Exposure to the antihistamine diphenhydramine affects thermoregulation and increases righting time in the freshwater snail *Planorbarius corneus*
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Abstract

Antihistamines have been shown to degrade poorly, and should be considered as contaminants that may pose risks to the aquatic ecosystem. Diphenhydramine (DPH) is a first generation antihistamine detected up to lower micrograms per litre downstream of wastewater treatment facilities. Freshwater snails like Planorbis corneus are ectotherms and behaviour plays an important role for the regulation of snail body temperature. In a laboratory experiment, it was tested if DPH affects the behavioural traits thermoregulation and righting time in P. corneus. Righting time was measured as the time snails took to right themselves from an upside down position. After a 24 hour exposure to three different sublethal concentrations (nominal concentrations: 10, 100, and 1000 µg/L) of DPH two thermoregulatory experiments (thermal preference (T_{pref}) and maximum critical temperature (C_{max})) and one righting time experiment were performed. C_{max} increased significantly from 37.5 °C to 39.7°C after exposure to 949 µg/L DPH. Minimal righting time was significantly increased in the lowest exposure concentration (8.21 µg/L DPH). No significant results were found in the T_{pref} analyses. Collectively these result suggest that exposure to non-lethal concentrations of DPH affect behavioral traits like thermoregulation and righting time in freshwater snails.

Keywords: Planorbarius corneus, Diphenhydramine, Thermoregulation, Righting time
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Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPH</td>
<td>Diphenhydramine</td>
</tr>
<tr>
<td>RT</td>
<td>Righting time</td>
</tr>
<tr>
<td>CT_{max}</td>
<td>Maximum critical temperature</td>
</tr>
<tr>
<td>T_{Opt}</td>
<td>Temperature at which performance is maximized</td>
</tr>
<tr>
<td>T_{Pref}</td>
<td>Preferred temperature</td>
</tr>
<tr>
<td>PPCPs</td>
<td>Pharmaceuticals and Personal care products</td>
</tr>
<tr>
<td>SSRI</td>
<td>Serotonin reuptake inhibitor</td>
</tr>
</tbody>
</table>
**Introduction**

Improvements in analytical detection methods and extensive use of pharmaceuticals and personal care products (PPCPs) have resulted in the detection of hundreds of different compounds in the environment (Glassmeyer *et al.* 2005, Bartelt-Hunt *et al.* 2009, Golovko *et al.* 2014). Pharmaceuticals are developed to specifically interact with their target molecule, preferentially at low concentrations. Unfortunately this characteristic can turn into unwanted consequences when persistent pharmaceuticals reach freshwater systems. A lot of pharmaceuticals are not fully metabolized in their target organism or sometimes reactivated after passing the sewage treatment facility. In addition, due to their persistence and high binding affinity, effective concentrations of pharmaceuticals can be reached and affect ecosystems that are exposed to effluents of urban wastewater (Boxall *et al.* 2012).

Diphenhydramine (DPH) is a first generation antihistamine drug used to reduce allergic reactions. In response to an allergic trigger, mast cells release histamine. Target cell-receptors of histamine are located in blood vessels, the gastrointestinal tract and the respiratory tract. Receptor binding induce increased vascular permeability and contraction of the bronchial and intestinal smooth muscles. The dilated vessels allow blood and immune cells to permeate into the affected area causing swelling and redness typically associated with an allergic reaction. In case of a systemic response, this mechanism can lead to an anaphylactic shock (Abbas *et al.* 2014). DPH competes with histamine for the H1-receptors on the effector cells and lower the allergic induced effects by preventing histamine binding. DPH can cross the blood-brain barrier and is known to inhibit the serotonin (5-hydroxytryptamine or 5-HT) uptake in the synaptic cleft. This side effect has turned out to be useful in treating depressions. Due to the sedative side effects triggered in humans, DPH is also used in sleep-inducing drugs. The synthesizing and testing of several analogues of DPH, has led to the development of the first selective serotonin-reuptake inhibitor (SSRI) called fluoxetine hydrochloride. (Wong *et al.* 2005) More characteristics of DPH are given in Table 1.

Antihistamines have been shown to be poorly degraded in common sewage treatment facilities and are therefore continuously discharged into freshwater systems. Due to their high biological activity, the permanent efflux and persistency, antihistamines should be considered as contaminants that may possess risks to the aquatic ecosystem (Kosonen & Kronberg 2009). Interestingly, DPH was one of four pharmaceuticals, which showed no discernible loss in a three year outdoor mesocosm study where the degradation of 72 PPCPs was tested in biosoils (Walters *et al.* 2010). DPH has been detected in wastewater downstream sewage treatment plants in concentrations up to 1.4 µg/L (Bartelt-Hunt *et al.* 2009).

