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Behavioural effects of psychoactive pharmaceutical exposure on European perch (*Perca fluviatilis*) in a multi-stressor environment

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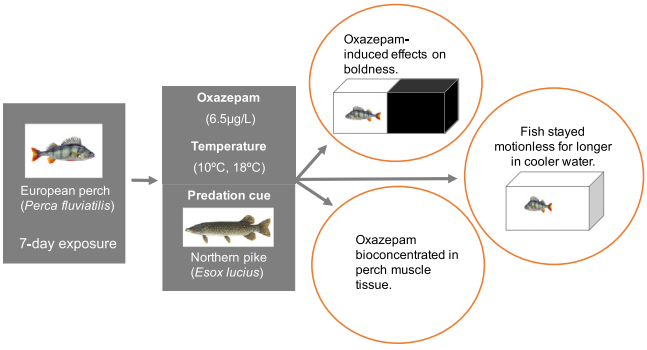
HIGHLIGHTS

- Juvenile European perch were exposed to oxazepam at two temperatures and under two predation risk regimes.
- Exposure altered anxiety-related behaviour and boldness of fish.
- Fish in the low temperature treatments froze for longer. Predator cue treatment affected perch risk-taking behaviour.
- We found no interaction effects of oxazepam and temperature on the studied behaviours.
- Highlights ecologically important sub-lethal effects of pharmaceutical contamination.

GRAPHICAL ABSTRACT



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ABSTRACT

With the ability to resist biodegradation and exert therapeutic effects at low concentrations, pharmaceutical contaminants have become environmental stressors for wildlife. One such contaminant is the anxiolytic oxazepam, a psychoactive pharmaceutical that is frequently detected in surface waters globally. Despite growing interest in understanding how wildlife respond to anxiolytics, synergistic effects of pharmaceuticals and other abiotic (e.g. temperature) and biotic (e.g. predation risk) stressors remain unclear. Here, using a multi-stressor approach, we investigated effects of 7-day oxazepam exposure (6.5 µg/L) on anxiety-related behaviours in juvenile European perch (*Perca fluviatilis*). The multi-stressor approach was achieved by exposing perch to oxazepam at two temperatures (10 °C and 18 °C), and at two predation risk regimes—generated using chemical cues from the northern pike (*Esox lucius*). Our exposures resulted in a successful uptake of the drug from the water, i.e., oxazepam was measured in perch muscle tissue at 50 ± 17 ng/g (mean ± SD). We found significant oxazepam-induced effects on boldness, with 76.7% of the treated fish entering the white background (i.e. 'exposed' area where exposure to presumed risks are higher) within the first 5 min, compared to 66.6% of the control fish. We also found a significant effect of temperature on total time spent freezing (i.e. staying motionless). Specifically, fish in the low temperature treatments (oxazepam, predation) froze for longer than fish in high temperatures. Our multi-stressor study is the first to uncover how anxiety-related behaviours in wild juvenile fish are altered by changes in water temperature and perceived predation risk. Importantly, our findings highlight the

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need to focus on multiple stressors to improve understanding of how organisms not only survive, but adapt to, human-induced environmental change.

1. Introduction

Pharmaceutical contaminants, which enter environments via wastewater effluents, are a major environmental concern due to rising pharmaceutical consumption with a growing and ageing human population (Boxall, 2004; Arnold et al., 2014). In fact, >600 pharmaceutical substances have now been detected in the environment worldwide (aus der Beek et al., 2016). Despite increasing research interest in understanding how wildlife responds to pharmaceutical contaminants (reviewed in Brodin et al., 2014; Saaristo et al., 2018), the synergistic fitness effects of pharmaceuticals and their interaction with temperature remain unclear. In particular, in aquatic environments, changing temperature due to anthropogenic activity (e.g. seasonal extremes due to climate change), impacts ecosystems at multiple levels of biological organisation.

At the individual level, temperature affects physiology, from metabolic activity to protein damage and organ function (Hochachka and Somero, 2002). Moreover, increased water temperature is known to stimulate metabolism and, thus, uptake of chemical contaminants (Pörtner, 2002). Indeed, elevated temperature changes toxicokinetics by enhancing bioavailability and toxicity of chemical contaminants (Buchwalter et al., 2003; Lydy et al., 1999; Maruya et al., 2005). For example, increased water temperature (from 15 °C to 25 °C) magnified acute toxicity of the pain reliever acetaminophen 8.3-fold in a freshwater invertebrate (*Daphnia magna*, Kim et al., 2010). As changing water temperature affects thermoregulation, the associated metabolic costs are directly linked to resources available for growth, reproduction, and development (Kooijman, 2001; Angilletta, 2009). These energetic trade-offs become especially costly when individuals are chronically exposed to a combination of environmental stressors (Gaw et al., 2014). Not all individuals are equally affected, however, and species differ in their ability to adjust their physiology with increasing water temperature. For example, mid-intertidal crabs are more tolerant to rising temperatures than their subtidal congeners (Tomanek and Somero, 1999; Stillman, 2002). Susceptibility to thermal pollution also depends on ontogenic stage (Pörtner, 2002) and thermal tolerance of the organism (Patra et al., 2007; Noyes et al., 2009). Because synergistic effects between rising water temperatures and chemical contaminants are likely to exacerbate these changes (Noyes et al., 2009), it is not surprising that a growing number of studies suggest that, in aquatic habitats, alterations in chemistry may be more important than changes in water temperature alone for the performance and survival of wildlife (Harvey et al., 2006; Gaw et al., 2014).

