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Protecting the environment from psychoactive drugs: Problems for regulators illustrated by the possible effects of tramadol on fish behaviour

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HIGHLIGHTS

- Fish were exposed to the psychoactive drugs tramadol and fluoxetine.
- Alteration of fish behaviour was assessed in a novel tank diving test.
- The results were not easy to interpret with confidence.
- Ecotoxicological experts reached different conclusions based on the same results.
- Determining whether or not psychoactive drugs alter the behaviour of fish is difficult.

GRAPHICAL ABSTRACT

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ABSTRACT

There is concern that psychoactive drugs present in the aquatic environment could affect the behaviour of fish, and other organisms, adversely. There is considerable experimental support for this concern, although the literature is not consistent. To investigate why, fish were exposed to three concentrations of the synthetic opiate tramadol for 23–24 days, and their anxiolytic behaviour in a novel tank diving test was assessed both before and after exposure. The results were difficult to interpret. The positive control drug, the anti-depressant fluoxetine, produced the expected results: exposed fish explored the novel tank more, and swam more slowly while doing so. An initial statistical analysis of the results provided relatively weak support for the conclusion that both the low and high concentrations of tramadol affected fish behaviour, but no evidence that the intermediate concentration did. To gain further insight, UK and Japanese experts in ecotoxicology were asked for their independent opinions on the data for tramadol. These were highly valuable. For example, about half the experts replied that a low concentration of a chemical can cause effects that higher concentrations do not, although a similar number did not believe this was possible. Based both on the inconclusive effects of tramadol on the behaviour of the fish and the very varied opinions of experts on the correct interpretation of those inconclusive data, it is obvious that more research on the behavioural effects of tramadol, and probably all other psychoactive drugs, on aquatic organisms is required before any meaningful risk assessments can be conducted. The relevance of

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these findings may apply much more widely than just the environmental risk assessment of psychoactive drugs. They suggest that much more rigorous training of research scientists and regulators is probably required if consensus decisions are to be reached that adequately protect the environment from chemicals.

1. Introduction

Many human pharmaceuticals and their metabolites are present in the aquatic environment, usually at concentrations in the low or even sub-ng/L range (Petrović et al., 2014; Batt et al., 2016). The challenge now is to identify which, if any, of these pharmaceuticals pose a threat to aquatic organisms, and to what degree compared to the threat posed by the many other chemicals present in probably every river of most countries (Malaj et al., 2014; Johnson et al., 2017).

Amongst the drugs that have been detected in the aquatic environment are many psychoactive drugs, both legal (Schlüsener et al., 2015; Fick et al., 2017) and illegal (Kasprzyk-Hordern et al., 2008). This is not surprising, given that there are many (a few hundred) different psychoactive drugs on the market and that some of them are amongst the most widely prescribed of all drugs. The legal psychoactive drugs include those given to induce anaesthesia (e.g., ketamine), manage pain (e.g., opioids), and treat a wide variety of mental disorders (e.g., antidepressants). The range of psychoactive recreational drugs is equally large, including as it does stimulants (e.g., cocaine, methamphetamine) and hallucinogens (e.g., D-lysergic acid diethylamide). By far the most comprehensive assessment to date of the presence of psychoactive drugs in the aquatic environment has been provided by Kasprzyk-Hordern and her colleagues. They have shown that a large number of legal and illegal psychoactive drugs are present in a typical river of a developed country (Baker and Kasprzyk-Hordern, 2013; Petrie et al., 2016). Active metabolites of some of these parent drugs are also present (Petrie et al., 2016).

Most psychoactive drugs are hydrophobic because to have their desired effects they need to cross the blood-brain barrier to act in the brain. Unfortunately their hydrophobicity means that once in the aquatic environment they will be readily taken up by aquatic organisms. Wild fish from the United States (Brooks et al., 2005; Arnnok et al., 2017), Europe (Huerta et al., 2013; Álvarez-Muñoz et al., 2015) and Japan (Tanuoe et al., 2015) have been shown to contain various psychoactive drugs, with most research demonstrating the presence of the anti-depressants fluoxetine and sertraline and the anti-epileptic carbamazepine. The highest concentrations of psychoactive drugs in wild fish are often reported to be in the brain (e.g., Arnnok et al., 2017), which is the main site of action for most of these drugs.

Understandably, biologists have become interested in determining whether or not psychoactive drugs alter behaviours of aquatic organisms. The targets of psychoactive drugs are highly conserved throughout the animal kingdom (Gunnarsson et al., 2008), and hence based on the read-across hypothesis (Rand-Weaver et al., 2013), similar effects of psychoactive drugs might be expected to occur in both humans and aquatic organisms (especially vertebrates such as fish) if plasma concentrations in these organisms reach those that cause therapeutic effects in humans. Ecotoxicologists have studied the effects – if any, of course – of a few psychoactive drugs on a wide range of species including both aquatic vertebrates (i.e., fish) and invertebrates (i.e., molluscs and shrimps). The majority of this research has focused on possible effects of anti-depressants, as reviewed in Sumpter et al. (2014), although there is also a growing body of research on the possible effects of the anti-anxiety drug oxazepam on the behaviour of fish (e.g., Brodin et al., 2017; Lagesson et al., 2018). Because studying the behaviour of wild fish in their natural environment would be extraordinarily difficult, in most cases the research has involved exposing fish to the drugs in laboratories, then investigating the behaviour of those fish. A variety of different behaviours, including feeding rate (e.g., Stanley et al., 2007), predator avoidance (e.g., Painter et al., 2009), capture of prey (e.g., Bisesi Jr. et al., 2016), aggression (e.g., Kohlert et al., 2012), shelter-seeking (e.g., Valenti Jr. et al., 2012), time spent in the upper half of the aquarium (e.g., Margiotta-Casaluci et al., 2014) and boldness (e.g., Brodin et al., 2017) have been chosen as endpoints. In almost all cases it has been reported that the psychoactive drugs altered the behaviour of the fish in a manner that would likely have reduced their fitness. Yet there are two major problems with this research. One is that some papers contradict the results of other papers, making it impossible to know which, if either, provide reproducible results. To provide just one example, whereas Thompson et al. (2017) report that the anti-depressant venlafaxine had pronounced adverse effects on young fish, Parrott and Metcalfe (2017) observed no effects of that drug over a full fish life cycle. The other is that different authors report that very different concentrations, ranging from low ng/L to high μg/L, are required in order to affect behaviour (Sumpter et al., 2014).

The current uncertainty over whether or not psychoactive drugs affect the behaviour of fish, and if so, at what environmental concentrations, and exactly how behaviour is affected, makes it impossible to decide whether or not these drugs constitute a significant threat to aquatic wildlife. Regulators, whose job it is to protect the environment from the many chemicals entering it, cannot reach a consensus position that is defensible when confronted with such variability and uncertainty. To highlight these current problems, we conducted an experiment in which fish were exposed to a psychoactive drug, tramadol, and their behaviour assessed both before and after that exposure. In order to anchor any behavioural effects of tramadol against the established behavioural effects of another psychoactive drug, we chose to use fluoxetine as a positive control. Then the results of that experiment were shown to a large group of very experienced ecotoxicology researchers and regulators, who were asked to provide their interpretations of the results.