Additionally to the induction of allergic reactions in humans, histamines are known to be neurotransmitters in different species from mammals to invertebrates. In *Drosophila*, histamine plays an important role in temperature preference and tolerance to low and high temperature (Hong *et al.* 2006). Visual-, mechanosensory reception and sleep were also affected after modulating genes involved in histamine signalling (Hong *et al.* 2006). The importance of histamine as a neurotransmitter has also been shown in aquatic snails (Habib *et al.* 2015), and it is suggested that antihistamines interacting with histamine receptors might interfere in the snails’ temperature regulation system.
Temperature is an important environmental variable that affects the organisms’ life. Some organisms can tolerate extreme temperatures although most organisms show their maximum performance within a narrow thermal range (Tansey & Brock 1972). Depending on which temperature range an organism has adapted to, there will be a trade-off between performance and the temperature range. This can be represented as a bell shaped performance curve with an optimum temperature at which performance is maximized ($T_{\text{opt}}$) and critical thermal limits at each end (Angilletta Jr. 2009). The upper critical thermal limit, also called maximum critical temperature ($CT_{\text{max}}$), of the bell shaped performance curve is defined as: “the arithmetic mean of the collective thermal points at which locomotory activity becomes disorganized” (Cox 1974).

Insects and gastropods are ectotherms and rely on environmental heat sources. Beside morphological traits, behaviour plays an important role for regulating their body temperature (Miller & Denny 2011). Snails are excellent study organism for examining thermoregulatory behaviour. $CT_{\text{max}}$ can be estimated by observing the attachment of snails to a surface during a slow temperature change directed to one critical thermal limit. The temperature at which snails lose their attachment from the surface is defined as their critical thermal limit (Angilletta Jr. 2009). To find their $T_{\text{opt}}$ and avoid critical thermal limits snails use thermoregulatory behaviour. Therefore, the preferred temperature ($T_{\text{Pref}}$) chosen is often used as an estimation for the $T_{\text{opt}}$ (Angilletta Jr. 2009).

Pharmaceuticals disturbing thermoregulatory behaviour might be a threat to ectotherms that only have limited ability to adjust their temperature via physiological adjustment. For example, Hoppe et al. (2012) showed that antihistamines lead to reduced growth and reproduction in *Gammarus fasciatus*. *Daphnia magna* was tested to be very sensitive to DPH in an acute 48 hours and a subchronic 10 days study (Table 1). In a seven day study *Pimephales promelas* changed their feeding behaviour after DPH exposure (Table 1). Comparing the lowest observed effective concentrations (LOECs) of the studies made in *P. promelas* in Table 1, it can be seen that the alternative behavioural test was more sensitive than the standardized toxicity tests: survival and growth (Berninger et al. 2011). Behavioural alterations induced by DPH have also been found in crucian carp, where swimming activity and feeding rates were decreased after a 7 day exposure to 21.7 µg/L DPH (Xie et al. 2016).

Since only limited data exists on sublethal effects of DPH additional studies on ecotoxicological effects of antihistamines are needed, and to assess ecological risks in a more accurate way, different sublethal end points should be studied in multiple aquatic species (Li 2013).
Table 1: General information on the antihistamine diphenhydramine (DPH)

<table>
<thead>
<tr>
<th>Physical properties</th>
<th>Structure:</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAS</td>
<td><img src="structure.png" alt="Structure" /></td>
</tr>
<tr>
<td>DPH</td>
<td>58-73-1</td>
</tr>
<tr>
<td>DPH-HCL</td>
<td>147-24-0</td>
</tr>
<tr>
<td>Formula</td>
<td>C₁₇H₂₁NO</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>DPH-HCL: 255.36 g/mol</td>
</tr>
<tr>
<td>Solubility (37 °C)</td>
<td>3.06 mg/mL</td>
</tr>
<tr>
<td>Log P</td>
<td>3.27</td>
</tr>
<tr>
<td>pKₐ</td>
<td>8.9</td>
</tr>
</tbody>
</table>

**Diphenhydramine**
Common brands: Benadryl®, Bayer Aspirin PM®, Desentol®, Unisom®, Chattem®, Sominex®

**Pharmacodynamics**

**Acute Toxicity**

<table>
<thead>
<tr>
<th>Pimephales promelas (48h)¹</th>
<th>2.09 mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC₅₀ (pH 8.5)</td>
<td>59.28 mg/L</td>
</tr>
<tr>
<td>Daphnia magna (48h)¹</td>
<td>0.37 mg/L</td>
</tr>
<tr>
<td>LC₅₀</td>
<td></td>
</tr>
<tr>
<td>Ceriodaphnia dubia (48h)³</td>
<td>3.94 mg/L</td>
</tr>
<tr>
<td>LC₅₀</td>
<td></td>
</tr>
<tr>
<td>Dugesia japonica (48h)²</td>
<td>9.8 mg/L</td>
</tr>
<tr>
<td>LC₅₀</td>
<td></td>
</tr>
</tbody>
</table>

