Behavioural and transcriptional changes in the amphipod *Echinogammarus marinus* exposed to two antidepressants, Fluoxetine and Sertraline

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Abstract

In the past decade, there have been increasing concerns over the effects of pharmaceutical compounds in the aquatic environment, however very little is known about the effects of antidepressants such as the Selective Serotonin Re-uptake Inhibitors (SSRIs). Many biological functions within invertebrates are under the control of serotonin, such as reproduction, metabolism, moulting and behaviour. The effects of serotonin and fluoxetine have recently been shown to alter the behaviour of the marine amphipod, *Echinogammarus marinus* (Leach, 1815). The purpose of this study was to observe behavioural and transcriptional modifications in this crustacean exposed to the two most prescribed SSRIs (fluoxetine and sertraline) and to develop biomarkers of neurological endocrine disruption. The animals were exposed to both drugs at environmentally relevant concentrations from 0.001 to 1 µg/L during short-term (1 hour and 1 day) and medium-term (8 days) experiments. The movement of the amphipods was tracked using the behavioural analysis software during 12 min alternating dark/light conditions. The behavioural analysis revealed a significant effect on velocity which was observed after 1 hour exposure to sertraline at 0.01 µg/L and after 1 day exposure to fluoxetine as low as 0.001 µg/L. The most predominant effect of drugs on velocity was recorded after 1 day exposure for the 0.1 and 0.01 µg/L concentrations of fluoxetine and sertraline, respectively. Subsequently, the expression of several *E. marinus* neurological genes, potentially involved in the serotonin metabolic pathway or behaviour regulation, were analysed in animals exposed to various SSRIs concentrations using RT-qPCR. The expression of a tryptophan hydroxylase (*Ph*), a neurocan core protein (*Neuc*), a Rhodopsin (*Rhod1*) and an Arrestin (*Arr*) were measured following exposure to fluoxetine or sertraline for 8 days. The levels of *Neuc*, *Rhod1* and *Arr* were significantly down-regulated to approximately 0.5, 0.29 and 0.46 fold respectively for the lower concentrations of fluoxetine suggesting potential changes in the phototransduction pathway. The expression of R*hod1* tended to be up-regulated for the lower concentration of sertraline but not significantly. In summary, fluoxetine and sertraline have a significant impact on the behaviour and neurophysiology of this amphipod at environmentally relevant concentrations with effects observed after relatively short periods of time.

Keywords: Antidepressants, SSRIs, Neuro-endocrine disruptor, Behaviour, Biomarker, Crustacean

1. Introduction

The issue of anthropogenic contaminants released in the aquatic environment acting as endocrine disruptors has been well studied, but the research effort has mainly consisted of the study of estrogenic substances and their effects on vertebrates (Hutchinson 2007; Weltje and Schulte-Oehlmann 2007). The increasing use (over 60% in just the past decade) of antidepressants, the improper disposal of unused pharmaceuticals, and their limited biodegradability has raised concerns about their potential effects in the aquatic environment (Demeestere et al. 2010; Santos et al. 2010). Antidepressants represent about 4% of the therapeutic drugs found in the environment, and are present in coastal waters and estuaries (Santos et al. 2010). Indeed, 30 to 90% of ingested drugs are excreted and released in the environment in an active form (Kashiyama et al. 2010) which can potentially have an impact on the organisms that inhabit these areas.

Much is still unknown about the ecotoxicological effects of pharmaceutical and personal care products in aquatic organisms (Crane et al. 2006; Santos et al. 2010). However, recent concerns regarding the impact of antidepressants, especially selective serotonin re-uptake inhibitors (SSRIs), on aquatic organisms has been increasing (Johnson et al. 2007; Minagh et al. 2009; Demeestere et al. 2010; Guler and Ford 2010; Styrishave et al. 2011). SSRIs inhibit the serotonin re-uptake into the pre-synaptic nerve inducing an increased neuro-stimulation of the post-synaptic nerve (Stahl 1998). These compounds act by modulating or mimicking the effects of serotonin (Santos et al. 2010). Since its approval by the US Food and Drug Administration in 1987, fluoxetine has become one of the most widely prescribed antidepressants, being in the top five psychiatric drugs prescribed in 2011 after citalopram and sertraline (Grohol 2012). Fluoxetine and sertraline are both SSRIs, primarily prescribed for depression but also used to treat compulsive behaviour, social anxiety, panic and personality disorders (AHFS 2013).

These drugs have been detected in the surface water and in wastewater effluent respectively at levels up to 0.54 μg/L and 0.929 μg/L for fluoxetine and up to 0.08 μg/L and 0.087 μg/L for sertraline (Brooks et al. 2003; Metcalfe et al. 2010; Styrishave et al. 2011; Silva et al. 2012). Fluoxetine has also been detected in groundwater at 0.056 μg/L (Silva et al. 2012). The only record of fluoxetine in seawater is in the Chesapeake Bay (Maryland, Virginia, USA) at 0.0026 μg/L (Pait et al. 2006). These findings make it clear that animals inhabiting aquatic ecosystems impacted by sewage effluent can be/are subjected to chronic exposure to SSRIs. Concentrations of fluoxetine and its metabolite norfluoxetine has been found at

extremely high level (10 μg/kg) relative to the environmental background in the tissues of fish collected near a municipal wastewater treatment plant, suggesting that these compounds have the capacity to bioaccumulate (Orem and Dolph 2002). The chronic effects of SSRIs on aquatic life are diverse (Brooks et al. 2003). For example, negatives impacts of fluoxetine have been found on the reproduction and growth of invertebrates, vertebrates as well as algae (Péry et al. 2008; Lister et al. 2009; Santos et al. 2010). The effects of sertraline on aquatic organisms have been less studied. According to several studies comparing the effects of SSRIs on diverse species, sertraline is the most toxic, seemingly more potent on daphnia species than on fish (Christensen et al. 2007; Paterson and Metcalfe 2008; Minagh et al. 2009).