Due to the conservative nature of physiological processes among vertebrates, pharmaceutical contaminants, which remain biochemically active in the environment, are a threat to non-target organisms. Among pharmaceuticals, psychoactive drugs—such as benzodiazepines—are the most commonly used globally, being prescribed to treat stress and anxiety-related disorders, as well as chronic insomnia (Rieman et al., 2015; Kurko et al., 2018). In the central nervous system, benzodiazepines bind to the gamma amino butyric acid $(GABA)_A$ receptor, which is highly conserved across animal taxa (Gunnarsson et al., 2008), and increase natural activity of the inhibitory neurotransmitter GABA (Pritchett et al., 1989). This, in turn, enhances inhibitory products to all of the major cell groups in the brainstem and hypothalamus that would otherwise stimulate arousal (Revel et al., 2009). One of the most widely used benzodiazepines is oxazepam. As a result of inadequate removal during wastewater treatment processes, oxazepam is commonly detected in surface (up to 61 ng/L) and effluent (up to 1.8 μg/L) waters around the world (Loos et al., 2013; aus der Beek et al., 2016; Fick et al., 2017). The estimated half-life of oxazepam in laboratory conditions is approximately 50–60 days (Loffler et al., 2005; Patterson et al., 2011), but it can withstand microbial degradation and exist in its bioactive form in lake sediment for decades (Klaminder et al., 2015). Furthermore, since psychoactive drugs, such as oxazepam, have been designed to treat behavioural disorders, it is not surprising that laboratory and field studies have found oxazepam to alter behaviour and survival of non-target animals at field-relevant concentrations (European perch, *Perca fluviatilis*: Brodin et al., 2013; Klaminder et al., 2014; Atlantic salmon, *Salmo salar*: Hellström et al., 2016).

The first response of an organism to environmental change is often to alter its behaviour (Nagelkerken and Munday, 2016). In this regard, behaviour is the result of numerous complex developmental and physiological processes (Wong and Candolin, 2015) and, thus, provides a comprehensive measure of exposure to multiple stressors. Importantly, behaviour has proven to be a sensitive early warning sign of contamination (e.g. Bell, 2001; Martinovic et al., 2007; Saaristo et al., 2009a, 2009b; Hallgren et al., 2011; Tomkins et al., 2016; Martin et al., 2017; Bertram et al., 2018a, 2018b) that can detect effects at much lower concentrations than traditional ecotoxicological endpoints (reviewed in Scott and Sloman, 2004; Söffker and Tyler, 2012; Melvin and Wilson, 2013; Arnold et al., 2014). One such behaviour is anxiety, which can be quantified using a well-established behavioural assay called scototaxis or 'white/dark assay' (Maximino et al., 2010). In short, this test utilises time spent in a white (i.e. 'exposed' environment) versus dark (i.e. protected black substrata environment) compartment to evaluate anxiety versus anti-anxiety behavioural attendance of the fish. This is particularly relevant when examining impacts of a drug prescribed to treat anxiety in human patients, such as oxazepam. In addition, the scototaxis assay tests for exploratory tendency and boldness in fish, because the white compartments represent an environment where the fish lacks camouflage and is 'exposed' to heightened predation risk (Maximino et al., 2010; Brodin et al., 2017).

In addition to predation, warmer temperatures have often been associated with increased activity and boldness in fish (e.g. Biro et al., 2010; Ojanguren and Braña, 2000; Forsatkar et al., 2016). However, this is not always the case and more recent studies have reported that elevated temperatures can actually decrease swimming speed, activity, boldness and foraging (Johansen and Jones, 2011; Nowicki et al., 2012; Colchen et al., 2017; Davies et al., 2017). Very few behavioural studies to date have investigated interactions between temperature and chemical contamination (see Lagesson et al., 2018), and even fewer in an ecologically realistic context, such as under predation pressure.

To date, no study has investigated interactions between temperature and oxazepam contamination. Therefore, we investigated responses of fish behaviour to multiple stressors, not only including oxazepam and temperature treatments, but also simulated predation pressure. More specifically, the objectives of this study were to 1) examine how exposure to a combination of temperature (10 °C or 18 °C) and oxazepam impacts the behaviour of European perch (*Perca fluviatilis*), 2) unravel if exposure to multiple stressors, such as temperature and predation threat, would increase or decrease the impact (if any) of oxazepam exposure, and 3) determine if exposure to multiple stressors impacts the level of bioaccumulation of oxazepam in fish muscle tissue. We hypothesised that, 1) oxazepam would reduce anxiety and make fish bolder, and 2) colder temperature would cause reduced activity due to decreased metabolism. With the multi-stressor (oxazepam, temperature, and predation) approach, we expected the behavioural outcome

to either strengthen the single stressor treatment due to additive and/or synergistic effects or to be less than the sum of the independent effects (i.e. antagonistic effect) (Folt et al., 1999; Alton and Franklin, 2017).

2. Methods

2.1. Study species

The European perch is a common freshwater fish native to Europe and Asia that has been introduced in Australia, New Zealand, and South Africa (Thorpe, 1977). It is an eurythermal species (i.e. tolerates wide range of temperatures) that inhabits clear rivers and lakes (Thorpe, 1977). Importantly for their use as a relevant species in behavioural ecotoxicology, the biology and behaviour of perch is well understood (Thorpe, 1977; Christensen and Persson, 1993) and previous studies have reported that perch are exposed to oxazepam in their natural habitats (Brodin et al., 2013).

2.2. Animal collection and housing

One-year-old perch ranging from 5.5 to 7 cm were collected in Bjännsjön, 16 km southeast of Umeå, Sweden, during the first week of June 2016, using a fine-meshed (5 mm mesh size) beach seine. The fish were transported in oxygenated containers, within 2 h of capture, to a holding tank (150 × 85 × 150 cm; length × width × height) at the Department of Ecology and Environmental Science, Umeå University. In the holding tank, the perch were held in oxygenated, aged tap water at ~13 °C, with a constant flow-through of water, and under a light:dark regime of 12:12 h. They were fed thawed chironomid larvae (~10% of perch body weight) daily during an acclimation period of 3 weeks prior to the behavioural trials.

