“Early-life manipulation of cortisol and its receptor alters stress axis programming and social competence”

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Supplementary Information (SI)

The supplementary information contains the ethogram of the behaviours recorded in this study (§1), supplementary methods including hormone sample preparation and RT-qPCR protocols (§2) and statistical tables with the results of the GLMMs and LMMs (§3).

**1. Ethogram**

**Table S1**. Ethogram used for behavioural recordings during experience and test phases.

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| --- | --- | --- |
| **Category** | **Behaviour** | **Description** |
| Attacks (Overt aggression) | Ramming; bow swimming | Rapid linear approach towards another fish ending with physical contact (ramming); may be a dash to and fro, with hitting the other fish at the apex of a bow-shaped swimming trajectory (bow swimming) |
|  | Biting, chasing | Biting another fish or attempting to do so (with physical contact) |
|  | Mouth fight | Grasping an opponent on the jaw and intensive pulling or pushing |
| Displays (Restrained aggression) | Fin spread, + head down display | All fins, particularly the unpaired fins, are maximally spread, body kept in a stiff posture; body may be tilted with head pointing downwards (head down display) |
|  | Frontal approach; operculum spread | Linear approach towards another fish that is abruptly stopped before physical contact; usually in combination with spreading of opercula and lowering the branchiostegal membrane. Both components, frontal approach and opercula spread may also occur without one another |
|  | S-bend; tail beat | Body kept stiffly in an S-shaped posture; may switch in its inverted shape. Tail beat looks like a dynamic form of S-bend repeatedly exhibited towards an opponent in parallel or antiparallel body position |
|  | Head jolting | Lateral jolting of the head, typically occurring in series and often combined with circling around an opponent |
| Other Agonistic | Avoid | Fish makes a small movement away or out of way of approaching conspecific |
|  | Flee | Fish moves speedily away from a chasing aggressor |
| Submissive | Tail quiver | Caudal peduncle, tail fin and back end of dorsal fin are intensively vibrating while the unpaired fins are folded; body may be pressed on the ground |
|  | Hook display | Bow swimming towards another fish usually with light touch at the apex of the bow (no ramming) and subsequent pausing close to the other fish |
|  | Zig-zag swimming | Swimming in short bursts in a zig-zag pattern in front of a conspecific, usually a dominant fish |
|  | Head-up posture | A fish takes up a head-up position with folded fins |
| Affiliative | Joining | Individual swims in proximity of another fish without any signs of aggression or avoidance behaviour. |
|  | Bumping (also called 'soft touch') | Linear approach towards another fish ending with a light touch with the mouth (open or closed); often the momentum is slowed down shortly before the touch |
|  | Following | A fish swims at + constant distance together with another one without any signs of aggression |

**2. Supplementary methods**

**Hormone sample preparation**

The water samples were filtered (paper filter 1/2 595 grade, diameter 320 mm, Whatman, Sigma-Aldrich, Switzerland) and the hormones extracted using a solid phase extraction method [1–4]. A cartridge (SPE column Isolute C18 (EC), 500 mg/6ml, Biotage, Sweden) was conditioned with MetOH 99.8% of HPLC grade (Sigma-Aldrich, Switzerland) and equilibrated with 2% MetOH solution in water. Samples (500 ml) were spiked by adding (i) 9 ml methanol 99.8% HPLC grade (MetOH, Sigma-Aldrich, Switzerland) and (ii) 100µl cortisol-D4 (B&J Brand, USA) [40 ng/ml] in MetOH of HPLC grade, as internal standard. The sample was loaded onto the cartridge using a 24-Port Vacuum Manifold (Grace™ Alltech™, USA) connected to a vacuum pump (77 mm Hg, Millipore Aschroft, USA). After the sample was completely loaded, the cartridge was washed twice: first with 6 ml of 10% MetOH solution and second with 6 ml hexane 99% (Sigma-Aldrich, Switzerland), after which the cartridge was allowed to dry for approximately 2 min. The samples were stored at -20 ºC and further processed at the Neuchâtel Platform of Analytical Chemistry (University of Neuchâtel). The hormones were eluted from the cartridges with 6 ml of acetyl acetate (HPLC grade, Fisher Chemical, USA), a common solvent used for eluting free corticosteroids in water samples[5,6], and collected in glass tubes (13 x 100 mm). The samples were evaporated at 35 ºC until dry using a CentriVap centrifugal evaporator (Labconco, USA), re-suspended in 500µl MiliQ H2O:MetOH HPLC (50:50), and finallyfiltered on 20 mm PTFE hydrophilic syringe filters (BGB Analytik AG, Switzerland) into vials containing 250 µl conical glass inserts. Samples were stored at -20 ºC until analysis. Cortisol content was measured by ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS).