The majority of studies on the impact of antidepressants within invertebrates have focused on reproduction and growth effects but few data sets are available on their behavioural effects (Fong 1998; Péry et al. 2008; Gust et al. 2009; Minagh et al. 2009; Campos et al. 2012b). Behavioural studies provide a link between physiological and ecological impacts, providing a major endpoint to assess population health and fitness (Craddock and Sklar 2013). Light is critical to a diverse range of behavioural and physiological processes such as diurnal rhythms, reproduction and predator avoidance (Henry et al. 2004). Indeed, light exposure regulates several neuro-modulatory systems; the activation of diverse photo-receptors modulates neurological components which in turn adjust behaviour. Serotonin, also named 5-hydroxytryptamine (5-HT), acts as a neurotransmitter or a hormone depending on its location and is a common modulator of animal behaviour in response to light. It is involved in many biological endpoints in invertebrates, such as growth, maturation, reproduction, visual perception and behaviour (Cezilly et al. 2000; Campos et al. 2012a). It has recently been demonstrated that exogenous serotonin and fluoxetine in amphipods increase phototaxis activity (Guler and Ford 2010). Acanthocephalan and trematode parasites can also act by increasing the serotonergic activity leading to an increase in phototaxis activity (Tierney et al. 2004; De Lange et al. 2006; Guler and Ford 2010; Underwood et al. 2010) which can increase susceptibility to predation (Cezilly et al. 2000; Lagrue et al. 2007; Perrot-Minnot et al. 2007). Guler and Ford (2010) suggested that altered phototaxis behaviour in amphipods following SSRI exposure could then conceivably make them more prone to predation.

Gammarid amphipods are fundamental to many food chains and have an important role in ecosystem dynamics (Donner et al. 1994). Therefore, they have often been used in ecotoxicology studies, being considered as excellent bioindicators to monitor the health of

aquatic biotopes and the effects of anthropogenic contaminants (De Lange et al. 2006; Felten et al. 2008; De Lange et al. 2009; Guler and Ford 2010; Issartel et al. 2010). *Echinogammarus marinus* (Leach, 1815) is a ubiquitous intertidal marine amphipod which is widely found throughout the coasts of northwest Europe. The aim of this study was to develop behavioural biomarkers of SSRI antidepressants exposure and elucidate the molecular mechanism of action through components of the serotonin pathway.

2. Materials and methods

2.1. Animals and exposure experiment

Echinogammarus marinus were collected on the intertidal zone beneath seaweed and stones at low tide from Langstone Harbour, Portsmouth, UK (50º47'23.13N 1º02'37.25W). This area is used for light recreational sailing and is a Special Protection Area (SPA), Site of Special Scientific Interest (SSSI) and Special Area of Conservation (SAC) due to the use of expansive mudflats by wading birds. Animals were sorted and adult males with no visual sign of infection by trematodes (incorrectly reported as acanthocephalans by Guler and Ford, 2010) were isolated. These parasitised individuals were excluded for their known impacts on host behaviour in response to light and modulation of serotonin in some species. The amphipods were kept individually in plastic containers filled with 80 mL of mechanical-filtrated natural seawater (from Langstone Harbour) at 10 °C under a 12 hrs light/12 hrs dark photoperiod and fed with fucoid seaweed.

After a week of acclimation, amphipods were exposed to the antidepressants fluoxetine and sertraline. In addition to an unexposed control group of 30 animals, groups of 15 animals were exposed to four nominal concentrations (0.001, 0.01, 0.1 and 1 µg/L) of each compound. Mortality was recorded and water was renewed every 3 d. Both fluoxetine (CAS no. 56296-78-7) and sertraline (CAS no. 79559-97-0) were obtained from Sigma-Aldrich® (St. Louis, MO, USA).

2.2. Behavioural analysis

Behavioural assays were performed after 1 hr, 1 d and 8 d of exposure to each condition using DanionVision™ (Noldus Information Technology, Wageningen, The Netherlands) and its software EthoVision® XT. Animals were put in 6-wells plates and placed within the DanioVision hardware for 2 min to allow settling prior to recording. The velocity (mm/s)

measurements of amphipods were recorded every 0.1 second (s) during 3 cycles of 2 minutes (min) dark and 2 min light, thus for a total period of 12 min (Fig. 1).

Due to the complexity of the dataset, an average velocity of every 10 s of the raw data were used to make heat maps for each condition by highlighting in green the 5th percentile, in black the 50th percentile and in red the 95th percentile (percentile calculated on the entire pool of data). This enabled a visual representation of periods when the amphipods were very active (red) or inactive (green). Statistical analyses were conducted using SPSS® Statistics v.20.0.0 software (IBM®) on the velocity of each amphipod during the 3 time periods (1 hr, 1 d and 8 d) of behavioural assays. The data was normalised with a cube-root transformation and tested using a Kolmogorov-Smirnov test. Repeated Measure Analysis of Variance (ANOVA) with Dunnett multiple comparison tests was used to determine whether significant differences occurred over the 12 min recording period and between concentrations for both drugs. This enabled us to determine whether the velocity of the amphipods changed over the 12 min dark/light regime, with SSRI concentrations or an interaction occurred between time and concentration. Within subject factors (time over the dark-light cycles and interactions between time and concentration) were tested using the Greenhouse-Geisser adjustments whereby sphericity of data is not assumed. All statistical analysis used a significance level of $p < 0.05$.

2.3. DNA/RNA isolation, purification and reverse transcription

After the 8 d behavioural assays, animals were anaesthetised using a mixture of clove oil and seawater (0.2 µL/mL). The head of each amphipod was rapidly dissected, snap frozen in liquid nitrogen and stored in Tri Reagent® (Ambion®, Life Technologies, Carlsbad, CA, USA) at -80 °C before the extraction. DNA and RNA were extracted according to the manufacturer protocol and used for the infection screening and real-time PCR, respectively. After a DNAse step using DNAse I (RNAse free) (New England Biolabs, Ipswich, MA, USA), RNA samples were cleaned on RNA clean and concentrator 5 columns (Zymo Research, Orange, CA, USA) following the manufacturer instructions. Quantification of total RNA and genomic DNA was performed with a NanoDrop® ND-100 Spectrophotometer (Nanodrop Technology Inc., Wilmington, DE, USA) and the integrity was checked using 1.5% agarose gel electrophoresis. For each sample, 250 ng of total RNA isolated was used to obtain cDNA by reverse transcription using the GoScript™ Reverse Transcription System

(Promega, Fitchburg, WI, USA) following the manufacturer protocol and using Oligo(dT)$_{15}$ primers and recombinant RNasin® Ribonuclease Inhibitor.

2.4. Infection screening

The infection of *E. marinus* by parasites capable of inducing an increase in the serotonergic activity (Guler and Ford 2010) might interfere with the response of this species to SSRIs or create additional variation within controls. The *E. marin*us population used for this study has been comprehensively screened and found to contain a single trematode species capable of neurological modulation of its host (Yasmin Guler, unpublished data). Therefore, an infection screen using PCR was performed to enable the removal of amphipods infected by this trematode from the dataset used for the behavioural and transcriptomic analysis. PCR assays were conducted on genomic DNA using primers designed to amplify the Internal Transcribed Spacer (ITS) region of the ribosomal RNA gene for this trematode species (Yasmin Guler, unpublished data). To check the quality of DNA sample, amplification of the glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) gene was used as a control (Table 1). PCR reactions were performed in a final volume of 25 µL containing 1X GoTaq® Flexi Buffer, 2.3 mM of $MgCl_2$, 0.8 mM of each dNTPs, 0.4 µM of each primer, 1 U of GoTaq® DNA polymerase (Kit GoTaq® Flexi DNA polymerase, Promega) and 30 ng of genomic DNA. The PCR conditions were: initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 45 s, 59 °C for 45 s 72 °C for 2 min and 20 s and a final incubation at 72 °C for 5 min. The PCR products were then analysed using agarose gel electrophoresis to check the presence of the amplified trematode ribosomal sequence.