**Chronic toxicity**

<table>
<thead>
<tr>
<th>Pimephales promelas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival (7 days)¹</td>
</tr>
<tr>
<td>NOEC</td>
</tr>
<tr>
<td>LOEC</td>
</tr>
<tr>
<td>Growth (7 days)¹</td>
</tr>
<tr>
<td>NOEC</td>
</tr>
<tr>
<td>LOEC</td>
</tr>
<tr>
<td>Feeding rate (7 days)¹</td>
</tr>
<tr>
<td>NOEC</td>
</tr>
<tr>
<td>LOEC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Daphnia magna</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reproduction (10d)¹</td>
</tr>
<tr>
<td>NOEC</td>
</tr>
<tr>
<td>LOEC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ceriodaphnia dubia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reproduction (7d)³</td>
</tr>
<tr>
<td>LOEC</td>
</tr>
</tbody>
</table>

**Pharmacokinetics¹**

<table>
<thead>
<tr>
<th>Bioavailability</th>
<th>43 - 72%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma half life</td>
<td>3 - 9 h</td>
</tr>
<tr>
<td>Vₜ</td>
<td>3.3 - 14.6 L/kg</td>
</tr>
</tbody>
</table>

1: (Berninger et al. 2011); 2: (Li 2013); 3: (Goolsby et al. 2013)

The used study organism was the freshwater snail Planorbarius corneus, which is widespread and abundant all over the world (Strong et al. 2008). This planispiral pulmonate is also known as the Great Ram’s Horn snail and is used in aquarium trade. This species can be found in slowly moving waters where there is abundant growth of many different pond weeds (Seddon & Van Damme 2011). Planorbids such as P. corneus have secondary gills and use the respiratory pigment haemoglobin which allows them to exploit oxygen-depleted environments. P. corneus has previously been used successfully for thermoregulatory
experiments (Zbikowska et al. 2013a). No studies were found that have tested the effects of antihistamines in freshwater snails.

To analyse behavioural changes induced after antihistamine exposure, two thermoregulatory experiments and one righting experiment were performed. $CT_{\text{max}}$ and thermal preference ($T_{\text{Pref}}$) tests have been performed many times to answer ecological questions in snails with different origins (Gerald & Spezzano 2005, Tepler et al. 2011, Salas et al. 2014, Johansson 2015, Ermold 2016), and snails should therefore be a suitable organism to study the effects of antihistamines.

The righting activity is the ability of the animal to maintain natural posture. The righting time ($R_T$) is defined as the required time an organism takes to right itself from an upside down position (Fei et al. 2007). Righting time experiments have been performed in *Ilyanassa obsoleta* after exposure to SSRI-type antidepressants (Fong et al. 2016). Righting is important for animals to orient properly, and the inability to do so indicates toxicity or stress (Lawrence & Cowell 1996). To maintain orientation and mobility the time a snail needs to right itself should be minimized (Lawrence & Cowell 1996).

**Objective**

The objective of this work was to analyse the effects of the antihistamine diphenhydramine on the thermoregulation and mobility of the freshwater snail *P. corneus* after a 24 hour exposure period to three different sub-lethal concentrations of the antihistamine diphenhydramine. Therefore two thermoregulatory experiments ($T_{\text{Pref}}$ and $CT_{\text{max}}$) and one righting time experiment were performed to assess the orientation and mobility ($R_T$).
Methods

Three different experiments were performed to evaluate if diphenhydramine (DPH) affects the mobility or temperature regulation of freshwater snails. To analyse the orientation and mobility the righting time (RT) before and after DPH exposure was measured. To assess the temperature regulation a temperature preference (Tpre) test and a critical temperature test (CTmax) were performed after the snails were exposed to DPH for 24 hours.

Snail housing and handling

The *P. corneus* used in the study were hatched from egg clutches laid from ten adult snails from a laboratory culture. The snails were kept in a 50 L aquarium and maintained on an *ad libitum* diet of spinach (Ekologisk hackad spenat- ICA), spirulina (Super Nature) and fish food (Sera vipan). The diet was supplemented with calcium carbonate (Merck- 1.020660250) to support the shell growth. Twice a week, faeces were removed and 1/3 of the water was changed to maintain good water quality and a pH of 8. The water in the aquaria was kept at a constant temperature of 21±1 °C and the light/dark cycle was 16:8. Snails were used for the experiments when they started to lay egg clutches, which happened approximately at a size of 9 mm and a weight of 300 mg.