2.3. Exposure design

Prior to the application of treatments, behavioural trials (see details in Section 2.4) were conducted to gain a baseline of behaviours across all individuals. To do that, fish were randomly taken from their holding tanks and individually placed into containers (28 × 19 × 14 cm) with a starting water temperature of 13 °C. Then, each container was placed in one of the temperature-controlled rooms letting the temperature of the water slowly increase or decrease to the corresponding treatment temperature (10 or 18 °C). Note that fish were not exposed to oxazepam or predation cues during these baseline trials. After 12 h in individual containers fish were introduced into the experimental arenas and allowed an acclimation period of 5 min before they were video-recorded from above using a single camera for 30 min (see details in Section 2.4).

Straight after the baseline trials, the same fish were randomly assigned, using a 'random number-draw-method' on excel, to one of eight treatments: freshwater control at 18 °C ($n = 30$), freshwater control at 10 °C ($n = 30$), freshwater control at 18 °C with predator cues ($n = 30$), freshwater control at 10 °C with predator cues ($n = 30$), oxazepam exposure at 18 °C ($n = 30$), oxazepam exposure at 10 °C ($n = 30$), oxazepam exposure at 18 °C with predator cues ($n = 30$), or oxazepam exposure at 10 °C with predator cues ($n = 30$). Temperatures were selected as they represent water temperatures of two different seasons (18 °C = summer, 10 °C = fall) in the area from which the fish were collected (Thorpe, 1977). Fish from each treatment were housed individually in identical exposure containers (28 × 19 × 14 cm) filled with 4 L of aged tap water, and with an air-stone for aeration. As with baseline behavioural trials, each container had a starting water temperature of 13 °C. Fish were acclimated to their specific temperature treatment by placing each container in one of the temperature-controlled rooms letting the temperature in the water slowly increase or decrease to the corresponding treatment temperature (10 or 18 °C). We staggered the exposure to enable us to collect behavioural data for 240 fish (each fish tested before and after exposure). Specifically, a set of 48 fish (1–48) were introduced into their individual exposure container on day one, the next set of 48 fish (49–96) on day two, and so on, until the total number of 240 fish had been introduced.

A stock solution with a nominal concentration of 10 μg/L oxazepam (Sigma-Aldrich, O5254, CAS 604-75-1) was prepared in dissolved water before being added to exposure containers at the start of the experiment. Exposure containers were spiked only once because oxazepam concentration in the water has previously been found to remain stable at least for 7 days (Brodin et al., 2014; Klaminder et al., 2014; Heynen et al., 2016a). We selected 10 μg/L oxazepam as an experimental exposure concentration since it represents a level high enough to generate behavioural effects in perch at 18 °C (Brodin et al., 2013; Klaminder et al., 2014), and our main objective was to see if behavioural effects are modified by temperature and/or predator regime. The predator cue treatment was achieved by adding 12 mL of water daily (3 mL added to each corner of the individual containers), which was taken from a 1 m^3 holding tank (150 × 85 × 150 cm) housing two 40 cm long pike (*Esox lucius*) that were fed perch every third day. Two temperature-controlled rooms were used, with 50% of the containers being stored at a low temperature, resulting in a 10 °C ± 0.32 °C (mean ± SD) water temperature, and 50% of the containers stored at a higher temperature, resulting in a water temperature of 18 °C ± 0.36 °C (mean ± SD). Each perch was fed 10 thawed chironomid larvae every second day during the exposure period. We monitored key water properties in exposure tanks throughout the exposure (ammonium [NH4], <0.004 mg/L; hardness, 2.8 ° dH; iron [Fe], <0.010 mg/L; nitrite [NO2], <0.003 mg/L; pH, 8.0).

2.4. Behavioural trials

After seven days of exposure, each perch was tested in a second set of behavioural trials, which followed the design of Brodin et al. (2017). Briefly, a scototaxis assay (light/dark preference; Maximino et al., 2010) was used to assess effects of oxazepam, temperature and predator cues on boldness. The behavioural trait 'boldness' is a well-established measure that defines how individuals respond to risky situations with direct fitness consequences (Frost et al., 2013). The scototaxis arena (80 × 50 × 42 cm) was divided equally into black (40 × 50 × 42 cm) and white (40 × 50 × 42 cm) halves. The depth of the water—which was clean, aged, and temperature treatment-matched—was 10 cm, yielding a volume of 40 L. Each test individual was introduced into the centre of the experimental arena and then given 30 min to swim freely, while its behaviour was video recorded (SONY Handycam HDR-PJ50VE) from above. Following behavioural trials, fish were euthanised with an overdose of MS-222 (Ethyl 3-aminobenzoate methanesulfonate) and stored at −20 °C for later tissue analysis of oxazepam.

The following behaviours were quantified: total time spent moving within, and frequency of visits to, the white compartment; total time spent moving within, and frequency of visits to, the black compartment; as well as the total time spent performing 'freezing' behaviour (i.e. staying motionless), total duration of the first freezing behaviour, and frequency of freezing events. The preceding behavioural variables were recorded with the intention of compiling them into an analysis that uses a multivariate synthetic axes approach. Additionally, we were interested in initial movement of the fish and measured the time fish took to cross onto the white and/or black background for the first time, and a binary score of whether the fish moved into a white or black region (i.e. recorded in a form suitable for a survival analysis). The behaviours were quantified blind to treatment from the 30 min video recordings using the event-recording software JWatcher V1.0.