2.5. Primer design and real-time PCR

The transcriptome of *E. marinus* has recently been sequenced (unpublished data). The generated expressed sequence tags were assembled to create a "transcriptome atlas" of contiguous sequences (or contigs) and these contigs were annotated by comparison to non-redundant sequences in the UniProt and FlyBase database (BLASTX, E-value cut-off of $1e^{-5}$). The contigs chosen for primer design were selected using the following criteria: (i) selection of contigs that potentially represent genes involved in behaviour modulation, even if they were not well annotated (E-value > e^{-5}), on the basis that such contigs might represent poorly conserved genes involved in neurological pathways; or (ii) selection of genes with a confident annotation (an E-value < e^{-5}) potentially involved in serotonin or neurological pathways; (iii) genes that appeared to show exclusively high expression in the head, on the

basis that these are more likely to represent genes with neurological functions. A pooled sample of cDNA was used to test the suitability of each set of primers. All primers used in this study, including those used as reference genes *Gapdh* and *Calreticulin* (Table 1) were designed using Primer-3 software (Koressaar and Remm 2007; Untergrasser et al. 2012) and synthesised by Eurofins MWG Operon (Ebersberg, Germany).

Quantitative real-time PCR (qPCR) analyses were performed using a real-time PCR cycler (Eco Illumina®, San Diego, CA, USA) on 12 samples per condition (or 3 pools of 4 head samples to test the primer pairs), using 7.5 µL of LabTAQ™ Green (LabTech International Ltd, Uckfield, UK), 1 µL of cDNA, 5.7 µL of ultra-pure water, 0.2 µL each of *Rhod1* forward and reverse primers and 0.4 µL each for all other primers (all primer volumes taken from a 10 µM stock). The PCR reactions were performed with an initial incubation at 95 °C for 2 min, followed by 45 cycles of 95 °C for 5 s and 60 °C for 30 s with Rox normalisation. Following the final cycle, the reactions underwent a 15 s, 95 °C denaturing step followed by a 15 s, 55 °C hybridisation step before PCR product melt curves were determined during a further temperature increase to 95 °C. Standard curve analysis was used to determine the efficiency of each primer pairs and melt curve analysis were performed for each gene to confirm the specificity of the PCR product in each reaction. Ultra-pure water was used in the place of template in the no template control reactions. Furthermore, minus RT reactions were performed to control for the potential presence of residual genomic DNA. The control group of animals (that were not exposed to antidepressants) were used as the reference sample. The relative expression of each gene was calculated using the ΔΔCt method (Livak and Schmittgen 2001) and normalised with both *Gapdh* and *Calreticulin* as reference genes. Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) is a reference (housekeeping) gene often used in several species (Barber et al. 2005) and particularly in crustaceans (Underwood et al. 2010; Leelatanawit et al. 2012). Statistical analyses were conducted on the square-rooted relative expression of each gene and results are expressed as the mean ± standard deviation (s.d.). The normality was tested using a Kolmogorov-Smirnov test and multiple comparisons and comparison of two mean values were performed following ANOVA using the Dunnett's multiple comparison test using SPSS® Statistics and at a significance level of $p < 0.05$.

3. Results

The mortality was very low during all exposure experiment with only 2 dead amphipods for the sertraline exposure at 1 µg/L and 1 ng/L. No trematode-infected amphipods (as detected by visual inspection and retrospective PCR) were used in the experiments.

3.1. Behavioural experiment

The average of each amphipods' velocity during the 3 times 2 min dark/2 min light cycle for each condition after 8 d exposure are shown in Fig. 1 as an example of the dataset generated. Generally, when the light was switched on, the amphipods react and the velocity increases almost instantly for each condition and time of exposure with the velocity gradually abating after 30 s.

Multiple comparison tests (Tukey's Multiple comparison; data not shown) revealed significant differences ($p < 0.001$) between 30 s time bins occurred overwhelmingly between the light and dark periods and were more pronounced for the 1st thirty seconds into the 2 min light cycles. This pattern was consistent for all concentrations, drugs and exposure periods. Interestingly, the 1st 30 s bin on the 1st of the 3 dark-light periods was also significantly different ($p < 0.001$) from all other periods within the light apart from after 1 d for both drugs (Fig. 2A.). For both drugs and at all exposure times, there was a significant effect of the varying dark-light cycles over the 12 min on the amphipods velocity ($p < 0.001$; Table 2; Fig. 2 and 3).

For fluoxetine after 1 hr exposure, there was no significant effect of the different concentrations ($p > 0.05$; Fig. 2 and Table 2) on velocity (mm/s) but there was a significant interaction between dark-light cycles and concentration ($p < 0.05$). This interaction appears to have occurred due to a divergence in velocity between concentrations over the three dark-light cycle which can be observed on the graph (Fig. 2A.). After 1 d exposure, a significant difference in the velocity was observed between concentrations ($p < 0.001$). Dunnett's 2-way multiple comparison tests revealed that significant differences occurred between the controls and all concentrations (0.001-1 µg/L: $p < 0.01$) with the highest velocities generally observed in the concentration 0.1 µg/L (about 78% higher than the control) and the lowest increase at 0.001 µg/L (about 43% higher than the control) (Fig. 2). Similarly, a significant interaction between concentrations and the dark-light cycles was observed ($p < 0.005$). After the 8 d exposure no significant difference was observed in the velocity of amphipods between treatments ($p > 0.05$) and the interaction tests failed to reach the significant cut-off ($p = 0.098$).

For sertraline, after just 1 hr exposure, significant differences were observed in the velocity between concentrations ($p < 0.05$; Fig. 3 and Table 2). Velocities were elevated in all concentrations, apart from the lowest (0.001 µg/L), relative to the control. However, Dunnett's multiple comparison tests revealed that significant differences occurred only between the controls and the 0.01 µg/L concentration (about 73% higher than control velocity; $p = 0.002$; Fig. 3). A significant difference was also observed after 1 d of exposure to sertraline with all (apart from 0.001 µg/L; velocity higher of about 69, 55 and 33% respectively for 0.01, 0.1 and 1 µg/L) exposed groups recording higher average velocities compared to the control ($p < 0.001$; Fig. 3). After 8 d exposure, no significant difference was observed in the velocity between exposed and control groups, with the associated p-value just failing to meet the significant criteria ($p = 0.057$; Fig. 3A.). For all sertraline exposure times, no interaction was observed between concentration and time ($p > 0.05$; Table 2).