**RT-qPCR protocol**

RNA from each sample and brain area was extracted using the miRNeasy micro kit (Qiagen, USA) according to manufactures instructions. Briefly, tissue samples were homogenized in 700 μl QIAzol lysis reagent, then the homogenate was incubated at room temperature (RT) for 8 min. After adding 140 μl of chloroform the samples were vigorously shaken for 15 sec, incubated at RT 10 min and centrifuged at 10’200 rpm at 4°C for 15 min. The upper aqueous phase was transferred to a new collection tube and 250 μl of Ethanol (EtHo) 70% was added, mixed thoroughly and immediately transferred into an RNeasy MinElute spin column (SC) (Qiagen, USA). The SC was centrifuged at 10’200 rpm for 1 min at RT and the flow-through discarded. DNA removal was done by adding 350 μl of buffer RWT (prepared with EtHO) to the column, centrifuged at 10’000 at RT for 1 min, the flow-through was discarded and 80 µl of DNase I incubation mix (10 µl DNase stock solution + 70 µl Buffer RDD, Qiagen) was pipetted onto the SC and the tube left at RT for 15 min. After the DNAse treatment, 500 µl of Buffer RWT was pipetted onto the SC, which was then centrifuged (10’000 rpm, 1 min, RT) and the flow-through discarded. Then 500 µl buffer RPE (Qiagen) was pipetted onto the SC, the SC centrifuged (10’000 rpm, 1min, RT) and the flow-through discarded. To collect total RNA instead of miRNA 500 µl of 80% Ethanol was pipetted onto the SC, the sample was incubated for 5 min at RT and then the SC was centrifuged (10’000 rpm, 2 min, RT) and the flow-through discarded. This step was repeated once more but without incubation. After the ethanol wash the SC was placed into a new 2 ml collection tube, centrifuged (10’000 rpm, 5 min, RT) with the lid open, then moved to a final 1.5 ml collection tube, 14 µl of RNase free water (Qiagen) added to the SC and the SC centrifuged (14’000 rpm, 5 min, RT) to elute the RNA.

The concentration of the extracted RNA was measured on Life Technologies Qubit 2.0® Fluorometer (Invitrogen, USA). Synthesis of cDNA was accomplished using iScript™ Reverse Transcription Supermix for RT-qPCR [7]; 200 ng of RNA was used as the input for the cDNA synthesis of each sample and brain area. The final cDNA was dissolved 1:50 in ultra-pure water for RT-qPCR analysis. We used the same primers for the RT-qPCR of *crf* (GenBank: EF363131.1), *gr1* (GenBank: EF661652.1), and *mr* (GenBank: EF661650.1) as in [8]. As reference gene, we used s18 (GenBank: AF337051.1, [9]. Quantification of gene transcript copy number was done with the kit MESA GREEN qPCR MasterMix Plus for SYBR® Assay No ROX (Eurogentec, Switzerland) in a Rotor Gene Q (Qiagen, USA). RT-qPCR conditions consisted of 40 cycles at 95 °C (10 minutes); 40 cycles of 95 °C (10 seconds) followed by 60 °C (15 seconds) and finally 72 °C (20 seconds). We then did a melt curve from 72 °C to 95 °C with increases of 1°C per step. The initial step took 90s and each subsequent step took 5 sec.

All reactions were run in triplicate; additional re-runs were added if the standard deviation of replicates exceeded 1 critical threshold (Ct). We used a standardized luminance threshold value of 0.10 to calculate Ct values.