Diphenhydramine exposure

To analyse sub-lethal toxicity at environmentally relevant concentrations of DPH (European Pharmacopoeia Reference Standard-ID:0054L8), exposure concentrations of 10, 100 and 1000 µg/L were chosen. Those concentrations based on a pilot study and a review of the relevant literature (Berninger *et al.* 2011, Kristofco *et al.* 2015, Nichols *et al.* 2015). The pH and the temperature (pH-meter: Orion Tamrolab-210A; Thermometer: Clas Ohlson, Modell: ST-9215C-300) of the aerated solutions used for exposure and during the experiment were monitored daily. Since results from a pilot study suggested that the snail behaviour was negatively affected by the use of de-chlorinated tap water, water from the animals’ aquaria (pH=8.2 ±0.1, T=21 ±1 °C) was used for preparing the DPH exposure solutions. Therefore, even snails were not fed during the exposure periods, some algae and traces of food were likely present in the exposure water. For the exposure solutions, 13.74 mg DPH-HCL (European Pharmacopoeia Reference Standard-ID: 0054L8; Sartorius-MC 210P) were dissolved in 100 mL aerated water. 25mL of this solution were filled up to 3000mL with aerated water to obtain the first exposure solution (1000 µg/L DPH). In two further dilution steps (1:10), the exposure solutions with 100 and 10 µg/L DPH were made. For all of the experiments, snails were exposed for 24 hours individually in glass jars each containing 100 mL of one exposure solution at 21±1 °C with a 16:8 light/dark cycle.

To verify the exposure solution concentrations, samples were taken each day during the experiments and frozen for later analysis. The analysis of the DPH concentrations were conducted at SWETOX (Astraallén, Södertälje, Sweden) using a Waters Acquity UPLC-MS/MS system (Waters, Milford, MA, USA). Chromatographic separation was performed using a Waters Acquity UPLC BEH C18 column (2.1x 50 mm, 1.7 µm; Waters, Milford, MA, USA). The column temperature was maintained at 40 °C with a flow rate of 0.5 mL/min and an injection volume of 1 µL. The mobile phase consisted of a 99.9/ 0.1% Water/ Formic acid solution (A) and a 99.9/ 0.1% Acetonitrile/ Formic acid solution (B). The gradient program
was as follows: 0 min, 5% B; 5.00 min, 70% B; 5.01 min, 95% B; 6.00 min, 95% B; 6.01 min, 5% B; 8.00 min, 5% B. To assure constant measurements during the analysis, an internal standard was added to each sample (mixture: 50 µL:50 µL). For the internal standard, a 1.2 µM warfarin (Fluka Analytical, lot: #MKBS1257V) solution (in Methanol) was used. The lower limit of quantification for this method is 0.01 µM (=2.6 µg/L). The measured values are provided in Table 2.

**Preferred temperature (T\textsubscript{pref})**

Preferred temperature of the snails was estimated using a temperature gradient. Three aluminium trays containing five (500 x 20 x 20 mm) channels each separated by 2 mm were used. To create an experimental temperature gradient, one end of the trays rested on a cooling plate and the other on a heating plate. The aluminium trays were isolated with Styrofoam plates and the top of the channels was covered with an acrylic glass plate to prevent evaporation (Figure 1). Each channel was filled with 150 mL of aerated tap water 2 hours prior to the experiments to create a constant temperature gradient with a difference of 25 °C (min: 8 ±1 °C, max: 33± 1°C, 0.5°C/cm).

![Diagram](image)

**Figure 1: Set-up for the assessment of preferred temperature.** A temperature gradient was constructed with aluminum channel trays with a heating unit below one end and a cooling unit at the opposite end. The heating unit was comprised of a flow through-heating plate connected via tubes to a water bath (MGW Lauda MT) resulting in a maximum temperature of 33± 1°C. The cooling unit was comprised of a cooling plate (Histo-lab CP-4) resulting in a minimal temperature of 8 ±1 °C. To keep the temperature gradient stable Styrofoam and aluminium foil was used for isolation.

To determine the T\textsubscript{pref}, 75 snails were divided into the four exposure treatments: n\textsubscript{control}=21, n\textsubscript{10, 100, 1000}=18 (Table 2). In each treatment snails were exposed for 24 hours in glass jars containing 100 mL of the different testing solutions. Due to the limited number of channels (15/experiment) in the temperature gradient, the exposure was staggered over seven days. After the exposure period the snails were evenly distributed through the channels and placed into the gradient at 21 °C. The temperature was measured at the snails’ position every three hours during the light period for 24 hours using a digital thermometer (± 1°C). After each experiment each snail was transferred into a bin containing aerated tank water to check for recovery.