2. Materials and methods

2.1. Selection of test chemicals

As our objective was to obtain a set of unique behavioural data for experts to assess, rather than replicate an existing set, we wanted to use a psychoactive drug that had not yet been studied for its effects on aquatic organisms. We chose tramadol because it is in widespread use across the world and because it appears to be present in the aquatic environment at higher concentrations than any other psychoactive drug. Its concentration in rivers is usually reported to be in the hundreds of ng/L range, and can be in the low μg/L range (Kasprzyk-Hordern et al., 2008; Baker and Kasprzyk-Hordern, 2013; Petrie et al., 2016). The toxic effects of tramadol on the early-life stage of zebrafish and common carp were investigated by Sehonova et al. (2016). They tested different concentrations ranging from 10 to 200 μg/L of tramadol hydrochloride. They reported that the drug had effects on hatching, early ontogeny, morphology, and histopathology. However, these effects appear unconvincing, mainly because these were often not concentration-related.

Tramadol is a synthetic opiate used to treat moderate to severe pain. In people, it undergoes demethylation to the active metabolite *O*-desmethyl tramadol, and this produces analgesia through μ-opioid receptor-mediated inhibition of pain transmission in the spinal cord (Minami et al., 2007; Myers, 2005; Vazzana et al., 2015). However, tramadol has multiple mechanisms of action. It also elicits sedation by

inhibiting serotonin and noradrenaline reuptake transporters in the central nervous system (Faron-Górecka et al., 2004; Minami et al., 2007; Myers, 2005; Vazzana et al., 2015), as antidepressant SSRIs do. Common side effects in people include drowsiness, dizziness, anxiety, and impaired thinking and motor skills. In rodent models, tramadol reduced anxiety and hence the animals explored their open environment more in the elevated plus maze test, which is one of the most widely used tests for assessing anxiety-like behaviour (Caspani et al., 2014; Jayasree and Rajeswaramma, 2015). In a recent study (Buřič et al., 2018), an aquatic invertebrate, the marbled crayfish, exhibited significantly lower velocity and moved shorter distances than controls following exposure to tramadol at water concentrations of 0.81–0.98 μg/L. The biological receptors which tramadol acts on are evolutionarily conserved across vertebrate species (Albrizio et al., 2014; Gunnarsson et al., 2008; Sanchez-Simon and Rodriguez, 2008). Given the "read-across hypothesis" (Rand-Weaver et al., 2013), it is hypothesised that tramadol elicits sedation and anxiolytic responses in fish, as shown in the rodent models and humans, when the plasma tramadol concentration is within the human therapeutic concentration range. The anxiolytic response is ecologically relevant because it affects variables important for survival and breeding. Thus, in the present study, anxiolytic behaviour in fish was assessed using the novel tank diving test. The test is commonly used to measure anxiolytic responses of fish to an unfamiliar environment (Cachet et al., 2010; Maximino et al., 2012), which is conceptually similar to the elevated plus maze test with rodents. Tramadol hydrochloride was purchased from Sigma-Aldrich (Dorset, UK) with a purity higher than 99% (product number 42965-5G-F, lot number BCBN4547V).

In order to anchor any anxiolytic responses of tramadol against the established anxiolytic behavioural effects of another psychoactive drug, we used the antidepressant SSRI fluoxetine as a positive control. Well-designed studies have been performed which indicate that fluoxetine affects the anxiolytic behaviour of fish in a reproducible, concentration-dependent manner, in the novel tank diving test (e.g., Margiotta-Casaluci et al., 2014; Ansai et al., 2016). Fluoxetine hydrochloride was purchased from Sigma-Aldrich (Dorset, UK) with a purity higher than 99.9% (product number PHR1394, lot number LRAA1901).

2.2. Experimental design

The basic experimental design was one we have used before to investigate the anxiolytic behavioural effects of two other psychoactive drugs, fluoxetine (Margiotta-Casaluci et al., 2014) and oxazepam (Huerta et al., 2016). The 23–24 days chronic exposure experiment was carried out using a continuous flow-through system comprising twelve 20.5 L glass tanks (dimensions: 45 cm (length) × 24 cm (width) × 19 cm (depth)). Ten days before the beginning of the experiment, sexually mature male fathead minnows (*Pimephales promelas*) were transferred into the flow-through systems for acclimation to the test conditions. The experiment was run with a photoperiod of 16 h light: 8 h dark. During the experiment the water temperature, pH, and dissolved oxygen concentration were 25 ± 1 °C, 7.8 ± 0.19 and 6.0–8.0 mg/L, respectively. Thermostatically-heated dechlorinated tap water flowed into 12 glass mixing chambers at a rate of approximately 10 L/h, which supplied approximately 12 tank volumes per day to each tank holding fish. The same mixing chambers also received concentrated stock solutions of the test drugs delivered via peristaltic pumps at a flow rate of approximately 2 mL/h. These stock solutions containing test drugs were prepared every 4 days in amber bottles with *N,N*-Dimethylformamide (DMF): Milli-Q water (1:4, v/v) as a carrier solvent. The final DMF concentration in the water of the fish tanks was approximately 0.004%.

The experiment consisted of 6 different treatments: water control (WC), solvent control (SC), 1 μg tramadol/L (TG-1), 10 μg tramadol/L (TG-10), 100 μg tramadol/L (TG-100) and 100 μg fluoxetine/L (FG-100). There were two replicate tanks for each treatment. Eight male fathead minnows (*Pimephales promelas*), approximately 7 months old, 2.6 ± 0.53 g weight, and 5.4 ± 0.30 cm length, were randomly allocated to each fish tank, giving a total of 16 fish per treatment. The concentrations of tramadol in the fish tanks were chosen to cover both environmentally and pharmacologically-relevant concentrations, with the highest concentration (100 μg/L) anticipated to produce fish plasma levels of tramadol proximate to the human therapeutic plasma range of 100–300 ng/mL. A schematic of the exposure aspect of the experiment is provided in Fig. 1A. Full details of the analytical technique used to determine drug concentrations in plasma and brain of fish as well as test water are provided in our previous paper (Tanoue et al., 2017).

The behaviour of all fish was assessed using the novel tank diving test. The test is based on the instinctive behaviour of fish to seek protection in a novel environment by diving to the bottom of the tank and staying there until the environmental conditions are perceived as safe enough to initiate exploration. In order to obtain both pre-exposure baseline behavioural data and post-exposure data, all fish were tested twice; once immediately before exposure to a test chemical began (i.e., on day 0) and again at the end of the exposure period. After testing each fish individually at the beginning of the experiment, fish were returned to the tank from which they came. That tank contained the seven other fish maintained throughout in that tank. This meant that it was not possible to link pre-exposure data with post-exposure data for individual fish.

At the initiation of the novel tank diving test, each individual fish was transferred from its exposure tank to a 8.2 L observation tank (dimensions: 28 cm (length) × 13 cm (width) × 22.5 cm (depth)), and the exploratory behaviour of the fish recorded for 18 min using a Fujifilm digital camera (FinePix JV300; 14.0 Mpix) positioned at the front of the tank. Nobody was in the room where the novel tank diving tests were conducted while filming was occurring. The offline analysis of fish exploratory behaviour was carried out using movement tracking software as described by Margiotta-Casaluci et al. (2014). The observation tank was divided visually into 3 areas of equal size (bottom, middle, top), and the following endpoints quantified: number of entries into the middle and top areas; percentage of time spent in each area; swimming speed in each area. Fig. 1B illustrates our experimental approach to assessing fish behaviour (top), as well as typical behaviour tracks (bottom) of inactive and active control fish and positive control (fluoxetine-exposed) fish.

