

## SUPPLEMENTAL DATA

### **“Anti-anxiety drugs and fish behaviour: establishing the link between internal concentrations of oxazepam and behavioural effects”**

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### *Method validation parameters*

Both MDL and MQL were determined in spiked samples ( $n = 3$ ) of all matrices considered as the minimum detectable amount of analyte with a signal-to-noise ratio of 3 and 10, respectively. Precision was calculated from five repeated injections of a spiked sample at 10  $\text{ng mL}^{-1}$  or  $\text{ng g}^{-1}$ , and was expressed as the relative standard deviation of the measured concentration. Calibration curves in both solvent and clean matrix extract were generated using linear regression analysis ( $R^2 > 0.98$ ) in the concentration range from 0.1 to 100  $\text{ng mL}^{-1}$  for water and plasma, or 0.1 to 50  $\text{ng g}^{-1}$  for tissues. When matrix-matched calibration curves were applied to the quantification, matrix effects (percentage of signal reduction or enhancement when comparing the compound in the matrix vs the solvent) were considered negligible (see Table 1).

**Table S1** SRM transitions and compound dependant MS parameters for target analytes

Compounds	Quantification			Confirmation		SRM1/2 ratio ( $\pm$ SD)n=3	
	Rt (min)	Precursor ion (m/z)	Q3	DP/CE/CXP	Q3		DP/CE/CXP
Oxazepam	1.33	287 [M+H] <sup>+</sup>	241	96/33/12	104	111/43/14	2.86
Diazepam-d5 (IS)	1.44	290 [M+H] <sup>+</sup>	198	101/47/26			

**Table S2** Concentration of oxazepam in plasma and tissues (wet weigh) of each of the fish individuals after 28-days exposure

Water Conc. ( $\mu\text{g L}^{-1}$ )	Gender	Plasma ( $\text{ng mL}^{-1}$ )	Brain ( $\text{ng g}^{-1}$ )	Liver ( $\text{ng g}^{-1}$ )	Muscle ( $\text{ng g}^{-1}$ )
30.6	M	63.0	75	111	48
30.6	M	122.6	109	215	184
30.6	M	135.1	166	184	154
30.6	M	86.6	136	266	113
30.6	M	151.6	82	74	81
30.6	M	53.0	97	101	60
30.6	M	112.9	238	370	63
30.6	M	313.1	177	272	38
30.6	M	91.1	144	256	79
30.6	M	10.6	117	224	98
30.6	M	208.3	219	420	181
30.6	M	232.4	314	439	193
30.6	M	60.8	271	63	33
30.6	M	87.3	160	216	56
30.6	M	166.0	323	388	140
30.6	M	245.7	262	337	62
30.6	F	51.8	88	0	80
30.6	F	16.8	57	61	20
30.6	F	104.6	99	120	49
30.6	F	75.5	97	94	123
30.6	F	68.7	122	199	37
30.6	F	146.1	132	61	50
30.6	F	32.2	97	61	42
30.6	F	10.0	178	93	177
30.6	F	121.0	127	119	64

30.6	F	75.3	70	137	73
30.6	F	76.4	89	71	29
30.6	F	19.1	107	71	7
30.6	F	52.6	85	68	81
30.6	F	24.6	192	65	17
30.6	F	98.2	131	70	85
30.6	F	66.5	137	230	96
4.7	M	40.2	76	36	22
4.7	M	26.9	41	25	44
4.7	M	25.1	65	47	10
4.7	M	37.8	50	52	28
4.7	F	21.1	44	72	24
4.7	M	57.5	81	34	26
4.7	F	39.6	29	31	9
4.7	M	29.3	56	59	21
4.7	M	46.3	70	37	27
4.7	M	52.8	91	45	17
4.7	M	39.0	46	28	12
4.7	M	51.4	41	58	39
4.7	M	80.1	61	49	28
4.7	F	24.5	37	33	40
4.7	M	32.3	42	96	96
4.7	M	41.8	63	51	27
4.7	F	17.6	39	75	30
4.7	F	17.2	36	20	20
4.7	F	23.1	51	80	60
4.7	F	34.0	53	21	16
4.7	F	35.5	41	18	55