3.2. The expression levels of neurologically-related genes in amphipods exposed to fluoxetine and sertraline

The expression level of reference genes, *Gapdh* and *Calreticulin*, did not change for any concentrations of both fluoxetine and sertraline: for *Gapdh*: df = 8, F = 1.380 and p-value = 0.239; for *Calreticulin*: df = 8, F = 1.648 and p-value = 0.148.

RNA pooled from 12 individuals for each exposure group were used to test the suitability of each primers pair associated to a set of 10 potential neurological biomarker genes (7 annotated with an E-value < e^{-5} and 3 unannotated). The serotonin receptor 1 (*5HT1*), the N-acetylserotonin O-methyltransferase-like protein (*Acser*), the inebriated neurotransmitter (*Ine1*) genes and contig 11430 presented very low expression, making it hard to determine expression from genomic contamination or the amplification of small amounts of artefact (results not shown). Those primer sets were then subsequently abandoned. For the two remaining unannotated genes (contig 9063 & 113810) the Ct value (the cycle number at which the fluorescent signal (ΔRn) crossed an arbitrary threshold set within the linear phase of amplification) for both genes, was less than 22 cycles and no contamination by dimers or hairpin hybridisation was evident. However, despite this high expression, no variation in their expression with pooled cDNA was observed between each exposure (results not shown). Four sets of primers, [Neurocan core protein (*Neuc*), Rhodopsin (*Rhod1*), Arrestin (*Arr*) and tryptophan hydroxylase (*Ph*)] did present evidence of both high and altered expression using

the pooled cDNA and were therefore used to quantify the variation of gene expression among each condition (drug and concentration after 8 d exposure).

The mRNA expression levels of these four genes in the head of *E. marinus* exposed for 8 d to 0, 0.001, 0.01, 0.1 and 1 μg/L of fluoxetine are illustrated in Fig. 4A. Significant differences were observed between expression of Neurocan core protein ($F = 6.632$, $df = 4$, $p = 0.007$), Rhodopsin ($F = 4.367$ $df = 4$, $p = 0.027$), and tryptophan hydroxylase ($F = 3.917$, $df = 4$, $p = 0.036$) but not for Arrestin ($F = 1.313$, $df = 4$, $p = 0.330$). Where significant differences were observed, these were predominantly found to be down-regulated in treated samples when compared to the control group for the lower fluoxetine concentrations (Dunnett's Multiple Comparison $p < 0.05$; Fig. 4A).

The mRNA expression levels of the four genes for 8 d exposure to sertraline are illustrated in Fig. 4B. Significant differences in expression were observed for Rhodopsin ($F = 7.868$, $df = 4$, $p = 0.004$) and Arrestin ($F = 3.527$, $df = 4$, $p = 0.048$) but not for Neurocan core protein ($F = 2.860$, $df = 4$, $p = 0.081$) and tryptophan hydroxylase ($F = 2.137$, $df = 4$, $p = 0.151$). Multiple comparison tests found no significant differences from the control, although it is worth noting that the expression of Neurocan core protein just failed to meet the significance criteria for the lowest concentration (0.001 μg/L, $p = 0.064$) as well as Rhodopsin for 0.1 μg/L ($p = 0.075$).

4. Discussion

4.1. Effect of light on amphipod behaviour

Amphipods naturally avoid well lighted areas and favour shadowed or dark regions in the intertidal zone where there is lower risk of predation (Cezilly et al. 2000). In this study, a significant increase in the velocity was observed in the first 30 s of light periods with a higher increase for the first of three light periods, at 1 hr and 8 d but not after 1 d of the beginning of the experiment. Sudden stimulation of the eyes could be interpreted by the amphipod as a reduction in cover and results in an escape-related behaviour in order to avoid predation. The decrease in the response to subsequent light periods indicates that the optic nerves may have been overstimulated and that a time of recovery from the first stimulation is needed. One day after the start of the experiment, the initial response to the light was reduced across all exposures indicating that more time may be necessary to recover.

4.2. Effect of fluoxetine and sertraline on amphipod behaviour

The first purpose of this investigation was to assess the effect of two SSRIs on the swimming behaviour of the amphipod *E. marinus*. In this study, amphipods were exposed to concentrations from 0.001 to 1 μg/L of fluoxetine and sertraline, these concentrations fall well within those currently being found in the aquatic environment (0.929 μg/L and 0.087 μg/L respectively) (Brooks et al. 2003; Metcalfe et al. 2010; Styrishave et al. 2011; Silva et al. 2012). Interestingly, a significant interaction between the dark-light cycling and concentration was observed for fluoxetine at short-term (1 hr and 1 d). This interaction was due to a divergence in the response to light between the animals exposed to various concentrations of fluoxetine and demonstrates that these antidepressants have an effect on amphipod behaviour. There was a significant increase in the velocity over the 12 min time period at 1 d exposure to 0.1 μg/L of fluoxetine (of about 78%) compared to the control, which is consistent with the concentration used in the experiment to produce maximum phototaxis behaviour of this species exposed to fluoxetine (Guler and Ford 2010). Guler and Ford (2010) highlighted the non-monotonic concentration response curve, noting a peak of phototaxis activity in the animals exposed at 0.1 μg/L of fluoxetine. The lack of significant or reduced effects in higher concentrations of fluoxetine could be due to the inhibition of a finite amount of endogenous serotonin or desensitisation, as also suggested by Guler and Ford (2010). Amphipods exposed to 0.01 μg/L of sertraline showed a significant higher velocity than the control after 1 hr exposure (about 69%), as well as from 0.01 to 1 μg/L after 1 d. Sertraline's mode of action is similar to fluoxetine, both being SSRIs. The effect of sertraline was most prominent for the 0.01 μg/L concentration compared to the higher concentrations for which the velocity was lower. This suggests that as well as for fluoxetine, higher concentrations of sertraline might tend to more quickly reach a maximum level of serotonin re-uptake inhibition or lead to a desensitisation. The larvae of the fish, *P. promelas* has a suppression of predator avoidance after less than a week of exposure to 0.025 μg/L of fluoxetine (Painter et al. 2009), although no alteration of this behaviour was found at higher concentrations. In adults, a decrease of the predator avoidance behaviour has also been demonstrated when exposed at a concentration of 3 μg/L of sertraline for 28 d (Valenti et al. 2012). However, contrary to fluoxetine, the response to higher concentrations (10 and 30 μg/L) of sertraline was the same as for 3 μg/L in *P. promelas*.