**Maximum critical temperature (CT\textsubscript{max})**

To determine the CT\textsubscript{max}, 96 snails were divided into four exposure treatments (Table 2). In each treatment snails were exposed for 24 hours in glass jars containing 100 mL of the different testing solutions. The experiment was staggered across 2 days (12 snails per treatment per day). After the exposure snails were rinsed in aerated tap water and placed into 30 mL glass jars filled with aerated water. The glass jars were put evenly distributed into a water bath (Julabo-F34) at a starting temperature of 21 °C. After an acclimatization period of
30 minutes the temperature was increased for one degree every 30 minutes. The snails were observed prior to each temperature increase to define the CT\textsubscript{max} at which the snails lost attachment to the glass wall. After losing attachment each snail was transferred into a bin containing aerated water with a temperature of 21 °C and checked for recovery. The experiments were performed at the same time period on both days.

**Righting time (R\textsubscript{T})**

To determine the R\textsubscript{T}, 60 snails were divided into the four exposure treatments (Table 2), and exposed for 24 hours in glass jars containing 100 mL of the different testing solutions. To control for between-animal variation, each individual was tested for righting ability before and after the exposure. Hence, each snail was placed into a flat 120 mL beaker containing 50 mL of aerated water and allowed to acclimate for five minutes. After manually flipping the snails on their back using a stick, the time each snail took to right was measured. A snail was considered completely righted when its foot was completely attached to the glass jar. The righting time was measured three times for each individual. After 24 hours of exposure, the righting time was measured of each individual again.

**Statistical analysis**

All graphs, tables and statistical analysis were performed in R (R Core Team 2013) and the data was imported via Microsoft Excel\textsuperscript{®}. The figures were drawn using Microsoft Paint\textsuperscript{®}.

For the T\textsubscript{Pref} experiment 75 snails (n\textsubscript{control}=21, each exposure treatment: n=18) were used within seven days. The mean, thermal range, maximum and minimal temperature visited was summarized for each snail and treatment. It was tested for significant difference between the exposure treatments using an ANOVA.

For the CT\textsubscript{max} experiment 96 snails (24/ treatment) were used within three days. To determine the CT\textsubscript{max}, the temperature at which 50% remain attached for each exposure treatment was calculated. To calculate the CT\textsubscript{50}, the percentage data was arcsine-transformed and a line was fitted to the data points as suggested by Salas et al. (2014). Using linear regression the CT\textsubscript{50} for each exposure treatment was calculated as in Salas et al. (2014). A Wilcoxon-Mann-Whitney test was used to determine differences between the exposure treatments and the control.

For the R\textsubscript{T} analysis 60 snails (15/ treatment group) were used within two days. Two different ways to analyse the results were used for the R\textsubscript{T}. In the first approach the means of the triplicates were used to calculate the delay of the righting time before and after the 24 hours exposure. In the second approach the minimal time out of three trials, each snail took to right, before and after the exposure was used to calculate the delay. Pairwise comparison (paired T-test) of the minimal and the mean time to right before and after the 24h exposure was performed. To test for significant difference between the exposure treatments and the control a Dunnett’s test and a Wilcoxon-Mann-Whitney test were performed.

Each exposure treatment in the T\textsubscript{Pref}, CT\textsubscript{max} and R\textsubscript{T} analyses was tested for outliers by comparing the distance from extreme values with the 1.5 interquartiles range of all values and using the modified Thompson Tau test. Outliers were removed if they were confirmed to be outliers in both tests. Additionally, all data was tested for normality using a Tukey Anscombe plot and a Q-Q plot.
Results

Analysis of the exposure solutions
The mean pH and the mean temperature for the aerated water and exposure solutions was 8.2 ± 0.1 and of 21 ± 1 °C respectively. According to the analysis performed at the SWETOX laboratory (Astraallén, Södertälje, Sweden) the following concentrations for the experimentally used exposure solutions were found, see Table 2.

Table 2: Analytically verified mean diphenhydramine concentrations of the exposure solutions. The exposure solutions were prepared and measured for three different experiments (CT<sub>max</sub>, T<sub>Pref</sub> and R<sub>T</sub>).

