

Article

Effects of the Antidepressants Citalopram and Venlafaxine on the Big Ramshorn Snail (*Planorbarius Corneus*) Histopathology

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Table S1. Detailed overview on the symptoms identified during the semi-quantitative histological evaluation. The experiment with big ramshorn snail with the respective treatment are listed in rows and the assessed symptoms are listed in columns. Each symptom is divided into three severity categories (white/0: not detected, light grey/0.5: detected in moderate frequency/severity, dark grey/1: detected in high frequency/severity). Values depict the absolute number of samples showing the symptom. CIT=citalopram, VEN=venlafaxine.

Treatment	Tubule Apex Protrusion			Tubule Irregular Basis			Dilated Tubule Lumen			Irregular Nucleus of Digestive Cells			Irregular Compartmentation of Digestive Cells			Irregular Nucleus of Crypt Cells			Irregular Cytoplasm of Crypt Cells		
	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
control	8	0	7	15	0	0	3	6	6	15	0	0	10	1	4	13	1	1	3	2	10
1 µg/L CIT	8	1	4	13	0	0	6	3	4	13	0	0	10	1	2	10	0	2	3	2	8
10 µg/L CIT	9	0	5	14	0	0	6	6	2	14	0	0	9	3	2	11	1	2	3	4	7
100 µg/L CIT	11	2	1	14	0	0	3	5	6	14	0	0	8	1	5	9	1	4	1	1	12
1000 µg/L CIT	5	1	9	15	0	0	0	9	6	15	0	0	3	1	11	11	2	2	0	2	13
control	5	4	4	13	0	0	3	5	5	13	0	0	6	2	5	12	1	0	1	7	5
1 µg/L VEN	5	3	6	14	0	0	5	6	3	14	0	0	3	3	8	10	2	2	0	7	7
10 µg/L VEN	7	5	3	15	0	0	8	6	1	15	0	0	7	5	3	13	2	0	2	6	7
100 µg/L VEN	7	3	5	15	0	0	7	7	1	15	0	0	8	4	3	14	1	0	1	9	5
1000 µg/L VEN	8	1	4	13	0	0	5	6	2	13	0	0	4	5	4	11	2	0	2	8	3

Water conditions

Table S2. Water quality parameters during the experiment with big ramshorn snail exposed to citalopram. Mean values are shown.

Sampling Date	Treatment Citalopram	pH	Conductivity (µs/cm)	Temperature (°C)	Oxygen Concentration (mg/L)	Oxygen Saturation (%)
26 October 2017	control	8.22	487.33	10.53	10.44	98.37
26 October 2017	1 µg/L	8.25	483.67	10.97	10.38	98.20
26 October 2017	10 µg/L	8.32	483.33	10.73	10.41	98.30
26 October 2017	100 µg/L	8.16	483.67	10.93	10.35	98.03
26 October 2017	1000 µg/L	8.33	484.67	10.77	10.41	98.33
24 November 2017	control	8.33	482.00	10.65	10.12	96.00
24 November 2017	1 µg/L	8.31	484.75	10.78	10.08	96.00
24 November 2017	10 µg/L	8.30	477.50	10.80	10.03	95.75
24 November 2017	100 µg/L	8.27	480.50	10.75	10.02	95.50
24 November 2017	1000 µg/L	8.30	480.00	10.73	10.04	95.80

Table S3. Water quality parameters during the experiment with big ramshorn snail exposed to venlafaxine. Mean values are shown.