The increased light-induced velocity of amphipods exposed to SSRIs is consistent with an increase of the serotonin amount. This study did not test the preference of the amphipods to

lit areas [although this was observed by Guler and Ford (2010)], but rather the velocity of shrimp within light or dark environments. The most consistent results from this experiment indicated that amphipods are significantly more active both in light and dark phases of the experiment (with some interactions between light and concentration observed) when exposed to SSRIs as compared to untreated amphipods. Furthermore the recovery time (time to return to the basal velocity level) to light stimulation is altered between exposures and control. It is possible that the increased activity could also be due to the influence of serotonin on other hormones [e.g. Crustacean Hyperglyceamic Hormone, CHH; (Fingerman 1997)] and/or locomotor activity (McPhee and Wilkens 1989). However, changes in the transcription of genes relating to phototransduction pathways measured during study add some weight for linking the behavioural and gene responses. It will be beneficial in future studies to lengthen the periods of light and dark to differentiate the behaviours further.

Studies investigating the effect of SSRIs on aquatic organisms have been mainly performed using concentrations higher than those found in the environment and used in this study. Impacts of fluoxetine on the reproduction of *C. dubia* were observed at 56 µg/L with a decrease of fecundity (Brooks et al. 2003), and around 10 µg/L in *D. magna* (Péry et al. 2008). The acute toxicity of sertraline on animals has been demonstrated with a LC50 of 380 µg/L in fish following 96 hr of exposure (Minagh et al. 2009) and change in the behaviour of fish was found from 3 µg/L (Valenti et al. 2012). Relatively few studies have been carried out using environmentally relevant concentration of SSRIs (Painter et al. 2009; Guler and Ford 2010; Fong and Hoy 2012). However, the current study has found significant impacts as low as 0.001 µg/L that fall well within concentrations considered environmental relevant in the aquatic environment close to wastewater effluent and inhabited by this species (about 0.0026 µg/L in US estuaries, Paint et al. 2006). Furthermore, the degree of degradability of these antidepressants in water is generally low and their half-lives is from 2 days to indefinite (Johnson et al. 2005; Kwon and Armbrust 2006). The benthos is a reservoir for these compounds as they tend to be absorbed by sediments or sludge (Kwon and Armbrust 2006). The amount of SSRIs in this compartment should also be investigated in order to better evaluate the effects of antidepressants on amphipods. In this study, fluctuations in fluoxetine and sertraline concentrations might have occurred due to the static renewal of water every 2 days and the potential binding to the exposure chamber. Furthermore, insignificant results from the lower concentration range need to be carefully interpreted in light of the nominal concentrations used and the potential for chemical breakdown.

The presence of antidepressants in the environment can be chronic due to a constant release from the sewage water (Santos et al. 2010), thus a long-term analysis is essential to truly understand the effect of prolonged exposure times on aquatic organisms. Our results indicated that the most enhanced effects of fluoxetine and sertraline were observed following short-term exposure, after 1 hr (sertraline only) and 1 d of exposure. Although, contrary to this, Guler and Ford (2010) found a significant and continued preference of lit arenas still after 3 weeks exposure to fluoxetine at 0.1 μg/L compared to controls. As suggested by our higher concentrations of SSRIs in this study, a longer term exposure might lead to a desensitisation effect or a lack of serotonin availability and explain why no significant effect of both drugs was found after 8 d exposure. In mammals, it has been shown that the responsiveness to fluoxetine decreases following chronic exposure due to a critical decrease in the tryptophan levels, the precursor of serotonin (Delgado et al. 1999). Therefore, after several days of exposure to SSRIs, the haemolymph tryptophan content might be nearly depleted, reducing the drug effect on amphipods. Another hypothesis could be a negative feedback loop in the serotonin pathways; amphipods might be compensating for the change by producing less serotonin to flood the synapse or by increasing the expression of serotonin re-uptake transporter (Pineyro et al. 1994). It would then be interesting to compare the impact of these drugs on the serotonin pathway at short-term and long-term in further research.

4.3. Effect of fluoxetine and sertraline on amphipod gene transcription

The second aim of this study was to elucidate the molecular mechanism by which behavioural changes may be taking place. The absence of variations in *Calreticulin* and *Gapdh* expression supports their utilisation as reference genes.

Rhodopsin (*Rhod1*) is involved in behaviour regulation and is a light receptor and signal for phototransduction in vertebrates and invertebrates (Orem and Dolph 2002). In invertebrates, phototransduction cascade is mediated by rhodopsin, a light receptor which is transformed into metarhodopsin by photo-isomerisation (Orem and Dolph 2002). The metarhodopsin activates a Gαq-type of G-protein, hydrolysing guanosine triphosphate (GTP) to guanosine diphosphate (GDP), which then activates a phospholipase C (PLC). Finally, the PLC opens a transient receptor potential (TRP) channels which induce a depolarisation of the cells. *Rhod1* was significantly down-regulated in amphipods exposed to low concentrations of fluoxetine (0.001 and 0.01 μg/L) and slightly up-regulated for those exposed to 0.001 μg/L of sertraline. One explanation for the opposite gene expression patterns observed in *E. marinus* when

exposed to these two antidepressants may be the differences in their mode of action. Therefore, one might speculate that the mis-regulation of *Rhod1* could then modulate the transduction of light stimulation and alter the behaviour of amphipods to light. However, further studies will be necessary to better understand the role of rhodopsin in modulating amphipod behaviour. The protein encoded by the arrestin (*Arr*) gene is also involved in the phototransduction. In fact, this gene contributes to the arrest of the phototransduction cascade (Kashiyama et al. 2010) by binding the active metarhodopsin and inhibits it by uncoupling rhodopsin from the Gα-subunit protein (Orem and Dolph 2002). An example of their role in crustacean is that arrestin and rhodopsin promote light-induced hatching in *Triops granarius* (Kashiyama et al. 2010). In our study, *Arr* is down-regulated only in animals exposed to 0.001 and 0.01 µg/L of fluoxetine, which could be potentially linked to the down-regulation of *Rhod1* and components of the phototransduction pathway if followed by a protein down-regulation.