2.5. Chemical analyses

Oxazepam concentrations in exposure water and perch muscle tissue (exposed and controls) were determined with a triple stage

Table 1
Standard deviations, proportion of variance and cumulative proportion of variance explained, and individual variable loadings for the behavioural PCA.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7
SD	1.91	1.11	0.95	0.78	0.58	0.43	0.32
Proportion of variance	0.52	0.18	0.13	0.09	0.05	0.03	0.01
Cumulative proportion of variance	0.52	0.7	0.82	0.91	0.96	0.99	1
Loadings							
Total time on white background (s)	−0.27	0.46	−0.7	0.1	−0.28	0.06	0.37
Total time on black background (s)	−0.41	0.12	0.59	0.15	−0.12	−0.32	0.58
Total time freezing (s)	0.44	−0.31	−0.17	−0.35	0.3	−0.02	0.69
Frequency of moving onto white	−0.41	0.23	−0.11	−0.53	0.56	−0.38	−0.17
Frequency of moving onto black	−0.44	−0.18	0.13	−0.46	−0.14	0.72	0.08
Frequency of freezing bouts	−0.28	−0.67	−0.26	−0.13	−0.43	−0.45	−0.08
Time elapsed until first freezing bout (s)	0.36	0.38	0.19	−0.58	−0.56	−0.19	−0.08

quadrupole MS/MS TSQ Quantum Ultra EMR (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Accela and a Surveyor LC pump (Thermo Fisher Scientific, San Jose, CA, USA) and a PAL HTC autosampler (CTC Analytics AG, Zwingen, Switzerland). For a detailed description of the pre-treatment and analysis see Brodin et al. (2013, 2014). For the muscle tissue samples, we measured bioconcentration factor (BCF) (Arnot and Gobas, 2006).

2.6. Statistical analysis

When examining the baseline behavioural assays (i.e. before exposure started) we used a non-parametric univariate approach. Specifically, univariate Kruskal-Wallis tests were performed for the six fundamental behaviours: 1) time spent on white background (s), 2) events moving onto the white background, 3) time spent on black background (s), 4) events moving onto the black background, 5) time spent freezing (s), and 6) number of freezing events. Because testing the baseline behaviours is intended to reveal potential confounding problems, Type II error should be controlled (i.e the β, not the α, is the prime concern) (Quinn and Keyough, 2002). We set $\beta = 0.9$ (i.e. $\alpha = 0.1$), which should be viewed as exploratory rather than a level suitable for hypothesis testing. Also, it is worth noting that the univariate approach inflates Type I error through multiple comparisons, but it would be misguided to view this as a concern. When checking for potential biases, the preference should always be to intentionally inflate Type I error in favor of restricting Type II error.

To examine effects of oxazepam, temperature (High/Low) and predator cue (Yes/No), we used a multivariate synthetic axis approach. This involved using a Principal Component Analysis (*prcomp* in R *stats* (R Core Team, 2018) to identify synthetic axes of positive and negatively correlated variation among measured behaviours (e.g. total time on white background (s), total time on black background (s), total time freezing (s), frequency of movement onto the white background, frequency of movement on the black background, frequency of freezing bouts, total time elapsed until the first freezing bout (s)). We used the standard method of accepting axes with Eigenvalues >1 as our threshold for variable reduction (Eigenvalues for axes PC1 = 3.6; PC2 = 1.2; PC3 = 0.9; PC4 = 0.6; PC5 = 0.3; PC6 = 0.2; PC7 = 0.1). The PCA axes were then used as synthetic behavioural responses in three-way ANOVAs with oxazepam, temperature, and predation, as two-level categorical predictors. As this is a hypothesis testing step, we set $\alpha = 0.05$, in line with standard methodology (Quinn and Keyough, 2002). However, the interpretation of a linear model can be misleading if the slopes of treatment groups are not equal (i.e. if significant interactions are present in the model). Therefore, we looked for significant interactions, although none were identified. Because the original hypothesis included the interaction of temperature × oxazepam, we retained this interaction term in our final models, despite it not showing any significance. Higher order interactions that did not specifically relate to a hypothesis, and were non-significant, were removed.

Finally, we were interested in initial movement of the fish, which was the time until a fish crossed onto the white and/or black background for the first time. These data were not suitable for inclusion in a PCA, but were suitable for survival analysis. We used a survival regression analysis (*survreg* in R library *survival*). We checked for the best