Equation 1 was used to calculate the PCR efficiencies (E) for each of the four primer pairs,

 (1)

where the slope is determined from a linear least squares regression fit to Ct data from a cDNA dilution series (1:10, 1:50, 1:100, 1:500, 1:1000).

The estimate of the initial amount of gene transcript (*T*i) was calculated for each individual and brain region using equation 2,

 (2)

where E is the PCR efficiency for a given gene calculated from equation 1 and Ct is the cycle number which first reached above the critical threshold for fluorescence. We calculated the gene expression for each candidate gene relative to the 18s rRNA reference gene. For each of the four genes we ran no template (Negative Control) and no reverse transcription controls in each set of reactions, in all cases there was no amplification detected. Melt curves and running of qPCR products on agarose gels indicated that there was no non-specific amplification for any of the primer pairs.

**3. Statistical tables**

Results of the statistical models for the effects of (i) early-life cortisol treatment and (ii) early-life mifepristone treatment compared to control treatment. For each factor estimates and standard errors were derived from summary tables of the respective models in R. Significance testing was done by model comparisons with likelihood-ratio tests (LRT) using a Chi square distribution; statistical values marked by a are *χ*2 values, all other values are either z or t-values. P-values < 0.05 are highlighted in bold (except for intercepts); in case of significant interactions the p-values for the main factors of the model are not bolded and main effects are not interpreted in the text.

**Table S2**. Results of GLMMs on spontaneous behaviour performed by individuals in their home tanks. Behavioural recordings were done always 9 days after the exposure to the hormonal or control treatment. Recordings were done four times between experimental Day 29 until Day 59 (factor ‘Day’). Treatments: mifepristone, n=10, cortisol, n=11, control, n=10 rearing groups; at each experimental Day three individuals per rearing group were recorded.

|  |  |  |  |
| --- | --- | --- | --- |
| **Affiliative behaviour** | | | |
| **Factor** | **Estimate ± SE** | **z or χ2** | **p-values** |
| Intercept | 2.147 ± 0.456 | 4.71 | <0.0001 |
| Cortisol | -1.725 ± 0.644 | -2.68 | 0.0074 |
| Mifepristone | 0.369 ± 0.369 | 0.58 | 0.56 |
| Day | -0.0375 ± 0.0100 | -3.75 | 0.00018 |
| Cortisol × Day | 0.0429 ± 0.0134 | 3.19 | 0.0014 |
| Mifepristone × Day | -0.00622 ± 0.0141 | -0.44 | 0.66 |
| LRT: Treatment x Day |  | 16.22a | **0.0003** |
| **Activity** | | | |
| Intercept | 4.139 ± 0.144 | 28.8 | < 0.001 |
| Cortisol | 0.0359 ± 0.10173 | 0.35 | 0.72 |
| Mifepristone | -0.0772 ± 0.105 | -0.74 | 0.46 |
| Day | 0.00712 ± 0.00284 | 6.09a | **0.014** |
| LRT: Treatment |  | 1.25a | 0.53 |

**Table S3.** Results of GLMM on the effects of cortisol and mifepristone treatments and ofs stress responsiveness on contest outcome (winning or losing). A total of 58 individuals were tested in the role of owners (mif. n=19, cort. n=20, control n=19) and 46 individuals in the role of intruders (mif. n=12, cort. n= 21, control n=13).

|  |  |  |  |
| --- | --- | --- | --- |
| **Factor** | **Estimated ± SE** | **t or χ2** | **p-value** |
| Intercept | -1.764 ± 0.919 | -1.92 | 0.055 |
| Stress responsiveness | -2.118 ± 0.913 | -2.32 | 0.02 |
| Role owner | 1.291 ± 0.744 | 1.74 | 0.083 |
| Treatment cortisol | 1.3973 ± 0.9364 | 1.492 | 0.1356 |
| Treatment mifepristone | 1.194 ± 1.052 | 1.14 | 0.26 |
| Treatment cortisol × Stress responsiveness | 1.729 ± 1.321 | 1.31 | 0.19 |
| Treatment mifepristone × Stress responsiveness | 5.679 ± 2.262 | 2.51 | 0.012 |
| LRT: Role owner |  | 3.5a | 0.061 |
| LRT: Treatment x Stress responsiveness |  | 13.71a | **0.0012** |