The neurocan core protein (*Neuc*) is a protein involved in cell adhesion and migration and is a factor in bipolar disorder, manic-depressive disorder and schizophrenia (Cichon et al. 2011; Mühleisen et al. 2012). In our study, *Neuc* mRNA expression significantly decreases for the two lower concentrations of fluoxetine. Assuming a similar function of *Neuc* in amphipods and mammals (Livak and Schmittgen 2001), a decrease in the expression of this gene if followed by a decrease in amount of its protein might lead to behavioural changes. It might then induce an increase of energy (Livak and Schmittgen 2001) which might tend to reduce the predator avoidance behaviour. The role of this gene should be investigated in further studies to define its function in amphipods. The enzyme tryptophan hydroxylase (*Ph*) catalyses serotonin biosynthesis in the serotonergic nerves (Hasegawa and Nakamura 2010). However, no significant variation in the expression of this gene has been found between each condition, which suggests that this gene is not involved in the serotonin regulation inducing the behavioural change observed when exposed to antidepressants.

The expression variations of these four genes were relatively low in *E. marinus* and it is unclear what impacts their down-regulation may have on amphipod behaviour. There is paucity of research regarding the molecular processes that underlie serotonin pathways and behavioural regulation in aquatic invertebrates. Further studies are essential in order to better understand the role of these genes in crustaceans and their relationship to the behaviour modification observed following antidepressant exposure. However, this study clearly demonstrates that exposure to SSRIs can be associated with alteration in the expression of

genes with plausible links to amphipod behaviour and serotonergic activity. Recently, it has been demonstrated in the crustacean *Gammarus pulex* that the histaminergic system is involved in the reaction to light in association with the serotonergic system (Perrot-Minnot et al. 2013). In the same study, looking at the influence of several 5-HT receptor antagonists and agonists, it has been suggested that the serotonin receptor 5-HTR2 subtype might be involved in the behaviour regulation of *G. pulex*. Furthermore, in *D. magna,* a transcriptomic analyses using a custom microarray showed that more than 1200 genes have a mRNA expression change when exposed to fluoxetine (Campos et al. 2013). Serotonin metabolism, neuronal development processes, carbohydrate and lipid metabolism functions were found to be differentially expressed when annotated by comparison to the functionally annotated *Drosophila* genome.

4.4. Summary

This study has provided evidence that a crustacean's behaviour and gene expression could be abnormally altered in waters receiving antidepressants at concentrations as low as 0.001 µg/L. The use of behavioural analysis has been demonstrated as good biomarker of the exposure of amphipods to antidepressants. The transcriptome of *E. marinus* is a rich resource for neurological genes that are potentially involved in behavioural regulation and serotonin related pathways. Therefore, future studies will be able to test an expanding number of amphipod genes for transcriptional change following exposure to antidepressants. This study has also provided further evidence for the non-monotonic concentration responses of some antidepressants, which should be taken into account when designing and evaluating toxicity tests. Whether other biological systems, for example: reproduction, moulting, metabolism and the immune system are impacted following low SSRIs exposure remains an important unanswered question. The effect of other SSRIs and their metabolites (Brooks et al. 2003; Stanley et al. 2007; Paterson and Metcalfe 2008; Metcalfe et al. 2010) on amphipods should also be evaluated along with other types of antidepressant such as the serotonin-norepinephrine re-uptake inhibitors (SNRIs) and the serotonin antagonist and re-uptake inhibitors (SARIs). The use of other types of antidepressants increases every year, with an increase of about 60 % for the SNRI duloxetine the last two years (HSCIC and Prescribing and Primary Care Services 2013). Considering that the mode of action for these other types of antidepressants is different from the SSRIs, it is important to also determine their potential

impact on aquatic organisms. How multiple antidepressants, with multiple modes of action, will act in mixtures is another challenge faced by ecotoxicologists. For example, it has been demonstrated that mixtures of antidepressants have additive effects in aquatic organisms (Christensen et al. 2007; Styrishave et al. 2011) and leads to a decrease in the predation avoidance behaviour in the larvae of the fish *P. promelas* (Styrishave et al. 2011). The organismal and ecological implications of these findings are difficult to deduce but coupled with previous studies suggest that SSRIs present in the aquatic environment could conceivably lead to population level effects through impacts on predation, feeding and reproductive associated behaviour.

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Table and Figures captions

Table 1. Primer sequences used in this study and target genes associate. The primers couple for serotonin receptor 1 have been design on alignment of several invertebrates' sequences of this gene and in very conserved area. *Italic*: the reference genes used to normalised the gene expression; † Four set of primers found relevant for quantification; * Target gene unknown, no annotation: E-value > e^{-5}.

Primer Name	Nucleotide sequences (from 5' to 3')	Target Gene	Uniprot or GenBank ID	Ref. Species	E-value
5HT1-F	CAA CGC AGA GTA CGG GGT TGG T	Serotonin receptor 1			
5HT1-R	GCA AAA CGG CGA AAT CGA ACG GG				
Acser-F	AAA CCC ACA AAC GAC GAC CA	N-acetylserotonin O-methyltransferase-like protein	O95671	*Homo sapiens*	7E-25
Acser-R	AAG GTT ACT CTC TGC CAC GC				
Arr-F	CTC CTT CGA CTC CAG GCT TG	Arrestin†	P32122	*Locusta migratoria*	5.00E-50
Arr-R	GGC TAA CCT GGG CAT CAA CA				
Calret-F	*AGA TCG GAG GCA TTG TTT TG*	*Calreticuline*	*Q7Z1E6*	*Bombyx mori*	*1.00E-155*
Calret-R	*AAC ACG TGG GCC GAG TAT AG*				
Gapdh-F	*ATA GTG TCC AAC GCC TCC TG*	*GAPDH*	*P56649*	*Panulirus versicolor*	*1E-164*
Gapdh-R	*CCA GTG GAG GAT GGA ATG AT*				
Ine1-F	CGT GGA GGA GCC GTT GCC TG	Inebriated neurotransmitter	NM057664.5	*Culex quinquefasciatus*	4.00E-05
Ine1-R	CCT GTG CGG CAT CCC TCT GC				
Neuc-F	CCC TAC CCT GTT TGC TCC AG	Neurocan core protein†	P55066	*Mus musculus*	7.00E-19
Neuc-R	CCA TTT TGG TAG TTC GCG GC				
Ph-F	GGT CAA GAC CTG GAG CGC GG	Tryptophan hydroxylase†	AY099427.1	*Aedes aegypti*	6.00E-142
Ph-R	GGT GCT GTG GAA CAC GCG GA				
Rhod1-F	CCC GCC AAC ATG CTG CCT GA	Rhodopsin†	DQ85259	*Neomysis americana*	4.00E-74
Rhod1-R	CGG GTG ACC GCA GGC TCT TG				
9063-F	TCA TCGACG AAC TTG GAG CC				*
9063-R	TCA TTG GCC TCT AGA AGC GC				
11381-F	TTC CGA ACT AAC GCC TGC TC				*
11381-R	CCA ACA GTG CAG CAA CAT CG				
11430-F	GTG AGG AGG AGG TGT GGG TA				*
11430-R	GGT ACA GGC GAG ACA ACA GG				

Table 2. Results of statistical analyses of velocity tracking during the 12 min of 2 min dark/2 min light periods in *Echinogammarus marinus* exposed to each concentrations of fluoxetine and sertraline for each time of exposure.