4.7	F	31.4	45	35	9
4.7	F	23.1	26	47	24
4.7	F	30.1	57	28	14
4.7	F	7.9	23	10	8
4.7	F	10.8	40	21	84
4.7	F	28.0	28	30	18
4.7	F	18.9	62	23	8
4.7	F	10.5	48	11	16
4.7	F	10.0	41	17	7
4.7	F	10.4	35	16	11
4.7	F	14.5	78	33	9
0.8	M	7.1	35	8	5
0.8	M	9.0	32	14	7
0.8	M	7.5	34	15	6
0.8	M	5.3	64	24	4
0.8	F	7.3	51	16	5
0.8	M	4.3	52	29	3
0.8	M	14.4	32	8	4
0.8	M	11.9	37	8	2
0.8	F	7.4	51	19	47
0.8	F	6.1	23	20	4
0.8	F	3.5	52	50	6
0.8	F	3.7	24	16	4
0.8	F	11.0	73	23	9
0.8	F	3.2	24	16	5
0.8	F	6.0	17	38	3
0.8	F	13.3	28	14	11
0.8	F	4.5	28	6	10

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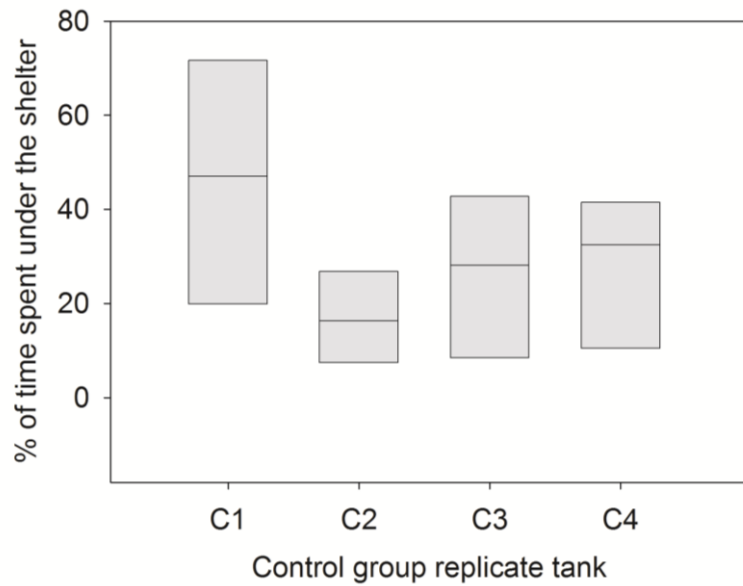
0.8	F	10.2	24	7	5
0.8	F	3.1	72	6	5
0.8	F	3.5	42	10	4
0.8	F	6.8	24	8	5
0.8	F	4.8	54	6	8
0.8	F	4.3	34	11	8
0.8	F	9.4	43	6	6
0.8	M	13.5	17	17	4
0.8	M	17.9	27	17	13
0.8	M	10.4	22	18	9
0.8	M	13.3	68	16	8
0.8	F	12.5	50	10	5
0.8	F	3.7	55	24	4
0.8	F	7.0	46	14	5
0.8	M	13.4	60	12	8

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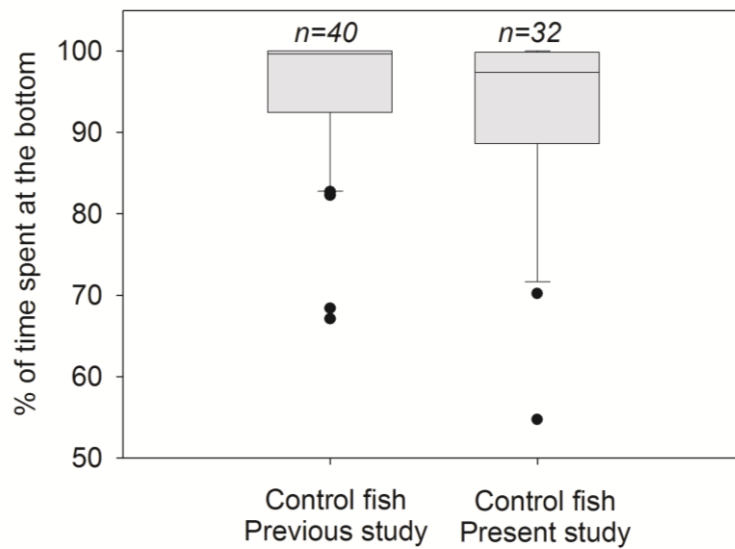
**Table S3** Inter-individual and intra-treatment variability of oxazepam plasma concentrations.