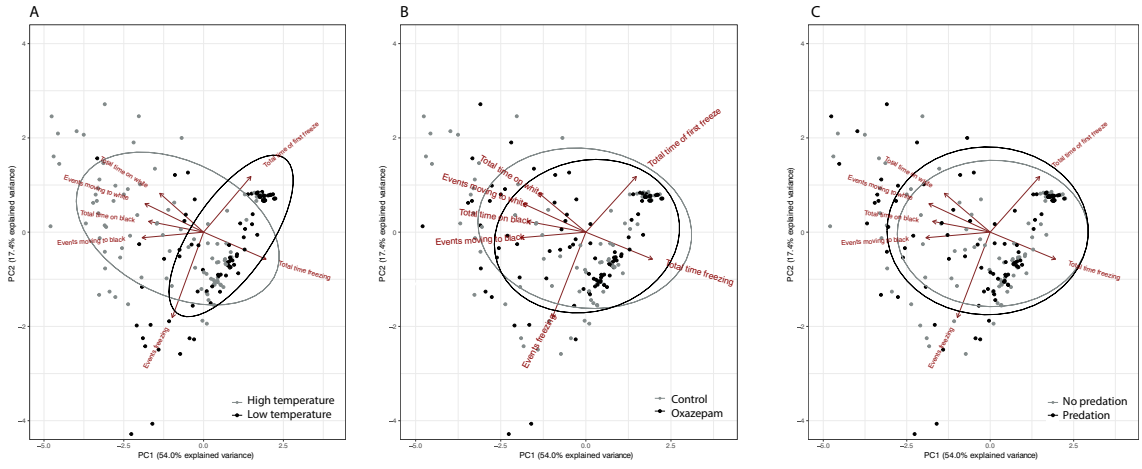


Fig. 1. Principal component analysis (PCA) of the selected behaviours across different treatment groups. PCA presents the level of similarity/dissimilarity between (A) high and low temperature, (B) control and oxazepam, and (C) no predation cue and predation cue. Total black = total time (sec) spent moving in the black background; Event black = number of times fish visited the black blackground; Total white = total time (sec) spent moving in the white blackground; Event white = number of times fish visited the white blackground; Total freezing = total time (sec) spent in staying motionless; Event freezing = total number of freezing events; Duration first freeze = length of the first freezing event (sec).

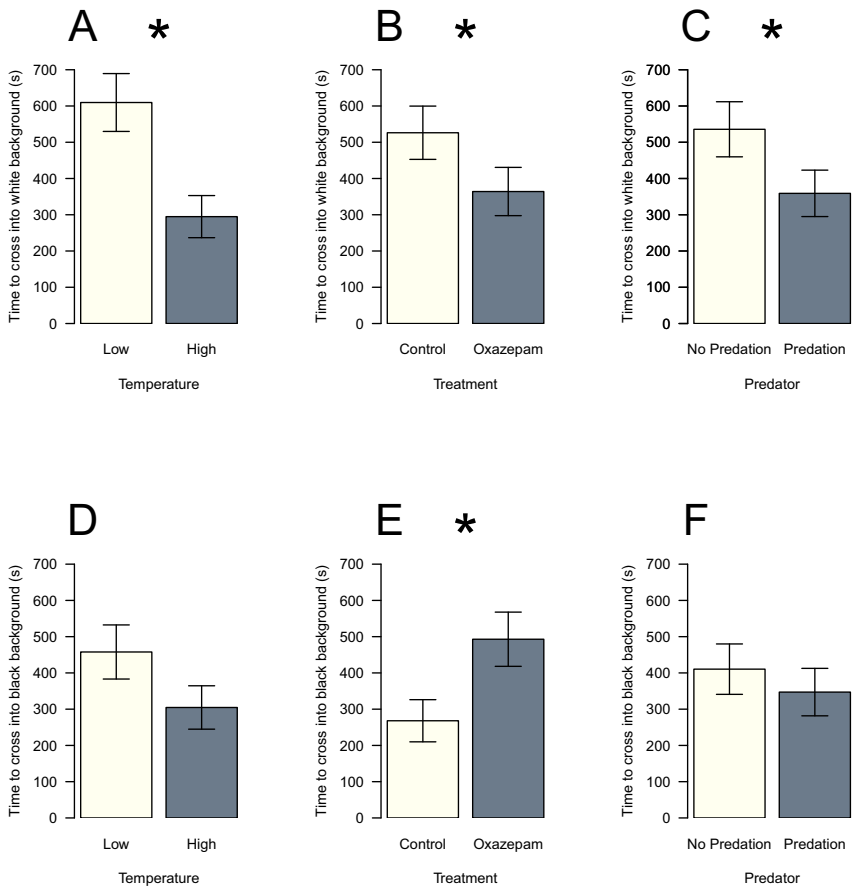


Fig. 2. The effect of temperature on the time (s) taken for fish to cross into (A-C) white background and (D-F) black background. Means and standard errors are shown. High = fish exposed to high temperature (18 °C); Low = fish exposed to low temperature (10 °C); Control = control treatment (freshwater); Oxazepam = oxazepam treatment (6.5 μg/L); No predation = no predation cue; Predation = predation cue. Significant differences ($p < 0.05$) are indicated by an asterisk (*).

distribution among Weibull, exponential, extreme, Gaussian and logistic options, but Weibull was always preferred (i.e. Weibull always had the lowest −2*LL; −2 multiplied by the log likelihood, as an indicator of model fit). As with the above ANOVAs, we checked for significant interaction terms, but no interaction terms were significant, and, therefore, we report main effects only.

To examine patterns of PCA response to the treatments, we used ANOVA. Specifically, we investigated how oxazepam, temperature and predator cue impacted fish behaviour (axis 1 and 2 of the behaviour PCA). All ANOVA assumptions were checked using standard diagnostic plots (i.e. residuals versus fitted, qq-plot) and no data transformations were needed. All significance levels (alpha) were 0.05 and all statistical analyses were conducted using R version 3.5.0 (R Core Team, 2018) and R Studio version 1.0.153 (R Studio Team, 2016).

3. Results

3.1. Baseline behavioural data

Before the exposures, there were no significant differences in any of the assayed baseline behaviours among the oxazepam and predation cue treatment groups (Kruskal-Wallis: $p > 0.130$ for all tests). There was, however, an effect of temperature on behaviour, which is not surprising given that the fish had been moved to the low and high temperature treatment tanks 12 h before the baseline assay and as such already had time to adjust their behaviour to the new environmental condition (Supplementary Figs. 1, 2, 3; Supplementary Table 1).