Compound	Exposure Period	Concentration			Time (Light-Dark Cycles)			Interaction: time* concentration		
		F	df	p	F	df	p	F	df	p
Fluoxetine	1 Hour	0.585	4	0.675	27.335	12.35	**<0.001**	1.482	49.412	**0.018**
	1 Day	7.199	4	**<0.001**	14.148	23	**<0.001**	1.694	53.017	**0.002**
	8 Days	1.087	4	0.368	13.787	23	**<0.001**	1.311	39.437	0.098
Sertraline	1 Hour	3.719	4	**0.008**	14.878	23	**<0.001**	1.061	53.725	0.358
	1 Day	7.966	4	**<0.001**	14.341	23	**<0.001**	1.307	43.656	0.407
	8 Days	2.373	4	<0.057	15.451	23	**<0.001**	1.321	46.337	0.076

F: ratio of the between and within group variance estimates; df: degrees of freedom; p: p-value, in bold when significant.

Fig. 1. Mean velocity (mm/s) of 15 *Echinogammarus marinus* per treatment exposed to fluoxetine and sertraline for 8 d recorded with DanioVision. 6-wells plates were used to track the velocity of 6 amphipods at a time every 0.1 s over a 12 min period of alternate 2 min dark/2 min light periods (A). Lines indicate mean values of replicates specimens. Black: control, gradation of blue: fluoxetine (FLU) concentrations (B), gradation of orange: sertraline (SER) concentrations (C).

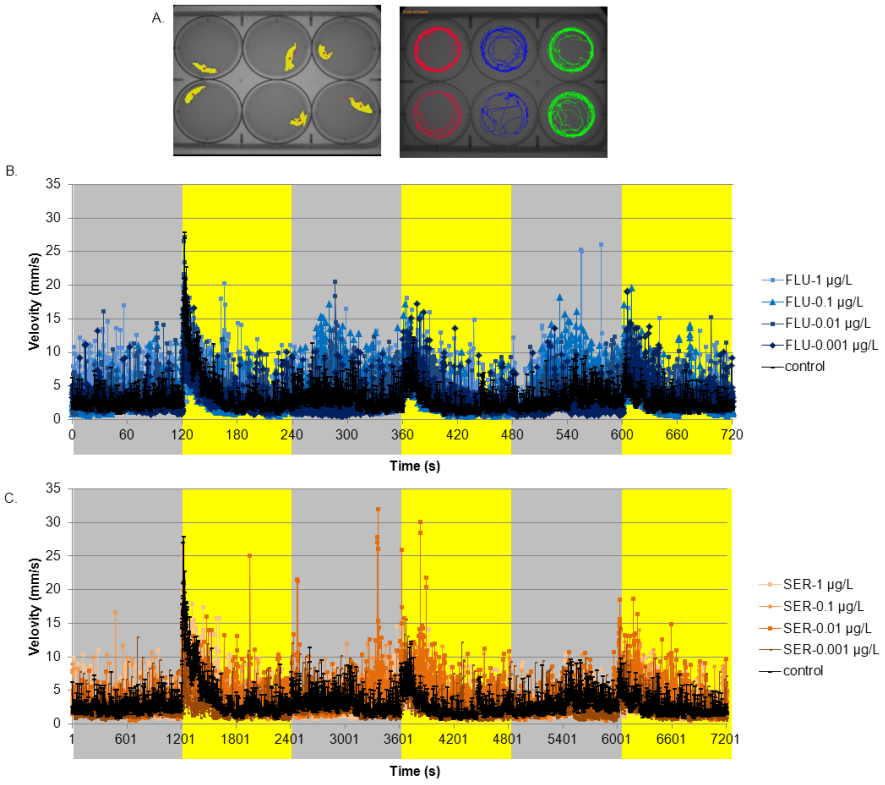


Fig. 2. Estimated marginal means (A) and heat map (B) of the velocity (mm/s) average every 10 s during the 12 min of 2 min dark/2 min light periods for each fluoxetine concentrations and time exposure. Heat map: green: the 5th percentile, black: the 50th percentile and red: the 95th percentile. Hr: hour, d: day(s). Asterisks indicate significant differences to the control ($p < 0.05$).