2.3. Interpretation of results by experts

Experts in ecotoxicology were asked to interpret the behavioural results. We sought the opinions of all participants of the 18th UK-Japan annual scientific workshop on research into environmental endocrine disrupting chemicals. This workshop was held in Weymouth, UK on 24th – 25th October 2016. It was attended by 41 scientists, many of whom could be considered experts in ecotoxicology. The UK provided 22 participants, Japan 17 and France 2. Approximately 40% of the participants were research scientists based in universities, 40% were scientists working in government-funded organisations, including regulatory bodies (e.g., Japan's Ministry of the Environment; UK's Environment Agency) and the remaining 20% came either from industry or non-governmental organisations. One of the authors of this paper (J.P.S.) gave a talk at the workshop entitled 'The possible effects of the analgesic tramadol on the behaviour of fish'. The talk consisted of one slide outlining the experiment conducted (this is reproduced here as Fig. 1) and two slides of the results (faithfully reproduced here as Figs. 2 and 3). During his talk the presenter did not provide any indication of his personal opinions on the interpretation of the results. At the end of the short talk the participants were each given a simple questionnaire to complete. It consisted of 5 straightforward questions (see Fig. 4 for those questions). The participants were instructed not to confer with each other, in order that independent opinions were obtained. To

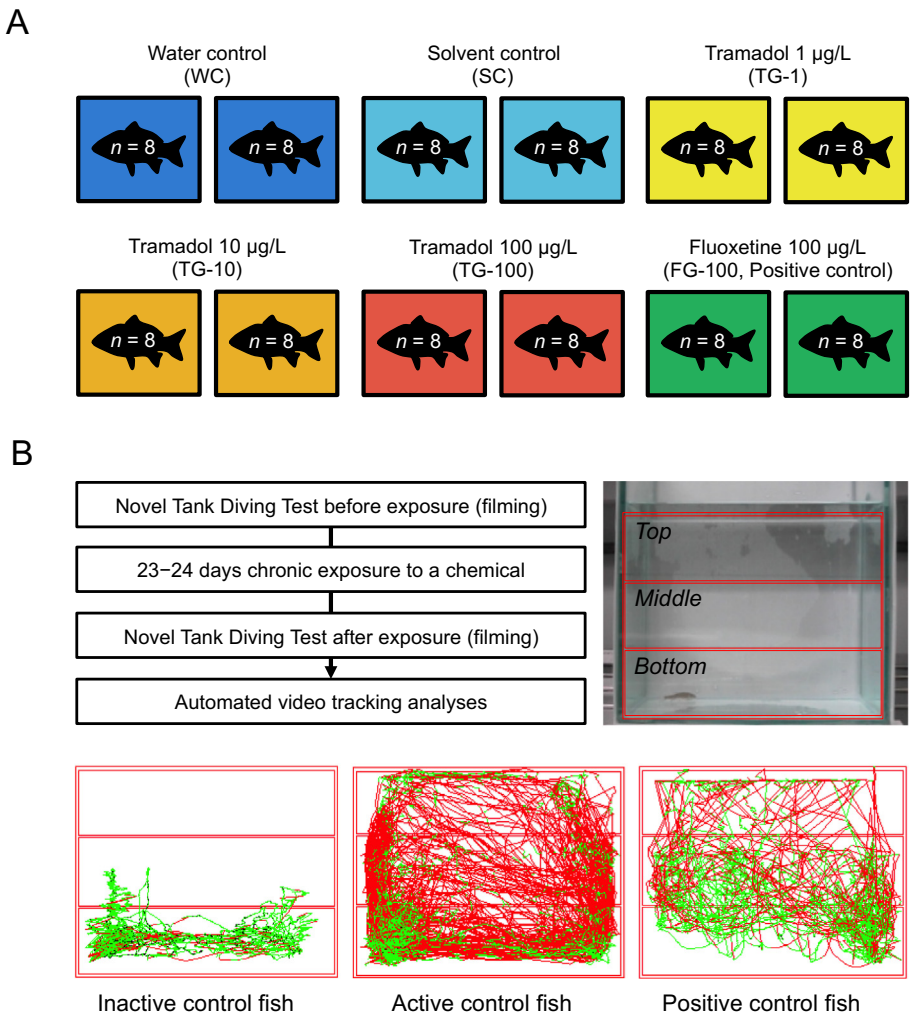

Fig. 1. Design of the experiment. Fish were exposed to various concentrations of tramadol (1, 10 and 100 μg/L) or a single concentration (100 μg/L) of the positive control fluoxetine (A). Their behaviour was assessed both before and after exposure to those drugs in the novel tank diving test (B), which investigates anti-anxiety behavioural endpoints. Observational tanks were divided into 3 areas (top, middle, bottom), fish behaviour videoed for 18 min, and VideoTrack software then used to quantify the exploratory behaviour of the fish. Behaviour varied substantially between individual fish, even within the same treatment group; typical behaviours of inactive and active control fish are represented at the bottom of B, as is the behaviour of a typical fish exposed to the positive control fluoxetine. Green indicates slow swimming and red fast swimming.

further aid in obtaining honest, independent opinions on the interpretation of the data, the participants were asked not to identify themselves on the forms. Hence, it is not possible to link any particular opinion to any individual or job (i.e. research scientist, regulator). The key slides displaying the data (Figs. 2 and 3 here) were on continuous display while participants were completing their questionnaires. The scientists had about 30 min to think about the results in front of them and complete the questionnaire. During that time two questions were asked of the presenter, both of which were about the number of fish in each treatment group. In response the presenter reiterated the number that he had stated earlier. Thirty-seven participants in the audience at the time, none of whom are authors of this paper, returned their questionnaires, having answered all 5 questions.

2.4. Statistical analyses

All statistical analyses were conducted using the open source statistical software R 3.5.1 GUI 1.70 for OS X 10.11 (El Capitan) and higher (http://www.r-project.org). As it was not possible to link pre-exposure data with post-exposure data for individual fish, non-parametric Wilcoxon rank sum tests were conducted to compare the behavioural data between pre-exposure and post-exposure periods. Treatment-related differences in all behavioural endpoints were analysed by the non-parametric Kruskal–Wallis test followed by Steel-Dwass post-hoc test (all-pairs multiple comparisons) and Steel post-hoc test (comparisons with SC). A p-value of <0.05 was considered statistically significant. A more in-depth analysis using logistic regression was also performed. Each behavioural parameter was dichotomized by its median into 2 even groups, and the probability of the behavioural occurrence was estimated in relation to the SC. Odds ratios were estimated by generalized linear model or Bayesian generalized linear model using the rstanarm package (Gabry, 2018). In addition, odds ratios were estimated by generalized linear mixed model or Bayesian generalized linear mixed model when including different tanks as factor variables with random effects (on the intercept) on binary data, by using glmmML (Broström, 2018) or rstanarm package, respectively, in statistical software R. In the Bayesian linear modelling, we ran four chains with each sample size of 2000. For each run, the first 1000 iterations were discarded for burn-in, and the next 1000 iterations were used for the estimation. The convergence was checked by ensuring R-hat was below 1.1 (Gelman et al., 2014). The reported empirical 95% credible intervals represent the 2.5th to 97.5th percentiles of the highest posterior density interval calculated from the posterior samples.