Sampling Date	Treatment Venlafaxine	pH	Conductivity ($\mu\text{S}/\text{cm}$)	Temperature ($^{\circ}\text{C}$)	Oxygen Concentration (mg/L)	Oxygen Saturation (%)
15 June 2018	control	7.03	486.67	12.40	10.09	99.67
15 June 2018	1 $\mu\text{g}/\text{L}$	7.00	486.33	12.20	10.12	99.60
15 June 2018	10 $\mu\text{g}/\text{L}$	7.08	482.67	12.20	10.12	99.53
15 June 2018	100 $\mu\text{g}/\text{L}$	7.18	488.00	12.13	10.12	99.60
15 June 2018	1000 $\mu\text{g}/\text{L}$	7.28	484.00	12.23	10.11	99.43
13 July 2018	control	7.37	490.33	12.97	10.06	100.20
13 July 2018	1 $\mu\text{g}/\text{L}$	7.41	486.00	12.73	10.05	99.93
13 July 2018	10 $\mu\text{g}/\text{L}$	7.48	484.67	12.80	10.04	99.90
13 July 2018	100 $\mu\text{g}/\text{L}$	7.54	487.00	12.73	10.06	100.10
13 July 2018	1000 $\mu\text{g}/\text{L}$	7.59	489.00	12.77	10.04	100.00

Chemical Analysis

Table S4. Operating parameters of the triple quadrupole MS (Agilent 6490 QqQ) in positive mode.

Parameter	Set Point
Gas temperature	250 $^{\circ}\text{C}$
Gas flow	14 L/min
Nebulizer gas pressure	45 psi
Sheath gas heater	300 $^{\circ}\text{C}$
Sheath gas flow	12 L/min
Capillary voltage	3500 V
Nozzle voltage	0 V

Table S5. Specific measurement parameters for venlafaxine and citalopram with LC-QqQ in water samples of the snail experiments. Intraday variations (RSD) is calculated with 1 $\mu\text{g}/\text{L}$ standard (10 μL injection volume and 4 replicates (n)). Limit of quantification= LOQ.

Name	Precursor	Product Ion (Quan/Qual) (m/z)	Collision Energy (Setpoint in V)	Retention Time (min)	RSD (%) n=4	LOQ (ng/L)
Venlafaxine	278	260/58	4/16	5.84	3.4	10
Citalopram	325	262/109	20/30	3.92	2.6	10

Water Concentrations

Table S6. Citalopram water concentrations during the experiment with big ramshorn snail exposed to citalopram. Concentrations are shown in $\mu\text{g}/\text{L}$. LOD= Limit of detection, a.w.e.= after water exchange.

	25 October 2017	10 November 2017 a.w.e.	24 November 2017
control	<LOD	<LOD	<LOD
1 $\mu\text{g}/\text{L}$	1,14	1,2	1,1
10 $\mu\text{g}/\text{L}$	8,5	8,93	7,98
100 $\mu\text{g}/\text{L}$	140	143	128
1000 $\mu\text{g}/\text{L}$	1213	1202	1101

Table S7. Venlafaxine water concentrations during the experiment with big ramshorn snail exposed to venlafaxine. Concentrations are shown in µg/L. LOD=Limit of detection, b.w.e.= before water exchange, a.w.e.= after water exchange.

	15 June 2018	04 July 2018 b.w.e.	04 July 2018 a.w.e.	13 July 2018
control	<LOD	<LOD	<LOD	<LOD
1 µg/L	0,79	0,70	0,72	0,72
10 µg/L	8,33	8,12	8,21	8,16
100 µg/L	85,21	78,32	77,70	77,27
1000 µg/L	881	866	846	865

CRED

Table S8. CRED-criteria of the experiment with big ramshorn snail exposed to citalopram and venlafaxine.

