleaners vs. cleaners, **C** non-cleaners control vs. non-cleaners opportunity, **D** cleaners control vs. cleaners opportunity, **E** opportunity non-cleaners vs. cleaners.

Table S9: Complete gene lists from DESeq2 analysis for differential gene expression without phenotype-by-condition interaction. The lists include Entrez gene ID, annotation, mean expression across groups, fold change, log2 fold change, SE of log2 fold change, Wald statistic, p-value and FDR-adjusted p-value. Each gene list is presented on a separate sheet: **A** control non-cleaners vs. cleaners, **B** non-cleaners control vs. non-cleaners opportunity, **C** cleaners control vs. cleaners opportunity, **D** opportunity non-cleaners vs. cleaners.

Table S10: Lists of Gene Ontology (GO) terms of genes found differentially expressed in comparisons of **A** control non-cleaners vs. cleaners, **B** non-cleaners control vs. non-cleaners opportunity, **C** cleaners control vs. cleaners opportunity.

12. References

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