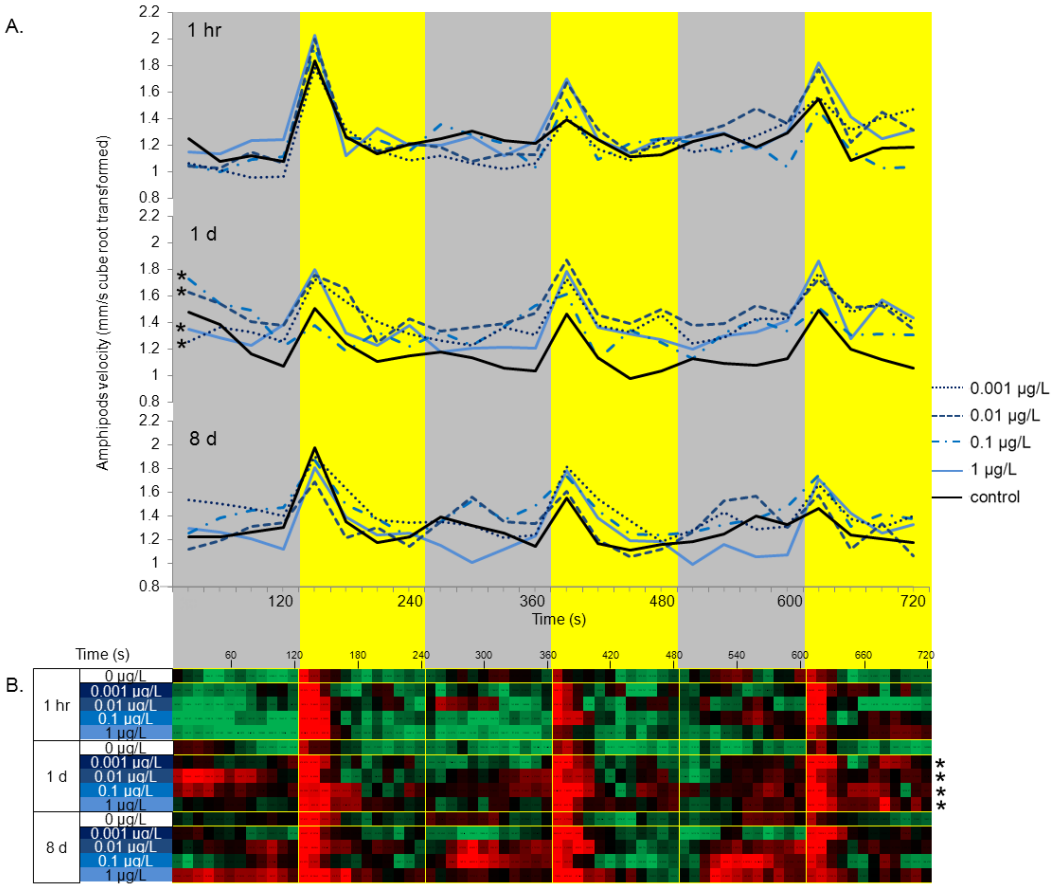
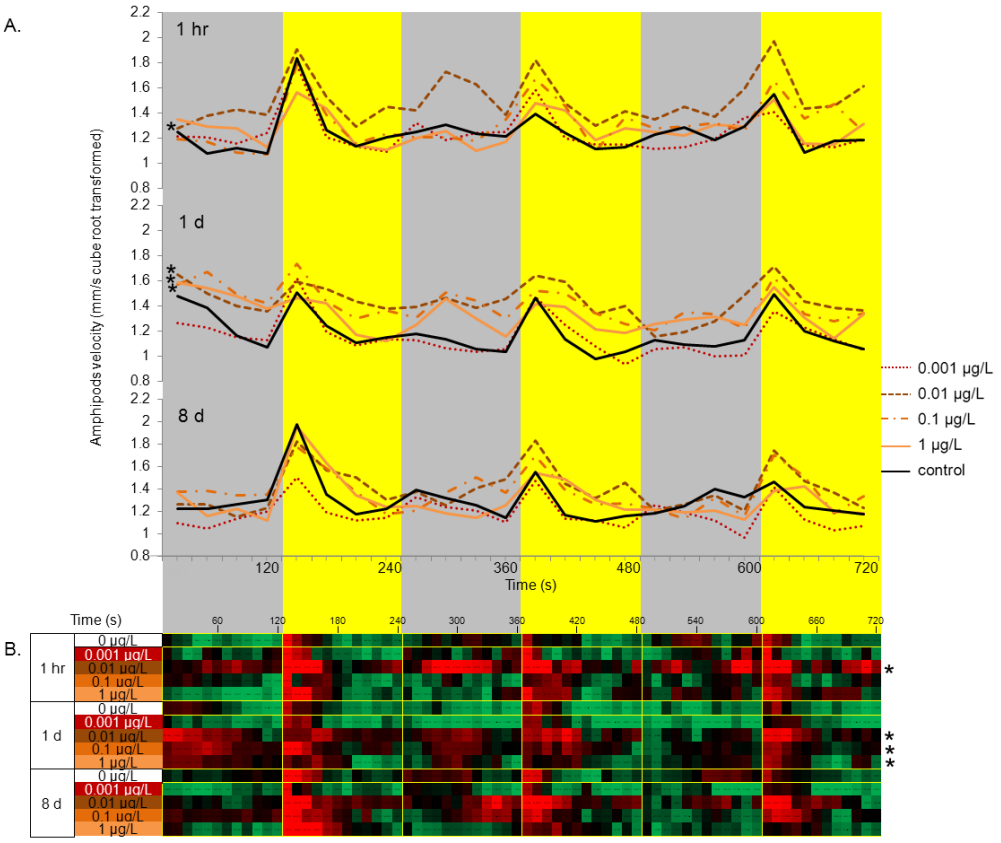


Fig. 3. Estimated marginal means (A) and heat map (B) of the velocity (mm/s) average every 10 s during the 12 min of 2 min dark/2 min light periods for each sertraline concentrations and time exposure. Heat map: green: the 5th percentile, black: the 50th percentile and red: the 95th percentile. Hr: hour, d: day(s). Asterisks indicate significant differences to the control ($p < 0.05$).



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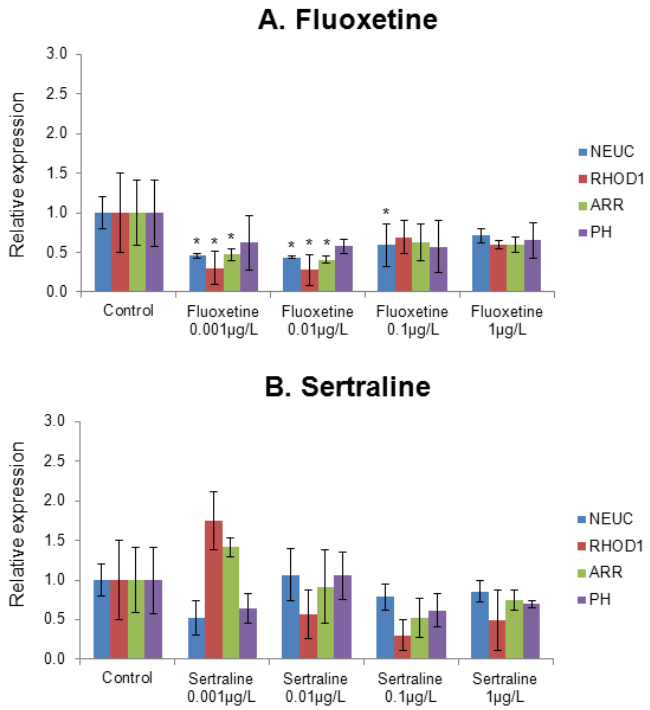
755 **Fig. 4.** Relative expression of Neurocan core protein (*Neuc*), Rhodopsin (*Rhod1*), Arrestin
756 (*Arr*) and tryptophan hydroxylase (*Ph*) mRNA in the head of *Echinogammarus marinus*
757 exposed to four fluoxetine (A) and sertraline (B) concentrations for 8 d. The expression was
758 normalised according to the expression of *Gapdh* and *Calreticulin*. n = 3 pools of 4
759 amphipods. Data are expressed as the mean ± s.d. Asterisks indicate significant differences to
760 the control ($p < 0.05$).


A. Fluoxetine
Relative expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
Fluoxetine 0.001µg/L
Fluoxetine 0.01µg/L
Fluoxetine 0.1µg/L
Fluoxetine 1µg/L
NEUC
RHOD1
ARR
PH
B. Sertraline
Relative expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
Sertraline 0.001µg/L
Sertraline 0.01µg/L
Sertraline 0.1µg/L
Sertraline 1µg/L
NEUC
RHOD1
ARR
PH

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