<table>
<thead>
<tr>
<th>Nominal Concentration [µg/L]</th>
<th>Measured concentration [µg/L]</th>
<th>T&lt;sub&gt;Pref&lt;/sub&gt;</th>
<th>CT&lt;sub&gt;max&lt;/sub&gt;</th>
<th>R&lt;sub&gt;T&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Mean  SD</td>
<td>0  0</td>
<td>0  0</td>
<td>0  0</td>
</tr>
<tr>
<td>10</td>
<td>7.87  0.33</td>
<td>9.58  0.43</td>
<td>8.21  0.018</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>92.1  7.6</td>
<td>97.0  3.9</td>
<td>96.7  12.0</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>925  51</td>
<td>949  44</td>
<td>952  82</td>
<td></td>
</tr>
</tbody>
</table>

Preferred temperature (T<sub>Pref</sub>)
No outliers were observed in the T<sub>Pref</sub> data, and the four different exposure treatments were normally distributed. The mean weight of the snails was 388 ± 12 mg and did not differ significantly between the groups (ANOVA: F<sub>3,71</sub> = 1.39, p = 0.254). The mean of the highest and the minimal temperature visited and the mean temperature overall during 24 hours was calculated for all treatments and compared using ANOVAs. These ANOVAs showed no significant differences among treatments (Mean: F<sub>3,71</sub> = 0.759, p = 0.521; Min: F<sub>3,71</sub> = 0.755, p = 0.523; Max: F<sub>3,71</sub> = 0.135, p = 0.939). There was also no significance in the thermal range visited (ANOVA: F<sub>3,71</sub> = 0.767, p = 0.517). The similarities between the different exposure treatments are shown in Figure 2.
Figure 2: Preferred Temperature of *P. corneus* during 24 hours observation after 24 hours exposure to the control and three diphenhydramine (DPH) concentrations. The black dots with standard error bars in the graph show the mean temperature snails visited in the temperature gradient for the four different exposure treatments (Control, 7.87, 92.1 & 925 µg/L DPH). The box around each black dot shows the thermal range the snails visited (maximum and minimal temperature visited) within the thermal gradient.

**Maximum critical temperature (CT<sub>max</sub>)**

One outlier was defined and removed in the highest exposure group (949 µg/L) using the two different outlier detection methods described. The four different exposure treatments were normally distributed according to the Tukey Anscombe and the Q-Q plot. The mean weight of the snails was 381 ± 10 mg and did not differ significantly between the groups (ANOVA: \( F_{3,92} = 0.668, \ p = 0.574 \)).

CT<sub>max</sub> is defined as the temperature at which 50% remain attached and was calculated for the four exposure treatments and was 37.5, 37.0, 37.9 and 39.7 °C for control, 9.58, 97.0 and 949 µg/L DPH respectively. Additionally, the CT<sub>50</sub> was calculated by arcsine-transforming the percentage data and fitting a line via linear regression (Figure 3). According to this evaluation the CT<sub>50</sub> values were 36.7 (\( R^2 = 0.964 \)), 36.4 (\( R^2 = 0.975 \)), 37.2 (\( R^2 = 0.972 \)) and 39.0 °C (\( R^2 = 0.975 \)) for control, 9.58, 97.0 and 949 µg/L DPH respectively. A Wilcoxon-Mann-Whitney test showed a significant difference (\( p=0.0179 \)) in CT<sub>max</sub> values between the control and the highest exposure treatment (949 µg/L DPH). All other pairwise comparisons were non-significant (Wilcoxon-Mann-Whitney: \( p > 0.05 \)).

![Graph showing the preferred temperature of P. corneus during 24 hours observation after 24 hours exposure to control and three diphenhydramine (DPH) concentrations.](image)

**Figure 3:** The percentage of *P. corneus* remaining attached after 24 hours exposure to the four different treatments (Control, 9.58, 97.0, 949 µg/L Diphenhydramine (DPH), n=24/group) plotted against temperature (°C). The y-axis shows arcsine transformed percentage of remaining snails. The CT<sub>max</sub> is defined as the value where 50% remain attached. The CT<sub>50</sub> (dashed line) was calculated after arcsine-transformation of the data and fitting a line to the data points (solid line) using linear regression. The \( R^2 \) of the fitted lines calculated is given in the top right of each exposure treatment.
**Righting time (Rₜ)**

Three outliers were defined for the mean time using the two different outlier detection methods described and therefore those data points were removed (Outliers found in control, 8.21, and 952 µg/L DPH). For the minimal time, two outliers (Outliers found in control and 96.7 µg/L DPH) were found and removed. The four different exposure treatments were normally distributed in both cases (mean and minimal righting time). The mean weight of the snails was 329 ± 7 mg and did not differ significantly between the groups (ANOVA: F₃,₅₆ = 0.423, p = 0.737). A pairwise comparison (paired T-test) showed that there was a significant increase in righting time measured in all groups (Control, 8.21, 96.7 and 952 µg/L DPH) after 24 hours (all p < 0.05). Due to this, the differences in righting time before and after the DPH exposure were used to compare between groups.