**Table S4**. LMMs to test for effects of treatment and opponent behaviours on restrained aggression and contest duration. Outcome of contest did not significantly affect any of these results and was therefore dropped from all models. A total of 58 individuals were tested in the role of owners (mif. n=19, cort. n=20, control n=19) and 46 individuals in the role of intruders (mif. n=12, cort. n= 21, control n=13).

|  |  |  |  |
| --- | --- | --- | --- |
| **Restrained aggression** | | | |
| **Factor** | **Estimated ± SE** | **t or χ2** | **p-value** |
| Intercept | 2.451 ± 0.302 | 8.12 | < 0.001 |
| Cortisol | 1.441 ± 0.418 | 3.44 | **0.0019** |
| Mifepristone | 0.131 ± 0.423 | 0.31 | 0.76 |
| Submissive behaviour, opponent | 0.691 ± 0.909 | 0.67a | 0.41 |
| LRT: Treatment |  | 13.49a | **0.0012** |
| LRT: Submissive behaviour, opponent |  | 0.6669a | 0.41 |
| **Contest duration** | | | |
| **Factor** | **Estimated ± SE** | **t or χ2** | **p-value** |
| Intercept | 14.156 ± 1.904 | 7.44 | < 0.001 |
| Cortisol | 3.867 ± 1.828 | 2.12 | **0.039** |
| Mifepristone | -1.429 ± 1.954 | -0.73 | 0.47 |
| Total aggression, opponent | -0.587 ± 0.298 | 4.04a | 0.046 |
| LRT: Treatment |  | 8.49a | **0.014** |
| LRT: Total aggression, opponent |  | 4.0364a | **0.045** |

**Table S5**. LMs on the effect of the treatment on the expression of *crf* and *mr* genes in the telencephalon and hypothalamus relative to the reference gene 18s. Treatments: cortisol, n= 11; mifepristone, n=10; control, n=10.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Corticotropin releasing factor, *crf*** | | | | |
| **Factor** | **Estimate ± SE** | | **t or *χ*2** | **p-values** |
| **Telencephalon** |  | |  |  |
| Intercept | 5.796e-06 ± 7.561e-07 | | 7.67 | < 0.001 |
| Cortisol | -2.175e-06 ± 8.480e-07 | | -2.57 | **0.017** |
| Mifepristone | -2.237e-06 ± 9.005e-07 | | -2.48 | **0.02** |
| Plate 2 | -4.714e-07 ± 8.260e-07 | | -0.57 | 0.57 |
| Plate 3 | -1.981e-06 ± 9.201e-07 | | -2.15 | 0.041 |
| LRT: Treatment |  | | -780.11a | **0.012** |
| LRT: Plate number |  | | -783.68 a | 0.07 |
| **Hypothalamus** |  | |  |  |
| Intercept | 0.518 ± 0.0116 | | 44.72 | < 0.001 |
| Cortisol | -0.000268 ± 0.009 | | -0.03 | 0.98 |
| Mifepristone | 0.0001690 ± 0.0093971 | | 0.018 | 0.99 |
| Plate 2 | -0.0152 ± 0.00878 | | -1.73 | 0.096 |
| Plate 3 | -0.0303 ± 0.00979 | | -3.09 | 0.005 |
| LRT: Treatment |  | | -228.24a | 0.1 |
| LRT: Plate number |  | | -216.45a | **0.0075** |
| **Mineralocorticoid receptor, *mr*** | | | | |
| **Factor** | **Estimate ± SE** | **t or *χ*2** | | **p-values** |
| **Telencephalon** |  |  | |  |
| Intercept | 0.661 ± 0.041 | 15.98 | | < 0.001 |
| Cortisol | 0.018 ± 0.009 | 2.06 | | **0.0497** |
| Mifepristone | 0.021 ± 0.009 | 2.33 | | **0.028** |
| Size | -0.041 ± 0.014 | -235.75 a | | **0.002** |
| Plate number 2 | 0.001 ± 0.008 | 0.13 | | 0.89 |
| Plate number 3 | 0.001 ± 0.009 | 0.074 | | 0.94 |
| LRT: Treatment |  | -240.28a | | **0.027** |
| LRT: Size |  | -232.21a | | **0.0023** |
| LRT: Plate number |  | -243.52 a | | 0.98 |
| **Hypothalamus** |  |  | |  |
| Intercept | 11.586 ± 3.535 | 3.28 | | 0.003 |
| Cortisol | 0.196 ± 0.741 | 0.26 | | 0.79 |
| Mifepristone | -1.031 ± 0.7804 | -1.32 | | 0.2 |
| Size | -0.044± 1.182 | -0.08 | | 0.94 |
| Plate number 2 | 0.304 ± 0.739 | 0.411 | | 0.68 |
| Plate number 3 | 1.8 ± 0.796 | 2.25 | | 0.034 |
| LRT: Treatment |  | 34.69 a | | 0.19 |
| LRT: Size |  | 33.39 a | | 0.97 |
| LRT: Plate number |  | 38.9 a | | **0.019** |