CRED-criteria: Exposure of Big Ramshorn Snail to Citalopram and Venlafaxine	
1. General information	
a. Purpose	Aim of the study was to investigate the effect of citalopram and venlafaxine on apical endpoints, the behaviour and biochemical biomarker in big ramshorn snail.
b. Endpoints	mortality, weight, behaviour during the exposure, biochemical determination of: B-esterase activity, SOD activity, Hsp70 level and histopathology of hepatopancreas
2. Test design	
a. Standard	No standard test procedure
b. GLP	Not GLP-accredited
c. Controls	Laboratory negative control
d. Validity	mortality in control was <10%
3. Test compound	
a. Identification	Citalopram hydrobromide 1-[3-(Dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro-5-isobenzofuran carbonitrile hydrobromide CAS: 59729-32-7 Venlafaxine hydrochloride (+/-)-1-[2-(Dimethylamino)-1-(4-methoxyphenyl)ethyl]cyclohexanol hydrochloride CAS: 99300-78-4
b. Physico-chemical characteristics	Citalopram: water solubility of 16.56 g/L (TOCRIS 2017) Venlafaxine: water solubility of 100 mmol/L (TOCRIS 2017)
c. Source	Citalopram: Sigma-Aldrich, Product Number: PHR1640-1G; Batch Number: LRAB6012 Venlafaxine: Sigma-Aldrich, Product Number: PHR1736-1G; Batch Number: LRAB7761
d. Purity	Citalopram: 99.8% according to the Certificate of Analysis from Sigma-Aldrich Venlafaxine: 99.8% according to the Certificate of Analysis from Sigma-Aldrich
e. Formulation	no formulation, no impurities
4. Test organism	
a. Scientific name	<i>Planorbis corneus</i>
b. Body weight	Citalopram experiment: 2.26 ± 0.87 g Venlafaxine experiment: 1.76 ± 0.40 g
c. Age/life stage	Adult
d. Reproductive condition	in reproductive condition
e. Sex	hermaphrodite

f. Strain/clone	no defined clone
g. Source	Lab cultured offspring of individuals obtained from Foerdefische (Handewitt, Germany)
h. Acclimatisation	1 day gradually cooling from room temperature to 11°C
5. Exposure conditions	
a. Schedule	semi-static design with water exchange of 50 % of the test medium once a week
b. System	closed
c. Test medium	Filtered tap water (iron filter, active charcoal filter, particle filter) cooled to 11°C and aerated
d. Temperature	Climate chamber set to 11°C. Watertemperature: Citalopram experiment: 10.76 °C Venlafaxine experiment: 12.52 °C
e. pH	Citalopram experiment: 8.28 Venlafaxine experiment: 7.3
f. Hardness	14.8 mg/L CaCO ₃ , 10.4 mg/L HCO ₃ ⁻
g. Conductivity	Citalopram experiment: 482.74 µS/cm Venlafaxine experiment: 486.47 µS/cm
h. Dissolved oxygen	Citalopram experiment: 10.23 mg/L Venlafaxine experiment: 10.08 mg/L
i. Light intensity/quality	10 h : 14 h light:dark cycle; aquaria shaded from direct light with black foil
j. Feeding	Every second day one algae tablet per tank
k. Aquaria	25 L glass aquaria filled with 10 L of medium. covered with glass plane, silicone tubing, aerated with airstones (JBL ProSilent Aeras Micro S2)
l. Sand/sediment	no sediment tested
m. Stock solutions	Citalopram: Stock solution 1 (100 mg/L) prepared from 124.94 mg citalopram hydrobromide in 1 L dest. Water, stock solution 2 (1 mg/L) produced from stock solution 1 via 1:100 dilution. Venlafaxine: Stock solution 1 (100 mg/L) prepared from 113.14 mg venlafaxine hydrochloride in 1 L dest. water, stock solution 2 (1 mg/L) produced from stock solution 1 via 1:100 dilution
n. Nominal concentrations	0. 1. 10. 100. 1000 µg/L
o. Measured concentration	Water samples were taken and analysed at the beginning, after 2 weeks before and after water exchange and at the end of the experiment. Mean citalopram: <LoD, 1.15, 8.47, 137, 1172 µg/L Mean venlafaxine: <LoD, 0.73, 8.2, 79.63, 864.15 µg/L
p. Method	HPLC-MS (QQ-MS) (LoQ = 1 ng/L)
q. Duration	Citalopram experiment: 26 October 2017–23 November 2017 Venlafaxine experiment: 15 June 2018–13 July 2018
r. Observations	Mortality, behaviour during the exposure. body weight, at the end of the experiment samples were taken for biochemical analyses
s. Results	summary table in article
t. Biomass loading	Citalopram experiment: mean= 1.13 g/L Venlafaxine experiment: mean=1.23 g/L
6. Statistical Design and Biological Response	
a. Replicates	Three replicate aquaria per test concentration and temperature
b. Number of organisms	Citalopram: 5 snail per replicate Venlafaxine: 7 snail per replicate
c. Design	three blocks, one replicate per treatment present in each block, arranged in randomized order