3.2. Oxazepam exposure

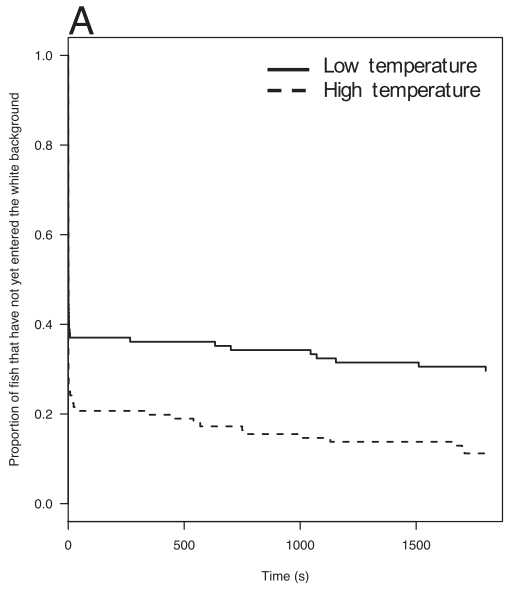
Measured oxazepam concentrations in our exposure treatments were as follows (mean ± SD): oxazepam high temperature = 6.1 ± 0.3 μg/L ($n = 14$), oxazepam high predation = 6.2 ± 0.5 μg/L ($n = 15$), oxazepam low temperature = 6.7 ± 0.7 μg/L ($n = 15$), and oxazepam low predation = 6.9 ± 0.8 μg/L ($n = 15$), respectively. No oxazepam was detected in the control tanks (<1 ng/L; $n = 40$). Further, oxazepam bioconcentrated in perch muscle tissue (mean ng/g ± SD ng/g), as follows: oxazepam high temperature = 49 ± 14.1 ($n = 31$), oxazepam high predation = 50 ± 11.5 ($n = 27$), oxazepam low temperature = 47 ± 14.8 ($n = 29$), and oxazepam low predation = 54 ± 23.0 ($n = 28$). All samples in the control group had concentrations below the method detection limits (<LOQ, $n = 40$) (Supplementary Table 2).

3.3. Behavioural effects: interactions and main effects

After 7-day exposure, we found a number of significant main effects for oxazepam and predation cue treatments. Because there were no statistical interactions among the predictors (oxazepam, temperature or predation cue treatments), interpretation of these main effects is most sensibly presented and interpreted independently for each predictor.

3.3.1. Multivariate synthetic axes

Using the standard threshold of keeping axes with Eigenvalues >1, we identified two potential axes of importance. Loadings that contributed >0.3 are reported for each principal component axis that we



examined (Table 1). The first axis (PCA1, variance explained = 54.0%) was contributed to negatively by total time spent on white (loading = −0.315) and black (loading = −0.395) backgrounds, and frequency of entering white (loading = −0.421) and black (loading = −0.442) backgrounds, implying a significant axis of variation associated with movement around the tanks as a whole. In comparison, total time spent freezing (loading = +0.449) and total time of first freezing event (loading = 0.345) associated positively with PCA1 (Fig. 1a, b, c). We found that there was a strong negative temperature effect for PCA axis 1 (i.e. PC1 values were lower for the high temperature treatment; ANOVA: $F_{1,223} = 49.9$, $p < 0.001$). This implies that the higher temperature treatment associated with less freezing and greater time spent moving around the tank (i.e. time spent on white and black backgrounds, and frequency of entering black and white backgrounds). The second axis (PC2, variance explained = 17.4%), on the other hand, was negatively associated with frequency of freezing events (loading = −0.734). Total time spent on white background (+0.335) and total time of first freezing event (loading = +0.470), associated positively with PC2. There were no significant treatment interactions or main effects on PC2 (all $F < 1.3$, all $p > 0.260$).

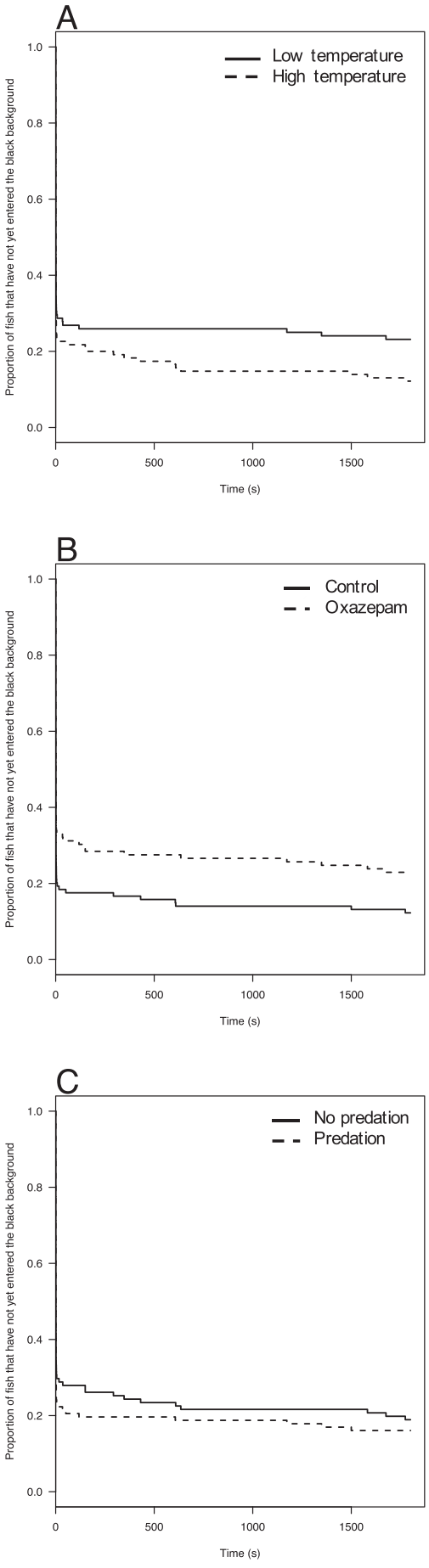
3.3.2. Time to enter white/black background