<b>Treatment</b>	<b>Mean</b>	<b>Median</b>	<b>Min</b>	<b>Max</b>	<b>25%</b>	<b>75%</b>	<b>SD</b>
1 µg/L - Rep. A Males	8.4	7.4	4.3	14.4	6.7	9.7	
1 µg/L - Rep. B Males	11.5	12.9	3.7	17.9	9.5	13.5	
1 µg/L - Rep. C Females	5.8	4.7	3.1	10.2	4.1	7.4	
1 µg/L - Rep. D Females	9.3	6.0	3.2	31.0	3.7	8.9	
1 µg/L - Males	10.9	11.1	5.3	17.9	7.5	13.5	3.8
1 µg/L - Females	7.4	5.4	3.1	31.0	3.7	7.9	6.3
1 µg/L - All Fish	8.7	7.2	3.1	31.0	4.5	12.1	5.7
5 µg/L - Rep. A Males	34.7	33.6	21.1	57.5	26.4	39.8	
5 µg/L - Rep. B Males	46.0	44.1	24.5	80.1	37.3	51.8	
5 µg/L - Rep. C Females	13.9	10.7	7.9	28.0	10.3	15.6	
5 µg/L - Rep. D Females	26.5	26.6	17.2	35.5	21.7	32.1	
5 µg/L - Males	43.1	40.2	25.1	80.1	32.3	51.4	15.0
5 µg/L - Females	21.5	21.1	7.9	39.6	12.7	29.0	9.6
5 µg/L - All Fish	30.3	28.7	7.9	80.1	18.6	39.2	16.1
25 µg/L - Rep. A Males	136.6	128.6	1.6	245.7	80.7	214.3	
25 µg/L - Rep. B Males	129.7	117.8	53.0	313.1	80.7	139.2	
25 µg/L - Rep. C Females	62.1	60.2	1.0	146.1	28.4	82.8	
25 µg/L - Rep. D Females	66.7	70.9	19.1	121.0	45.6	81.8	
25 µg/L - Males	133.2	117.8	1.6	313.1	80.7	176.6	82.6
25 µg/L - Females	64.4	67.6	1.0	146.1	30.3	81.8	40.2
25 µg/L - All Fish	98.8	81.5	1.0	313.1	52.9	125.7	72.9

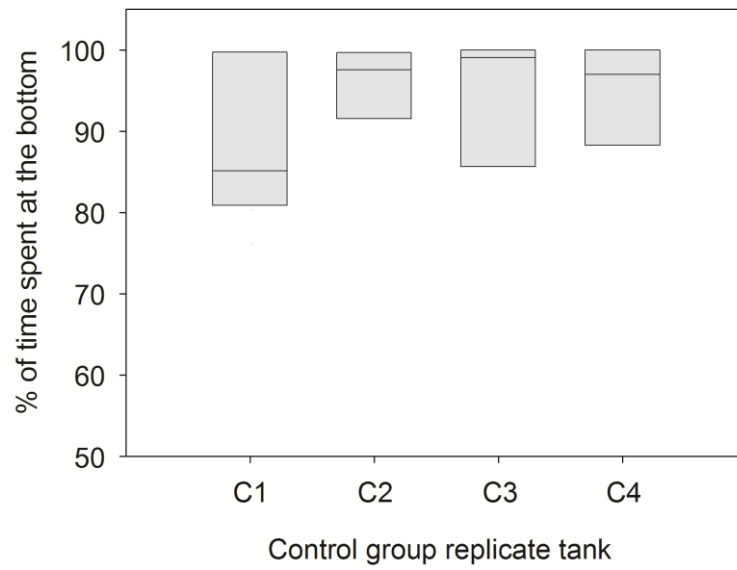




**Figure S1.** Behaviour of fathead minnows in the control group. The box-plot show the percentage of time spent at the bottom of the tank during a Novel Tank Diving Test. Each group refer to one of the four replicate tanks. Boxes represent medians (full middle line), with 10th and 90th percentiles.