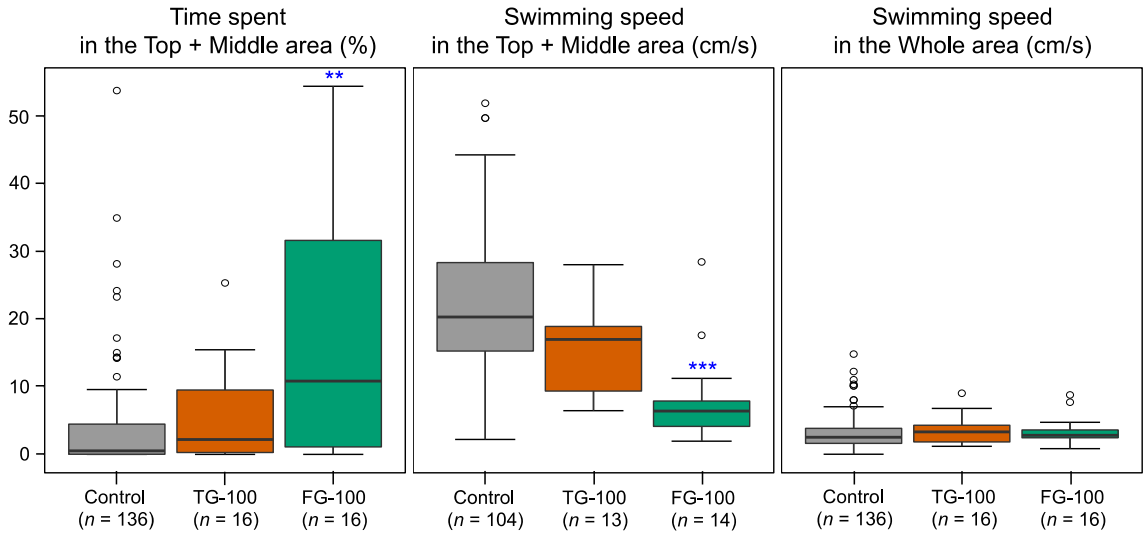

Fig. 2. The behaviour of male fathead minnows in a novel tank diving test following their exposure to either 100 μg/L of tramadol or the positive control drug fluoxetine for 23–24 days. Behaviour were recorded for 18 min after the fish were placed in the novel tank. After omitting the first 2 min and the last 1 min of data, the remaining 15 min of behavioural data was analysed. *n* denotes the number of fish assessed. The time that fish spent in the Top + Middle area (left), swimming speed in the Top + Middle area (middle), and swimming speed in the Whole area (right) are shown. The box-and-whisker plots show 25th percentile – 1.5 × IQR (lower whisker), 25th percentile (bottom edge of the box), 75th percentile (top edge of the box), and 75th percentile +1.5 × IQR (upper whisker). The horizontal line in each box is the median value. The small dots (open circles) are outliers. Asterisks denote statistically significant differences compared with the control (**$p < 0.01$; ***$p < 0.001$) based on the non-parametric Kruskal-Wallis test followed by Steel post-hoc test).

3. Results

3.1. Tramadol and fluoxetine concentrations in test water

Tramadol water concentrations (mean ± SD, $n = 18$) measured for TG-1, TG-10, and TG-100 treatments were 1.1 ± 0.053, 9.9 ± 0.65, and 98 ± 5.2 μg/L, respectively (Tanoue et al., 2017). Fluoxetine water concentration (mean ± SD, $n = 18$) measured for the FG-100 treatment was 94 ± 8.5 μg/L (Tanoue et al., 2017). Inter-tank variations were within ±20%.

3.2. Tramadol and fluoxetine concentrations in fish plasma

Tramadol fish plasma concentrations (mean ± SD, $n = 16$) measured after the exposure for TG-1, TG-10, and TG-100 treatments were 1.0 ± 0.32, 5.9 ± 2.9, and 46 ± 12 ng/mL, respectively (Tanoue et al., 2017). Plasma tramadol concentrations in fish following exposure to the highest water concentration of tramadol (TG-100) were slightly below the human therapeutic plasma concentration range (100–300 ng/mL). Fluoxetine fish plasma concentrations (mean ± SD, $n = 16$) measured after the exposure for FG-100 treatment were 6400 ± 1300 ng/mL (Tanoue et al., 2017), which is significantly higher than the human therapeutic plasma concentration range (120–500 ng/mL).

3.3. Behavioural assay

Behaviour was recorded for 18 min after the fish were placed in the novel tank. After omitting the first 2 min and the last 1 min of data, the remaining 15 min of behavioural data were analysed. The results of three behavioural endpoints, namely the time that fish spent in the Top + Middle area, swimming speed in the Top + Middle area, and swimming speed in the Whole area, are summarized in Fig. 2. All fish pre-exposure records as well as fish records from WC and SC groups at post-exposure were combined and shown as control (baseline), because there was no statistically significant difference between the three groups. Most of the control fish spent almost all of their time at the bottom of the tank and did not explore their new environment during the recording period (see 'Inactive control fish' in Fig. 1B). Whereas tramadol had no significant effect on where in the tank the fish spent their time, fish exposed to fluoxetine spent significantly more time ($p = 0.0030$) than either of the other two groups of fish in the middle and top areas of the tank; that is, they explored their novel tank more. However, there was a large amount of variation in the behaviour of fish in all groups (Fig. 2) – with some fish being relatively inactive whereas others explored their novel tank very actively.

Fish exposed to fluoxetine swam more slowly in the Top + Middle areas of their novel tank ($p = 0.000018$) than control fish (Fig. 2). Although the median swimming speed of tramadol-treated fish in the Top + Middle area of their tank was lower than that of control fish, the difference was not statistically significant ($p = 0.062$). When median values of swimming speeds in the entire tank (whole areas) were compared between treatments, there was no difference between the three groups of fish (Fig. 2). These results indicate that only when fish were exploring their novel tank for the relatively small percentage of their time that they did so, they swam at different speeds depending on the treatment.

Fig. 3 provides a more comprehensive picture of the behavioural results. Behaviour both before and after exposure to the drugs is compared, data for all three concentrations of tramadol are shown, and the data for the replicate tanks within each treatment are shown, allowing visualisation of tank to tank variation. One of the most striking results was the very considerable variation that occurred in the behaviour of fish within a group of 8 fish. This variation is most obvious when the time spent in the Top + Middle area of the tank is considered; for example, it was extremely variable in tank 2 of WC. Not only did time spent in the Top + Middle section of the tank often vary considerably within a group of 8 fish, it also often varied appreciably between the replicate tanks within a treatment; for example, compare the results of tank 5 with those of tank 6 in the TG-1 group (Fig. 3A). However, despite this high degree of variability in the time spent in the Top + Middle area of the novel tank, there was an obvious effect of fluoxetine. This drug increased the time that fish spent in the upper two thirds of the tank, when pre- and post-exposure behaviours were compared, although in only one tank was this statistically significant (Fig. 3A). In contrast there was no evidence that tramadol affected the time fish spent in the upper two thirds of the tank.