We found significant main effects in the initial movements of fish, but no significant interactions. Therefore, main effects of the temperature, oxazepam, and predation treatments are reported individually, for clarity. Regarding movements to the white background, there were significant temperature, oxazepam, and predation effects (Fig. 2a,b,c). Specifically, in the high temperature treatments, 79.4% of fish entered white background within the first 5 min while only 63.9% did so in the low temperature ($z = 4.15$, $p < 0.001$) (Fig. 3a). In the oxazepam treatment, 76.7% of the treated fish entered white background within 5 min compared to 66.6% control fish ($z = -2.10$, $p = 0.036$) (Fig. 3b). In the predation treatment, 76.2% of fish exposed to predation cues entered the white background within the first 5 min ($z = -2.77$, $p = 0.006$) compared to 67.1% of control fish (Fig. 3c). Regarding movements to the black background, there was only significant effect on oxazepam treatment ($z = 2.73$, $p = 0.006$) (Fig. 2e), with 70.8% oxazepam-exposed fish entering black background compared to 83.2% of control fish (Fig. 4a,b,c). Note that we are reporting the percentage of fish that moved onto a background in the first 5 min, rather than across the whole trial, because the drop in all survival curves is quite steep (Figs. 3a,b,c and 4a,b,c), implying that most of the difference in behaviour develops within the first 5 min of the start of a trial.

4. Discussion

Here, using a multi-stressor approach, we report that exposure to low levels of oxazepam affects the initial movements of perch. Specifically, although we found no significant interaction effects of oxazepam and temperature on the behaviours studied, we found that fish in the low temperature treatments (oxazepam, predation) froze for significantly longer than fish in the high temperature treatment. In addition, we found oxazepam-induced effects on boldness and a significant effect of predation cue on perch entering the white compartment. Finally, we provide evidence supporting earlier studies showing that oxazepam exposure can significantly bioconcentrate in perch muscle tissue (Brodin et al., 2013; Heynen et al., 2016b).

Fig. 3. Survival analysis presenting the time to first cross into the white background. (A) Temperature low = fish exposed to low temperature (10 °C); Temperature high = fish exposed to high temperature (18 °C); (B) Control = unexposed control fish; Oxazepam = fish exposed to oxazepam (6.5 μg/L); (C) No predation = no predation cue; Predation = predation cue.



4.1. Behavioural effects induced by oxazepam and temperature

In line with our first hypothesis, we found that the initial movements of fish were significantly affected by oxazepam treatment. Specifically, within the first 5 min, 60–93% of the oxazepam-treated fish entered the white background, compared to 59–79% control fish. The white background represents a riskier environment (Maximino et al., 2010) and thus demonstrates boldness, because fish are entering into an environment perceived as more dangerous (Brodin et al., 2017). Previous studies have shown that fish exposed to oxazepam become bolder (roach, *Rutilus rutilus*: Brodin et al., 2017), more active (European perch: Brodin et al., 2013; Klaminder et al., 2014; Chiffre et al., 2016) and migrate faster (Atlantic salmon, *Salmo salar*: Hellström et al., 2016). Our study provides further evidence that oxazepam increases boldness of fish, because a higher proportion of perch exposed to oxazepam entered the white 'more vulnerable' environment compared to controls, and took longer to enter the black 'protected' environment. This enhanced boldness, however, usually comes with a cost. Bolder individuals take more risks (Smith and Blumstein, 2008; Sih and Del Giudice, 2012) and are therefore more exposed to, and more likely to be targeted by, predators (Hulthén et al., 2017). Indeed, recent studies found that migrating salmon smolt exposed to oxazepam migrated faster (Hellström et al., 2016) but were also predated upon to a greater extent (Klaminder et al., in prep). Even though some studies have found that bolder individuals make decisions faster without compromising accuracy (Mamuneas et al., 2015), others have reported high activity to be tempered by poor accuracy (Raoult et al., 2017). Therefore, increased boldness caused by anxiolytic exposure might not lead to better decision-making, as was shown by Klaminder et al. (in prep), where bolder Atlantic salmon smolt seemed unable to respond plastically to the anxiolytic-induced increase in boldness and, hence, suffered increased predation.

Although it is clear that oxazepam is readily taken up by fish, as indicated by higher concentrations in the fish muscle than in the water, the results of the present study highlight that exposed fish are likely to be metabolically compromised, not only via a modified behaviour, but also via detoxification processes driving the depuration of the drug. This increased energy expenditure could, in turn, hamper the ability of individuals to respond adaptively to additional stressors (Vasseur et al., 2014; Barbosa et al., 2017). Future studies should explore this possibility further by examining if the metabolic rate of fish is also affected by multiple stressors.

At the lower temperature, fish spent more time in their first freezing event and, overall, froze for longer, regardless of the predation or oxazepam treatment—hence, supporting our second hypothesis. Staying motionless after being introduced to an unfamiliar environment is a well-established measure of anxiety in both rodents and fish (reviewed in Stewart et al., 2012). Activity, on the other hand, can be temperature-dependent (Fukuhara, 1990; Farrell, 1997; Pörtner, 2002; Biro et al., 2010) and colder temperatures are known to reduce metabolism, heart rate, respiration, and digestion (Farrell, 1997; Pörtner, 2002; Jensen et al., 2017). This could explain why fish in the cooler temperature reduced their activity and stayed motionless for longer. Indeed, a recent study reported that the winter-dormant fish cunner (*Tautogolabrus adspersus*) reduced activity under the cold treatment (0.6 °C), which translated into a reduced metabolic rate (Speers-Roesch et al., 2018). Thermal tolerance, however, is species-specific, and species living at the lowest and/or highest range of their temperature tolerance are more vulnerable and have limited adaptive capacity in a changing climate (Sandblom et al., 2016). In the current study, we

Fig. 4. Survival analysis showing the time taken to first cross into the black background. (A) Temperature low = fish exposed to low temperature (10 °C); Temperature high = fish exposed to high temperature (18 °C); (B) Control = unexposed control fish; Oxazepam = fish exposed to oxazepam (6.5 μg/L); (C) No predation = no predation cue; Predation = predation cue.

can rule out anxiety and/or stress caused by temperature because the chosen low temperature (+10 °C) treatment is not at the lowest thermal range for the European perch (~5–27 °C, Fiogbé and Kestemont, 2003; Jensen et al., 2017). Also, we used an extended acclimation period (3 weeks) to minimise stress caused by altered thermal environment. Overall, increased time staying motionless is likely to have fitness-related consequences because it translates to time not spent feeding and finding mates, thus underscoring the importance of determining the impacts of multiple stressors on behavioural endpoints.