**Figure S2.** Comparison of the percentage of time spent by non-exposed fathead minnows at the bottom of the tank during a Novel Tank Diving Test in two independent studies (present study vs Margiotta-Casaluci et al., 2104). In both cases, the Novel tank Diving Test was performed using the same protocol. Boxes represent medians (full middle line), with 10th and 90th percentiles. Outliers are represented by black dots.



**Figure S3.** Behaviour of fathead minnows in the control group. The box-plot show the percentage of time spent under the shelter during a Shelter-Seeking Test. Each group refer to one of the four replicate tanks. Boxes represent medians (full middle line), with 10th and 90th percentiles. The asterisk (\*) indicate a statistically significant difference ( $p < 0.05$ ).

**Table S4** Summary of all behavioural endpoints measure after 28 days of exposure to oxazepam (median %, 10-90<sup>th</sup> percentile)

		<b>Novel Tank Diving Test</b>						<b>Shelter-seeking Test</b>	
		<i>Time spent in the area (%) ( 10-90<sup>th</sup>)</i>			<i>Distance travelled m (10-90<sup>th</sup>)</i>			<i>Time spent in the area (%) ( 10-90<sup>th</sup>)</i>	<i>Distance travelled m (10-90<sup>th</sup>)</i>
		Bottom	Middle	Top	Bottom	Middle	Top	Under tile	Outside the tile
<b>C<sub>control</sub></b>	M	96.1 (80.8-99.9)	3.5 (0.02-14.8)	0.3 (0.0-5.0)	26.5 (11.8-49.5)	4.1 (0.04-27.2)	0.6 (0.0-7.0)	26.1 (7.3- 73.2)	21.6 (7.2-95.7)
	F	98.8 (66.-100.0)	1.1 (0.0-13.0)	0.0 (0.0-13.7)	24.1 (5.1-34.3)	1.4 (0.0-20.3)	0.004 (0.0-16.9)	32.4 (6.2- 45.1)	23.6 (4.9-58.5)
<b>C1</b>	M	99.6 (91.9-100.0)	0.3 (0.0-7.9)	0.0 (0.0-0.5)	25.0 (12.3-38.3)	0.2 (0.0-8.0)	0.0 (0.0-1.2)	8.9 (0.1- 51.9)	10.4 (0.5-71.7)
	F	99.6 (88.7-100.0)	0.3 (0.0-9.7)	0.0 (0.0-1.2)	27.1 (17.0-39.8)	0.3 (0.0-10.7)	0.0 (0.0-2.7)	10.2 (0.6- 49.4)	10.0 (1.3-52.9)
<b>C2</b>	M	100.0 (95.3-100.0)	0.0 (0.0-4.2)	0.0 (0.0-0.0)	17.5 (2.6-38.6)	0.0 (0.0-1.4)	0.0 (0.0-0.0)	10.6 (1.3- 58.5)	11.5 (1.3-65.6)
	F	100.0 (98.1-100.0)	0.0 (0.0-1.7)	0.0 (0.0-0.2)	18.0 (3.5-37.7)	0.0 (0.0-3.3)	0.0 (0.0-0.2)	12.1 (0.7- 44.8)	9.9 (0.2-57.0)
<b>C3</b>	M	99.6 (70.0-100.0)	0.3 (0.0-23.9)	0.0 (0.0-4.5)	25.3 (12.4-53.5)	0.4 (0.0-30.9)	0.09 (0.0-10.6)	9.9 (0.8- 62.9)	10.8 (2.2-71.5)
	F	95.3 (83.8-100.0)	4.1 (0.0-13.1)	0.0 (0.0-3.0)	23.8 (8.5-53.5)	1.7 (0.0-13.3)	0.0 (0.0-4.5)	33.1 (7.5- 53.3)	35.3 (4.2-70.7)