Swimming speed in the Top + Middle area of the novel tank was very similar pre- and post-exposure in both control groups (WC and SC) (Fig. 3B). In contrast, fluoxetine (FG-100) significantly reduced swimming

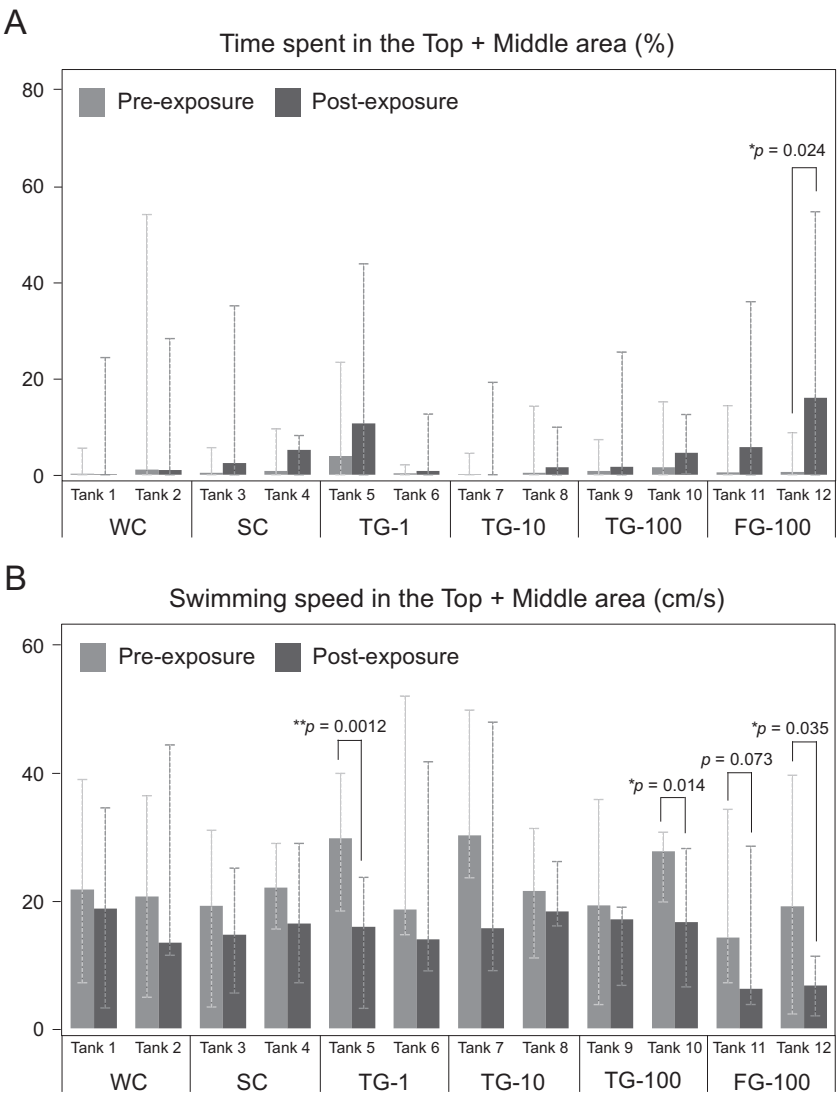

Fig. 3. A comparison of the behaviour of male fathead minnows in a novel tank diving test both before and after exposure to various concentrations of tramadol (1, 10, and 100 μg/L) and the positive control fluoxetine (100 μg/L). Data for replicate tanks for each treatment are shown. The time that fish spent in the Top + Middle areas (A) and swimming speed in the Top + Middle areas (B) are shown. For each replicate $n = 8$ fish. Bars represent median values, with whiskers representing the maximum values. Asterisks denote statistically significant differences between pre- and post-exposure for each tank (*$p < 0.05$; **$p < 0.01$; non-parametric Wilcoxon rank sum tests).

speed in the upper two thirds of the tank (Fig. 3B). The results for tramadol were less clear. In one of the replicate tanks of the highest concentration (TG-100), swimming speed was reduced significantly, but it was not in the other replicate. There was no effect of the intermediate concentration (TG-10), but in one of the replicates of the lowest concentration (TG-1), the swimming speed was significantly lower post-exposure, whereas it was unchanged in the other replicate (Fig. 3B).

3.4. Interpretation of results by experts

The results of the questionnaire are provided in Fig. 4. What is most obvious about the answers provided by the 'experts' is that there was relatively little agreement between them, whatever the question asked. A few participants replied 'maybe' to a question even though there was no box on the questionnaire for this equivocal answer (the questionnaire was designed to force the participants to answer 'yes' or 'no'). By answering 'maybe' the participants demonstrated how difficult they found it to reach a firm conclusion. A quarter of the participants thought that the highest concentration of tramadol had an effect (Fig. 4, Question 1), despite only one of the replicates showing a statistically significant effect, and for one endpoint only, namely swimming speed (Fig. 3). Five out of the 37 respondents (14%) considered that the lowest concentration of tramadol also had an effect. Interestingly, one of these 5 'experts' did not think that the highest concentration did, although the other 4 did. The most disparate opinion occurred when the participants were asked if they thought that low concentrations of chemicals can cause effects that are not observed when higher concentrations are tested. The participants were split nearly 50:50 in response to this question (Fig. 4, Question 3). They were split exactly equally when asked if they thought that a higher concentration of tramadol (1000 μg/L) would have produced significant effects on the behaviour of the fish, although 40% of the respondents answered by saying 'maybe', meaning that they could not decide one way or the other. The last question of the questionnaire asked participants for any of their thoughts on how to interpret the data. Many of their thoughts have been combined in Table 1. Their major thoughts were:

"More information on what normal behaviour is would be helpful."

"The high degree of variability compromises interpretation of the data."

"The experiment needed to be larger and a more comprehensive analysis of behaviour conducted."

"Were appropriate statistics conducted?"

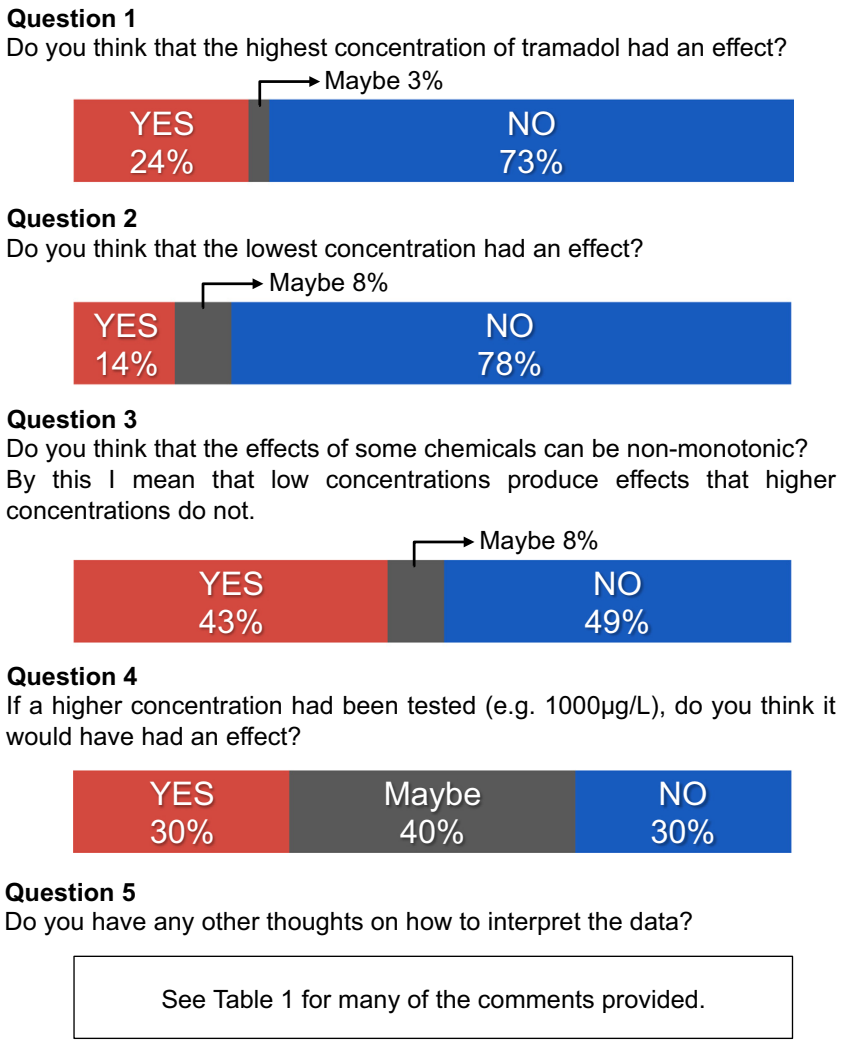

Fig. 4. A summary of the independent opinions of 37 UK and Japanese ecotoxicologists based on the data shown in Figs. 2 and 3. Although the questionnaire provided only two options, either 'yes' or 'no' to each question, some respondents nonetheless replied 'maybe'.