Using this difference, the mean righting time differed significantly between the control and 96.7 µg/L DPH treatment, and between the control and the 952 µg/L DPH comparison (Dunnett’s test: t = 3.046, p = 0.0099, and t = 3.093, p = 0.0087, respectively; Figure 4). Using the Wilcoxon-Mann-Whitney test the same significant difference (p = 0.012, and p = 0.004 for the 96.7 and 952 µg/L DPH respectively; Figure 4) was found. No other pairwise comparisons were significant: all p > 0.05 for both statistical test methods.

Analysing the minimal righting time using the Dunnett’s test showed significant difference between the control group in all three treatments: 8.21 (t = 3.67, p = 0.0016), 96.7 (t = 3.345, p = 0.0043) and 952 µg/L DPH (t = 4.396, p = 0.001). Using the Wilcoxon-Mann-Whitney test significant difference between the control group, the 96.7 (p = 0.022) and the 952 µg/L DPH (p=0.0024) exposure treatments was found, but not between the control and lowest exposure treatment of 8.21 µg/L DPH (p = 0.057).
Figure 4: Mean (A) and minimal (B) delay of righting time ± SE (sec) of 60 P. corneus after 24 hours exposure to one of four different treatments (Control, 8.21, 96.7, 952 µg/L Diphenhydramine (DPH), n=15/group). The four bars show the mean delay of (A) the mean time ± SE snails took to right themselves from three trials; (B) the minimal time ± SE snails took to right themselves within three trials. Stars (*) on the bars indicate significant difference from the control group according to Dunnett’s test.
**Discussion**

The primary objective of this work was to analyse if a 24 hour exposure to the antihistamine diphenhydramine (DPH) affects the thermoregulation and mobility of the freshwater snail *Planorbarius corneus*. Therefore three different sub-lethal concentrations of DPH were tested. The thermoregulatory behaviour of the snails was affected in the highest DPH exposure of the critical temperature (CT\text{max}) analysis. The result of the righting time showed that snails were affected in all three tested concentrations. In contrast, no significant effect was found in the temperature preference (T\text{Pref}) experiments, since the snails moved through the temperature gradient without showing any behavioural difference to the control group.

CT\text{max}-values increased with higher concentrations of DPH, and thus DPH affects the thermoregulatory behaviour of *P. corneus*. Increased CT\text{max} values (from 37.5 °C to 39.7 °C) were observed in the highest exposure treatment (949 µg/L DPH). Transforming the data and fitting a line via linear regression to the transformed data points, as suggested by Salas et al. (2014), resulted in lower CT\text{50} values compared to CT\text{max}. However, all the resulting values shifted down by approximately 0.7 °C, and therefore the results for CT\text{50} were qualitative similar as those for CT\text{max}.

The majority of past studies on thermal preferences and CT\text{max} in snails have compared within species variation using individuals from different thermal origins to answer questions about global warming (Diaz et al. 2011, Huey et al. 2012, Johansson 2015, Ermold 2016). Most studies that have tested environmental pollutants affecting the critical thermal maxima have been conducted on fish (Lydy & Wissing 1988, Patra et al. 2007). Patra et al. (2007) showed that exposure to endosulfan and chlopyrifos caused significant reduction in CT\text{max} values in fish. This is contrary to results in this study, where exposure to DPH increased the maximum thermal tolerance. Patra et al. (2007) argued that the CT\text{max} decreased in fish due to additional stress caused by the organic chemicals. However, the increase of the CT\text{max} in the used freshwater snails might be caused by a different mode of action. Since DPH inhibits serotonin reuptake and reduces stress, snails might not be exposed to the same stress levels as fish treated with organic chemicals. Although, to explain these contrary results of chemicals acting different on temperature tolerance, further studies are needed.

DPH exposure leads to a delay in righting time in *P. corneus*. Fong et al. (2016) analysed the righting time of mud snails after the exposure to four commonly used antidepressants. One of their antidepressant, Fluoxetine, is an analogous compound of DPH. These authors found a significant delay in mean righting time after a two hour exposure to 3.45 µg/L Fluoxetine. Surprisingly the righting time did not increase more at higher concentrations, 34.5 and 345µg/L Fluoxetine (Fong et al. 2016). The discovery of the sedative effects induced by DPH lead to the development of Fluoxetine, which was the first established serotonin reuptake inhibitor (SSRI) (Berninger et al. 2011). Since Fluoxetine and DPH are strongly related to each other they address a similar mode of action inducing delayed righting time in freshwater snails. Fluoxetine was developed to be a stronger and more specifically binding serotonin-reuptake inhibitor than DPH. Similar effective concentrations at longer exposure times were measured. This suggests that the righting time of freshwater snails is mainly affected due to the increase in serotonin, which is the mode of action addressed by Fluoxetine. Inhibition of
serotonin reuptake might lead to less stress in exposed snails. Reduced stress levels in a vulnerable posture might be a reason for the extended time snails took to right themselves.