**References**

1. Bender N, Heg D, Hamilton IM, Bachar Z, Taborsky M, Oliveira RF. 2006 The relationship between social status, behaviour, growth and steroids in male helpers and breeders of a cooperatively breeding cichlid. *Horm. Behav.* **50**, 173–182. (doi:10.1016/j.yhbeh.2006.02.009)

2. Earley RL, Edwards JT, Aseem O, Felton K, Blumer LS, Karom M, Grober MS. 2006 Social interactions tune aggression and stress responsiveness in a territorial cichlid fish (Archocentrus nigrofasciatus). *Physiol. Behav.* **88**, 353–363. (doi:10.1016/j.physbeh.2006.04.002)

3. Ellis T, James JD, Sundh H, Fridell F, Sundell K, Scott a. P. 2007 Non-invasive measurement of cortisol and melatonin in tanks stocked with seawater Atlantic salmon. *Aquaculture* **272**, 698–706. (doi:10.1016/j.aquaculture.2007.07.219)

4. Boulton K, Couto E, Grimmer AJ, Earley RL, Canario AVM, Wilson AJ, Walling C a. 2015 How integrated are behavioral and endocrine stress response traits? A repeated measures approach to testing the stress-coping style model. *Ecol. Evol.* **5**, 618–633. (doi:10.1002/ece3.1395)

5. Ellis T, James JD, Stewart C, Scott a. P. 2004 A non-invasive stress assay based upon measurement of free cortisol released into the water by rainbow trout. *J. Fish Biol.* **65**, 1233–1252. (doi:10.1111/j.1095-8649.2004.00499.x)

6. Koren L, Ng ESM, Soma KK, Wynne-Edwards KE, Soldin S. 2012 Sample Preparation and Liquid Chromatography-Tandem Mass Spectrometry for Multiple Steroids in Mammalian and Avian Circulation. *PLoS One* **7**, e32496. (doi:10.1371/journal.pone.0032496)

7. Rennison DJ, Owens GL, Heckman N, Schluter D, Veen T. 2016 Rapid adaptive evolution of colour vision in the threespine stickleback radiation. *Proc. R. Soc. B Biol. Sci.* **283**, 20160242. (doi:10.1098/rspb.2016.0242)

8. Taborsky B, Tschirren L, Meunier C, Aubin-Horth N. 2013 Stable reprogramming of brain transcription profiles by the early social environment in a cooperatively breeding fish. *Proc. Biol. Sci.* **280**, 20122605. (doi:10.1098/rspb.2012.2605)

9. Nyman C, Fischer S, Aubin-Horth N, Taborsky B. 2017 Effect of the early social environment on behavioural and genomic responses to a social challenge in a cooperatively breeding vertebrate. *Mol. Ecol.* **26**, 3186–3203. (doi:10.1111/mec.14113)