In response to some of these thoughts, after the UK-Japan workshop a more comprehensive statistical analysis of the behavioural data was undertaken, mainly to determine if that would provide more convincing answers as to whether or not tramadol affected the behaviour of the fish. In addition to examining the data on swimming speed and the proportion of their time the fish spent in the top, middle, and bottom areas of the novel tank, the number of entries fish made into the top and middle areas was also included in the analysis. Further, based on the suggestion of Melvin et al. (2017) that different lengths of observation time can impact on basic swimming parameters, we separated the behavioural data into two periods (2–10 min and 10–18 min) and analysed each time period separately. There is logic in doing so because fish might feel anxious when first placed in a novel tank, but after a while become less anxious and explore their surroundings more. The results of this more comprehensive analysis are presented in Fig. 5 and Table S1. Non-parametric Kruskal—Wallis test followed by Steel-Dwass post-hoc test (all-pairs multiple comparisons) were conducted on the behavioural data, as shown in Fig. 5, while non-parametric Kruskal—Wallis test followed by Steel post-hoc test (comparisons with SC) were also conducted, as shown in Table S1. The clearest result is the effect of fluoxetine on the behaviour of the fish: fish treated with this drug spent more time in the upper two thirds of the novel tank and swam more slowly in both the first (2–10 min) and second (10–18 min) periods, as well as the entire observational period (2–18 min), compared with pre-exposure data (Fig. 5). Nevertheless, when comparing with the SC group, these differences were not statistically significant ($p = 0.59$ for time spent in the upper two thirds of the tank; $p = 0.056$ for swimming speed in the upper two thirds of the tank, Steel-Dwass post-hoc test) (Fig. 5). Tramadol had no statistically significant effect on any behavioural endpoints in both the first (2–10 min) and second (10–18 min) periods. However, when data for the entire observational period (2–18 min) were analysed, a statistically significant ($p = 0.037$, Steel-Dwass post-hoc test) lower swimming speed in the Top + Middle area of the novel tank compared to the pre-exposure data was detected for the highest concentration (TG-100), whereas there was no statistically significant difference between the SC and TG-100 groups ($p = 1.0$, Steel-Dwass post-hoc test) (Fig. 5). When using the Steel post-hoc test (comparisons with SC), statistically significant lower swimming speeds of fluoxetine-treated fish than SC fish in the Top + Middle area of the novel tank was shown for all observation periods (Table S1). On the other hand, there was no statistically significant difference between SC and tramadol-treated fish for any behavioural endpoints (Table S1).

We further conducted logistic regression analyses for three behavioural endpoints (number of entries to, percentage of time spent in, and average swimming speed in, the Top + Middle area of the novel tank) recorded during the full observation period (2–18 min). These behavioural parameters were dichotomized by their medians into 2

Table 1
Representative comments by UK and Japanese experts on the difficulties associated with interpreting the behavioural data collected during an experiment investigating the possible effect of tramadol on fathead minnows.

A: Understanding normal behaviour
"One should pay attention to what is normal"
"Do we have background data on how fathead minnows would normally/typically behave?"
"More baseline data are needed"
B: The degree of variability in behaviour
"I'm not sure any effect of tramadol can be discerned, given the variation of controls (i.e. the difference between less-active and more-active control fish)"
"Were there enough replicates to understand the variability of behaviours?"
"Lack of consistency between tanks at same concentration – can't rule out tank effect"
"Control variability very high – not enough fish"
C: Issues with experimental design
"n is too small"
"The fluoxetine positive control showed similar responses in the replicate tanks. This did not occur with the replicate tramadol tanks. I wonder if there are other end-points one could examine, as this was only one? "
"Duplicate tanks is nowhere near enough for a study of this nature"
D: Issues with analysis of the results
"Are the statistical probabilities corrected for multiple testing?"
"If 20 statistical tests, one will be significant!"
""I don't think this study can conclude if tramadol had effect or not. Individual effects are probably small, so detecting effect is very difficult (underpowered)"
"Technically $n = 2$ with only two tanks, leading to pseudoreplication by treating fish separately (include tank as random factor in statistical modelling)"
"Need to be sure statistical model is the correct one. Very different results seen using very different statistics"

even groups, and the probability of the behavioural occurrence was estimated in relation to the SC group. The probabilities, which are expressed as odds ratios, were estimated by generalized linear (mixed) model or Bayesian generalized linear (mixed) model on the binary data. The results of these analyses are shown in Tables S2 and S3. The logistic regression analyses revealed the association between swimming speeds and treatments. Amongst all fish, those exposed to fluoxetine were 8.3–9.6 times (depending on the generalized linear model used) as likely to swim slowly in the upper two thirds of the novel tank as the SC fish. For the other two behavioural parameters - number of entries to the Top + Middle area and percentage of time spent in the Top + Middle area of the novel tank - there was no association with treatments.

The results obtained from these different statistical approaches to examining any behavioural alteration are summarized in Fig. 6. All statistical approaches detected the clear effect of fluoxetine on the swimming speed (decreased swimming speed) in the upper two thirds of the novel tank. Only a comparison between pre-exposure and post-exposure data suggested that the lowest concentration of tramadol (TG-1) and the highest concentration of tramadol (TG-100) had a statistically significant effect on the swimming speed in the upper two thirds of the novel tank. A statistically significant increase in the amount of time fish spent in the upper two thirds of the novel tank was shown for fluoxetine treatment, although only when comparing between pre-exposure and post-exposure behavioural data.

4. Discussion

We conducted what we would consider a reasonably well-designed study: in the experiment there was a relatively high number of fish in each treatment ($n = 16$), two control treatments (water and solvent), replicate tanks for each treatment, three concentrations of the psychoactive drug tramadol, and a positive control group. We also assessed behaviour both before and after exposure to the drugs, using the widely utilized novel tank diving test. In addition, the plasma and brain concentrations of the drugs in the fish were measured, thus demonstrating uptake of the drugs and their presence in the target organ, the brain (see Tanoue et al., 2017). The highest water concentration of tramadol (TG-100) produced fish plasma levels of tramadol (46 ± 12 ng/mL) proximate to the human therapeutic plasma range of 100–300 ng/mL. Yet despite this, an equivocal set of behavioural data was obtained, leading to 'experts' having great difficulty in interpreting it. The inclusion of the positive control drug, fluoxetine, confirmed that the experimental design was robust. Fluoxetine increased the amount of time fish spent exploring the novel tank and reduced their swimming speed in the upper two thirds of the tank, as has been demonstrated before both by us (Margiotta-Casaluci et al., 2014) and others (Cachet et al., 2010; Ansai et al., 2016). Thus reproducible behavioural results can be obtained. In contrast, the results with tramadol were unclear. There was limited statistical support for both the highest and lowest concentrations having modest effects on fish swimming speed in the upper two thirds of the tank, but no evidence that the intermediate concentration had any effect. A number of experts at the UK-Japan workshop suggested in their comments (Table 1) that a more rigorous statistical analysis of the results was required, and that different statistical approaches might lead to different conclusions being reached. We found a discrepancy between statistical results when the highest concentration of tramadol (TG-100) was compared with pre-exposure data or the SC group. A statistically significant decrease in swimming speed in the upper two thirds of the tank was found when the post-exposure data of the TG-100 group was compared to the pre-exposure data of the same group, whereas a statistically non-significant decrease in this parameter was found when the comparison was to the SC fish (Fig. 5 and Table S1). This discrepancy might be due to the slight difference between pre- and post-exposure values of the behavioural parameters in the two control groups (WC and SC). Although there was no statistically significant difference between pre-exposure values and post-exposure control (WC and SC) values, median values of swimming speed in the upper two thirds of the tank in post-exposure WC and SC groups were lower than that in pre-exposure group (Fig. 5). Nevertheless, when comparing between pre-exposure and post-exposure data, the lowest concentration of tramadol (TG-1) and the highest concentration of tramadol (TG-100) showed statistically significant differences in the swimming speed in the upper two thirds of the tank, while control (WC and SC) fish did not show statistically significant differences in the behavioural parameter between pre-exposure and post-exposure (Fig. 5). The number of fish per treatment and the number of tanks per treatment might not be enough to obtain statistically robust and reliable results, despite the experiment being a relatively large one involving nearly 100 fish.