Even though there was no statistical significance found in the $T_{\text{Pref}}$-analysis, the thermoregulatory behaviour of *Planorbarius corneus* might be affected by DPH exposure. Temperature preference analyses of snails have been performed many times, using different experimental set-ups, by different research groups (Gerald & Spezzano 2005, Diaz et al. 2011, Tepler *et al.* 2011, Zbikowska *et al.* 2013b, Johansson 2015, Ermold 2016). There is no standardised temperature gradient setup used or recommended since every experiment has focused on a different endpoint. The used experiment a setup was adapted from two previously performed experiments where $T_{\text{Pref}}$-values for *Galba truncatula* and *Radix balthica* were analysed (Johansson 2015, Ermold 2016). In a pre-experimental setup local temperature of *P. corneus* was measured every 15 minutes for 6 hours in four treatments. No significant difference was found between the exposure treatments. Observations during this experiment showed that the frequency of moving snails during the 6 hours might be too low. Due to that, the observation time was expanded to 24 hours. This resulted in a similar outcome, i.e., no significant effect. This might indicate that DPH does not affect the thermoregulatory behaviour of *P. corneus*. However, an odd behaviour was noticed through all exposure treatments and the control. Snails experiencing extreme temperatures in the gradient did sometimes stopped moving until removed from the gradient at the end of the experiment. This behaviour was also observed in snails which were directly transferred into the temperature gradient from the snail aquaria. Behavioural stress generally includes either a loss of grip and inability to remain attached to a substrate or increased movement, both followed by immobility (Dallas & Ketley 2011). The length of the used aluminium panels was rather short for the applied temperature gradient ranging from 8 to 33 °C (0.5°C/cm). Due to that it could be that the temperature gradient was too steep and the channels too short to give snails the opportunity to choose their $T_{\text{Pref}}$. Snails moving through the gradient might have noticed the change in temperature too late resulting in a stress response and immobility. Other snails did not move at all during the whole experiment, but started moving again in the recovery jar. In summary, it cannot be excluded that DPH has no effect on *P. corneus* $T_{\text{Pref}}$. To answer this question either longer aluminium panels or a smaller temperature range should be chosen for future experiments. This might reduce stress and give snails the opportunity to adapt and sense their surrounding temperature.

Repetitive effects in wastewater streams might lead to disruptive effects affecting thermoregulatory behaviour and orientation in freshwater organisms. The sub-lethal effects found, might have severe consequences for aquatic organisms if the organisms are repeatedly exposed to these concentrations. For example, the repetitive effects of longer righting time over an organisms life time might cause less time available for food acquisition and might also expose the snails to higher predation risk.

Beside DPH several other antihistamines and pharmaceuticals have been tested to be poorly degraded in wastewater treatment plants (Kosonen & Kronberg 2009). Therefore aquatic organisms are usually exposed to trace concentrations of different pharmaceuticals. Not many studies so far have measured adverse effects in aquatic organisms when testing those pharmaceuticals separately with naturally occurring trace concentrations. Interestingly, it
might turn out to be difficult to predict the ecological impact of this pharmaceutical cocktail when mixed together. Goolsby et al. (2013) showed by testing combinations of DPH and Sertraline that nontoxic tested concentrations affected health drastically when exposed in combination. Pharmaceuticals acting on similar mode of actions might be able to induce adverse effects already at trace concentrations due to additive effects. Adverse effects induced by additive effects on behaviour controlling thermoregulation and orientation cannot be excluded, and more research is therefore needed.

Behavioural studies are fast, sensitive and powerful tools and might not be utilized to their full potential for environmental monitoring. Due to relatively high power and sensitivity achievable, more research to expand current and evolve new behavioural techniques should be done (Melvin & Wilson 2013). In this thesis the behavioural endpoints thermoregulation and righting time were successfully used to evaluate the adverse effects of DPH. Due to the result in this thesis it can be confirmed that righting response might be a useful behavioural endpoint for aquatic toxicology (Fong et al. 2016). Using thermoregulation as a behavioural endpoint to test for pharmaceuticals addressing the nervous system might need further investigations.
References