We found that water temperature also affected the initial movements of fish when entering the white/black background, which gives further support to our second hypothesis. Specifically, in the high temperature treatment, 79% of fish entered the white background within the first 5 min, while only 64% did so in the low temperature. A recent study demonstrated that, in coral reef fish, individual boldness increases 2.5-fold as a function of temperature (Biro et al., 2010). The authors of that study observed dramatically increased levels of activity, boldness and even aggressiveness after only a few degrees increase in water temperature. Similarly, exposure to elevated temperature of 4 °C for ten days increased activity level of Siamese fighting fish (*Betta splendens*, Forsatkar et al., 2016). In the current study, low and high temperature treatments had a difference of 8 °C, which, according to Biro et al. (2010), would be very likely to demonstrate an increase in behaviours, such as boldness. Given that individuals will vary in their contextual plasticity—some will show the same levels of activity at all temperatures (Biro et al., 2010, 2013; Wong and Candolin, 2015; Killen et al., 2016)—and thermal tolerances vary from species to species (Pörtner, 2002), further studies are needed to confirm that increased temperature translates to boldness in European perch. However, it is important to point out that individual variation in responsiveness to temperature is likely to represent an adaptation to variable temperatures, and thus be subject to selection (Chevin et al., 2010; Reed et al., 2010; Biro et al., 2013). Future studies should, therefore, strive to incorporate variation in responsiveness to temperature when investigating impact of chemical contaminants.

4.2. Importance of predator cues

Our study revealed a significant effect of predation cue on perch entering the white compartment. Specifically, we found that up to 93% of the perch exposed to the predation cue treatment entered the white compartment within the first 5 min, compared to, an average of 67% of control fish, suggesting an effect on boldness. Indeed, the prolonged exposure to predators may have habituated or primed fish to cope better with risky situations (Brown and Braithwaite, 2004; Brown et al., 2007). As animals need to trade-off between predator avoidance, foraging, and reproduction to maximise their fitness (Sih, 1980; McNamara and Houston, 1987; Verdolin, 2006), by choosing a more vulnerable white background, perch chose the riskier environment. On one hand, this bolder behaviour could be explained by habituation (i.e., a loss of response in the absence of an actual predation threat; Ferrari et al., 2010; Imre et al., 2016). On the other hand, bolder behaviour could be due to variation in prey personality (Sih et al., 2004; Wolf and Weissing, 2012; Belgrad and Griffen, 2016). Indeed, a recent study showed that mortality of prey was dependent on its personality, with blue crab (*Callineuctes sapidus)* consuming primarily bold mud crabs (*Panopeus herbstii*), while toadfish (*Opsanus tau*) primarily predated upon shy mud crabs (Belgrad and Griffen, 2016). The predator used in the current study was the northern pike, which is an ambush predator (Savino and Stein, 1989). As bolder perch are more likely to be occupying front positions (Bumann and Krause, 1993; Ward et al., 2004) and keeping a greater distance to the school (Budaev, 1997; Wilson et al., 1993), it has been suggested that pike would be more selective, as well as, successful with bolder perch. A recent study, however, found that pike selected prey based on the prey's morphological traits (i.e., shallow bodied) rather than on their behavioural characteristics, such as boldness (Heynen et al., 2017). Interestingly, it has been surprisingly difficult to link the increased boldness induced by oxazepam to increased predation of perch by Northern pike (*Esox lucius*) in pond (Lagesson et al., 2018) and whole lake experiments (Klaminder et al., 2016). Lagesson et al. (2018) hypothesised that this mismatch between laboratory prediction and field verifications were most likely due to increasing abiotic factors (e.g. temperature). Our study is the first to support this idea as our assays suggest that temperature has a profound impact on behaviour, which is independent from exposure to oxazepam. Overall, while it is clear that oxazepam exposure is affecting prey behaviour, to determine whether or not oxazepam is also impacting their survival, further studies should explore the direct (e.g. predators less adept at capturing prey) and indirect effects (e.g. prey-switching due to changes in predatory behaviour) of chemical contaminants on predator-prey interactions (reviewed in Saaristo et al., 2018).

5. Conclusions

Here, we report that short-term exposure to oxazepam affects behaviour of juvenile perch. We found the initial movements of fish to be significantly affected by treatment, and fish became bolder (i.e. entered the white background) in the oxazepam, high temperature, and predation treatments. Importantly, we found no interaction effects of oxazepam and temperature on the studied behaviours, which suggests that temperature has a profound impact on behaviour that is independent from exposure to oxazepam. As wildlife are facing an increasing range of biotic and abiotic pressures, it is vital to investigate how organisms adapt and persist under chemically-induced environmental change. More generally, our study highlights the need to focus on multiple stressors to improve understanding of the risks and hazards posed by chemical contaminants.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.11.228.

Ethics

This research was approved by the Ethical Committee on Animal Experiments in Umeå (dnr: A18-15) and complied with Swedish law.

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