The expert participants of the UK-Japan workshop obviously reached different conclusions based on the same data. They did not agree whether or not tramadol had affected the behaviour of the fish, and were evenly split when asked if they thought a higher concentration of tramadol than any tested would have had an effect. Perhaps most surprisingly, almost half of the respondents believe that low concentrations of a chemical can produce effects that higher concentrations do not, although a similar number did not believe this was possible. We accept that we did not use a very sophisticated approach to obtaining the opinions of other scientists on the data they were shown. This was not our intention. We wanted to obtain the immediate opinions of a group of 'experts', and hence took the opportunity of the meeting of Japanese and UK scientists held in Weymouth, UK, to obtain those opinions. We are not aware of any other examples in the literature of similar surveys. Thus, although our survey was relatively small (37 respondents), and definitely should be considered preliminary, it appears to be the only one conducted to date. The closest the existing literature comes to attempting to do what we did is probably the paper by Kase et al. (2016), who asked a group of chemical risk assessors to judge the reliability and relevance of a small number of published ecotoxicity studies. Their risk assessors also provided very varied opinions, despite them being constrained (unlike those we surveyed) by having to make

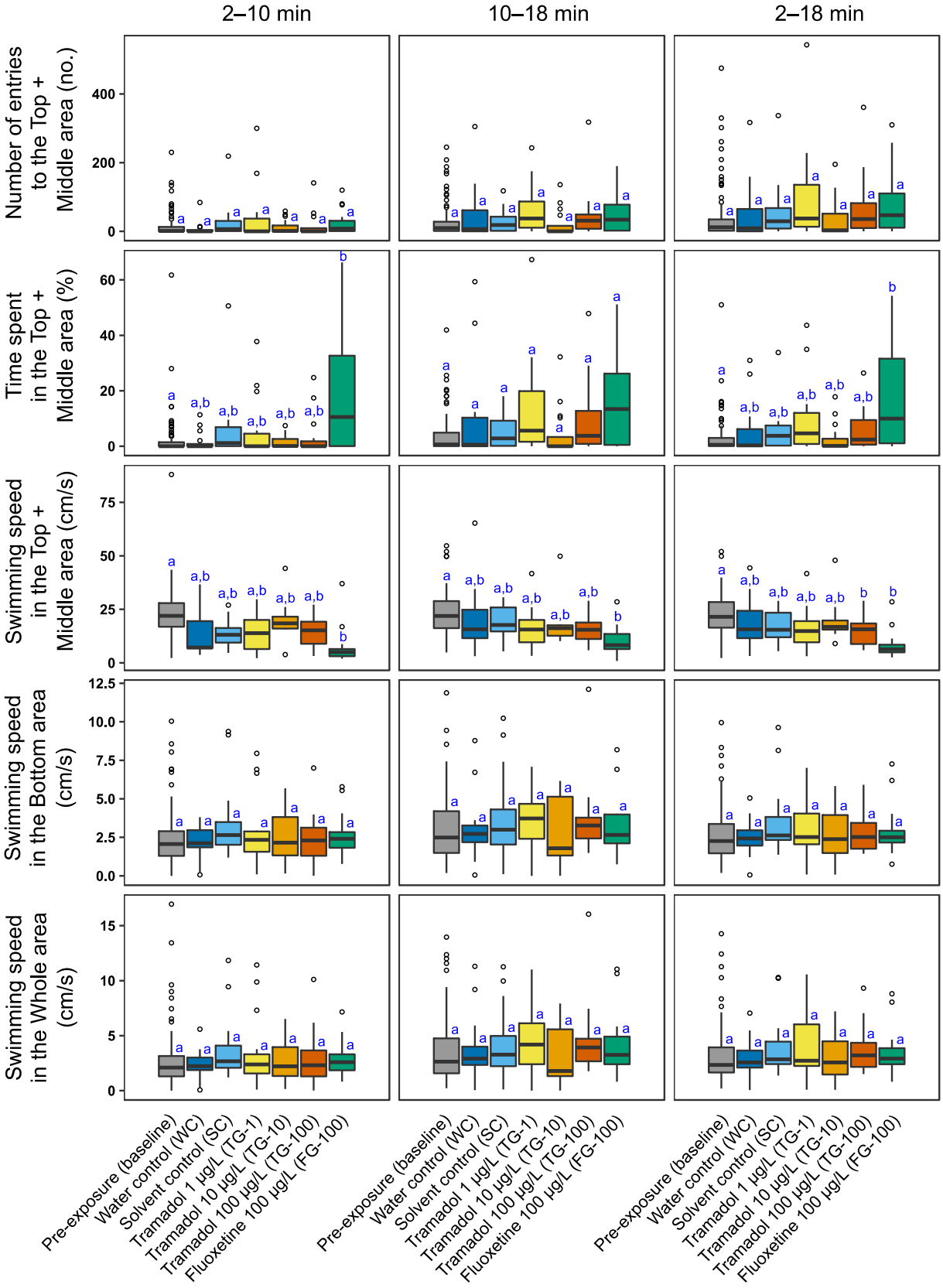

Fig. 5. The behaviour of male fathead minnows in a novel tank diving test following their exposure to either various concentrations of tramadol or the positive control drug fluoxetine. Behaviour was recorded for 18 min after the fish were placed in the novel tank. After omitting the first 2 min of data, when the fish were adjusting to their new surroundings, the remaining 16 min of behavioural data were divided equally into 2–10 and 10–18 min periods to allow comparison between these two periods. The pre-exposure group (baseline) consists of data from all fish records ($n = 96$) at pre-exposure period, whereas all subsequent treatment groups consist of data from 16 fish for each treatment. The box-and-whisker plots show 25th percentile − 1.5 × IQR (lower whisker), 25th percentile (bottom edge of the box), 75th percentile (top edge of the box), and 75th percentile +1.5 × IQR (upper whisker). The horizontal line in each box is the median value. The small dots (open circles) are outliers. Statistically significant differences ($p < 0.05$, non-parametric Kruskal-Wallis test followed by Steel-Dwass post-hoc test) between treatments are marked by different alphabetical letters.