d. Statistical methods	Mortality: nested Cox proportional hazards model; body mass, biochemical biomarkers: nested ANOVA; behaviour during exposure: Generalised Liner Mixed Model
e. Biological response	Citalopram: decreased weight at 1000 µg/L Qualitative assessment of the hepatopancreas revealed a higher proportion of pronounced irregular shaped apices, dilated lumen of tubules and irregular compartmentation of digestive cells.
f. Dose-response	Venlafaxine: increased sole detachment at 100 µg/L and higher Increased sole detachment at 100 and 1000 µg/L venlafaxine
g. Statistical significances	Citalopram: decreased weight at 1000 µg/L Venlafaxine: increased sole detachment at 100 µg/L and higher
h. Significance level	$\alpha = 0.05$, in cases of multiple comparisons adjusted via sequential Bonferroni
i. Variability	not estimated
j. Raw data	provided on https://effectnet-seek.bioquant.uni-heidelberg.de/

Statistical Details

Table S9. Detailed statistical information of the assessed parameters of both experiments.

Parameter	Experiment	Detailed Statistical Information
weight	Big ramshorn snail + citalopram	nested ANOVA: $df=4,10$; $F=2,6876$; $p=0,0398$; post-hoc Dunnett's test [control 1 µg/L] $p=0,0789$ [control 10 µg/L] $p=0,0977$ [control 1000 µg/L] $p=0,0616$ [control 1000 µg/L] $p=0,0136$
weight	Big ramshorn snail + venlafaxine	Logarithm transformation: nested ANOVA: $df=4,10$; $F=0,6776$; $p=0,6093$
mortality	Big ramshorn snail + citalopram	nested Cox proportional hazards model: $df=4,10$; $\chi^2=0$; $p=1$
mortality	Big ramshorn snail + venlafaxine	nested Cox proportional hazards model: $df=4,10$; $\chi^2=0,0632$; $p=0,9995$
behavior	Big ramshorn snail + venlafaxine	generalised linear mixed model: $df=4$; $F=6,8118$; post-hoc Dunnett's test [control 100 µg/L] $p=0,00932$ [control 1000 µg/L] $p<0,001$
AChE activity	Big ramshorn snail + citalopram	Logarithm transformation: nested ANOVA: $df=4,10$; $F=0,2707$; $p=0,8957$
AChE activity	Big ramshorn snail + venlafaxine	Kruskal-Wallis test: $df=4$; $\chi^2=3,8627$; $p=0,4249$
CbE activity (NPA-substrate)	Big ramshorn snail + citalopram	Logarithm transformation: nested ANOVA: $df=4,10$; $F=0,1822$; $p=0,9467$
CbE activity (NPA-substrate)	Big ramshorn snail + venlafaxine	nested ANOVA: $df=4,10$; $F=2,0917$; $p=0,093$
CbE activity (NPV-substrate)	Big ramshorn snail + citalopram	nested ANOVA: $df=4,10$; $F=2,0789$; $p=0,0955$
CbE activity (NPV-substrate)	Big ramshorn snail + venlafaxine	nested ANOVA: $df=4,10$; $F=1,7893$; $p=0,1431$
SOD activity	Big ramshorn snail + citalopram	nested ANOVA: $df=4,10$; $F=1,8002$; $p=0,1417$
SOD activity	Big ramshorn snail + venlafaxine	nested ANOVA: $df=4,10$; $F=0,7864$; $p=0,5385$
Hsp70 level	Big ramshorn snail + citalopram	nested ANOVA: $df=4,10$; $F=1,124$; $p=0,3563$
Hsp70 level	Big ramshorn snail + venlafaxine	nested ANOVA: $df=4,10$; $F=1,4562$; $p=0,2273$
histopathology	Big ramshorn snail + citalopram	likelihood ratio test: $df=12$; $\chi^2=13,661$; $p=0,3228$
histopathology	Big ramshorn snail + venlafaxine	likelihood ratio test: $df=12$; $\chi^2=14,602$; $p=0,2639$

Statistical Output

```
behaviour of big ramshorn snail exposed to venlafaxine:
[1] "summary(GLM.PHS.VEN)"
Generalized linear mixed model fit by maximum likelihood (Laplace
Approximation) [glmerMod]
Family: binomial ( logit )
Formula:
cbind(schwimmend, nicht.schwimmend) ~ Treatment + Tag.nach.Versuchsbeginn +
(1 | AquariumID)
Data: Data.PHS.VEN
Control: glmerControl(optimizer = "bobyqa", optCtrl = list(maxfun = 1e+05))
```

```
AIC BIC logLik deviance df.resid
360 384 -173 346 218
```

```
Scaled residuals:
Min 1Q Median 3Q Max
-1.3600 -0.5033 -0.3636 0.2158 3.6613
```

```
Random effects:
Groups Name Variance Std.Dev.
AquariumID (Intercept) 0.1375 0.3709
Number of obs: 225, groups: AquariumID, 15
```

```
Fixed effects:
Estimate Std. Error z value Pr(>|z|)
(Intercept) -3.63105 0.54840 -6.621 3.56e-11 ***
Treatment1 0.22522 0.62759 0.359 0.7197
Treatment10 0.90063 0.58091 1.550 0.1211
Treatment100 1.63430 0.54676 2.989 0.0028 **
Treatment1000 2.15881 0.53558 4.031 5.56e-05 ***
Tag.nach.Versuchsbeginn -0.01798 0.01519 -1.184 0.2364
---
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
Correlation of Fixed Effects:
(Intr) Trtmn1 Trtm10 Trt100 Tr1000
Treatment1 -0.625
Treatment10 -0.673 0.597
Treatment100 -0.721 0.631 0.681
Treatment1000 -0.741 0.642 0.693 0.743
Tag.nach.Vrsc -0.519 -0.002 -0.003 -0.010 -0.007
[1] "anova(GLM.PHS.VEN)"
Analysis of Variance Table
Df Sum Sq Mean Sq F value
Treatment 4 27.2473 6.8118 6.8118
Tag.nach.Versuchsbeginn 1 1.3792 1.3792 1.3792
[1] "summary(glht(GLM.PHS.VEN,linfct=mcp(Treatment=Dunnett)))"
```

Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: Dunnett Contrasts

```
Fit: glmer(formula = cbind(schwimmend, nicht.schwimmend) ~ Treatment +
Tag.nach.Versuchsbeginn + (1 | AquariumID), data = Data.PHS.VEN,
family = binomial, control = glmerControl(optimizer = "bobyqa",
optCtrl = list(maxfun = 1e+05)))
```

Linear Hypotheses:

Estimate Std. Error z value Pr(>|z|)

1 - 0 == 0 0.2252 0.6276 0.359 0.98310

10 - 0 == 0 0.9006 0.5809 1.550 0.29376

100 - 0 == 0 1.6343 0.5468 2.989 0.00932 **

1000 - 0 == 0 2.1588 0.5356 4.031 < 0.001 ***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

(Adjusted p values reported -- single-step method)