Behavioural endpoint in the Top + Middle area (2–18 min after fish diving into a novel tank)	Experimental group	Results of statistical analyses			
		Non-parametric Kruskal–Wallis test followed by Steel post-hoc test (comparing with SC)	Logistic regression (comparing with SC)		Wilcoxon rank sum tests (comparing between pre-exposure and post-exposure)
			Generalized linear mixed model	Bayesian generalized linear mixed model	
Number of entries	Tramadol 1 μg/L				
	Tramadol 10 μg/L				
	Tramadol 100 μg/L				
	Fluoxetine 100 μg/L				
Time spent	Tramadol 1 μg/L				
	Tramadol 10 μg/L				
	Tramadol 100 μg/L				
	Fluoxetine 100 μg/L				Increase (only 1 out of 2 tanks)
Swimming speed	Tramadol 1 μg/L				Decrease (only 1 out of 2 tanks)
	Tramadol 10 μg/L				
	Tramadol 100 μg/L				Decrease (only 1 out of 2 tanks)
	Fluoxetine 100 μg/L	Decrease	Decrease	Decrease	Decrease (only 1 out of 2 tanks)

Fig. 6. A summary of the results obtained from using different statistical approaches to analyse the behavioural data. Grey, red, and blue represent no significant effect, a significant increase, and a significant decrease, respectively.

their judgements against a set of established criteria. Conferring was not allowed – and did not take place – in order that we obtained independent opinions. We are not suggesting that experienced regulators, if presented with all the literature on the effects of psychoactive drugs on fish, and given as much time as they wanted, would reach the same conclusions that the group of scientists we asked reached. But again this was not the point of our approach. Irrespective of any perceived weaknesses of our approach, the results very clearly demonstrate that experienced scientists can reach very different conclusions when presented with a set of equivocal data, probably as a consequence of them possessing different knowledge, having had different experiences, and possibly even possessing different prejudices. In other words, their opinions on the interpretation of the data were influenced by confirmation bias. Confirmation bias is a tendency to interpret and/or recall information in a way that confirms one's pre-existing beliefs. Thus, for example, if a scientist already believes that low concentrations of a chemical can cause effects not produced by higher concentrations of that same chemical, then when shown the data presented here in Fig. 3, confirmation bias would lead them to conclude that the data are an example of a 'low dose' effect, leading to a non-monotonic dose-response relationship. Those scientists who do not believe in 'low dose' effects will conclude from the same data that the apparent effect of the lowest concentration tested is probably an artefact due to chance, and hence likely would not be reproducible. Both groups of scientists would be succumbing to confirmation bias if they let their pre-existing opinions unduly influence their interpretation of the data shown to them in this study. Formal training of risk assessors and research scientists, which is largely lacking presently, would probably improve their judgement of experimental results.

One feature of the results that stands out is the very variable nature of the response of a fish to a novel environment. This high degree of individual variability in behaviour, of both control and drug-treated fish, has been noted by others (e.g., Margiotta-Casaluci et al., 2014; Huerta et al., 2016). One way to deal with this high degree of variability in laboratory toxicity tests would be to substantially increase the number of fish (or other animals) used in an experiment, thereby increasing statistical power. This was suggested by some of the experts, who made comments such as "*n* is too small" and "duplicate tanks is nowhere near enough for a study of this nature". However, our experiment involved the use of 96 fish (16 fish per treatment), which is more than many authors have used in their studies investigating the possible effects of various psychoactive drugs on the behaviour of fish (e.g., Bisesi Jr. et al., 2016 used 5 fish per treatment; Kohlert et al., 2012 used 48 fish in total; Valenti Jr. et al., 2012 used 60 fish in total; Brodin et al., 2017 used 37 fish in total). Using a higher number of fish would also create ethical problems, as society tries to cease using live animals in research. A better strategy would be to improve the understanding of fish behaviour. Why, for example, when placed into a novel tank, do some fish dive to the bottom and 'freeze' there, whereas others explore their new tank? How repeatable are these behaviours? As a first step it would be very useful to analyse the behaviour of control fish, to determine what is normal and the degree of variation. Thus, we are strongly in support of the comments made by a number of our experts, such as "one should pay attention to what is normal" and "do we have background data on how fathead minnows would normally/typically behave?" The answer to that final question, not only for the fathead minnow but for all species of fish, is undoubtedly 'no'.

Two important issues arise from this study: one from the experimental component and one from the social science component. The experimental component demonstrates that not enough is known presently about how and why fish behave as they do to enable robust conclusions to be reached on how chemicals might affect that behaviour and the consequences of any changes in behaviour induced by chemicals. Far too often normal behaviour is not established; that is, the baseline is unknown (Harris et al., 2014). Very helpfully, recent studies have begun to address both the variation in behaviour between individual fish within a species as well as the repeatability of the behaviour of each fish (e.g., Thoré et al., 2018). Collaboration between ecotoxicologists and behavioural ecologists, which is currently very rare, would be extremely advantageous in this regard.

The social science component (the survey) very clearly shows that different scientists, whether they are researchers or regulators, can reach entirely different conclusions from the same set of data. This is, of course, more likely to be the case when the data are equivocal, as

here. That inference is probably not restricted to research on how psychoactive drugs might affect the behaviour of fish, but instead is likely to be true for any effect and any chemical. Currently formal training in behavioural toxicology and in the design and interpretation of animal behavioural studies is not common within the ecotoxicology community. Given the lack of formal training in this aspect of biology, perhaps it is not surprising that 'our' group of experts reached the very varied opinions that they did. Considering the recent growing concern about the alteration of wildlife behaviour potentially induced by chemicals in the environment, that situation suggests that formal training in behavioural biology would be advantageous to both ecotoxicology researchers and regulators. Such training should lead to a more appropriate interpretation of behavioural toxicology data and their potential ecological implications, and consequently be used to better protect the environment from chemicals.

5. Conclusions

The presence of psychoactive drugs in the aquatic environment should be taken seriously, and it is important to understand whether or not their presence constitutes a significant risk to aquatic organisms. Yet despite more than a decade of academic research, we still do not know. In this study, an experiment was conducted in which fish were exposed to the synthetic opiate tramadol for over 3 weeks, then their anxiolytic behaviour assessed in a novel tank diving test. It produced equivocal results that are difficult to interpret. There was weak but statistically significant support for an effect of the highest concentration tested, and also at the lowest. The intermediate concentration had no apparent effect. Thus there was no clear concentration-response relationship. When a large group of independent experts, comprising both research scientists and regulators, was asked for its opinion on the results, these were highly variable; some experts concluded that tramadol had affected the behaviour of the fish, whereas others did not. Half of these experts believe that low concentrations of a chemical can cause effects that higher concentrations do not. Given the very inconsistent literature on the effects of psychoactive drugs on aquatic organisms, and the equally variable thinking and opinions of ecotoxicology experts, it appears impossible presently to conduct a meaningful aquatic risk assessment for any psychoactive drug. This situation is unlikely to change until considerably more is known about the normal behaviour of fish and the degree of individual variability. Given the very considerable difficulties associated with understanding the effects of psychoactive drugs in humans (e.g., Cipriani et al., 2018 for anti-depressants), even after millions of people have been treated with these drugs for decades, it is perhaps not surprising that difficulties are being encountered in determining whether or not they affect the behaviour of aquatic organisms, and if so, at what concentrations.

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Author contributions

R.T. led the experimental component of the research. She was assisted by L.M-C., B.H. and T.J.R., who also trained her in the appropriate techniques. J.P.S. designed the questionnaire and got all participants of a scientific meeting to complete it. R.T. conducted statistical analyses using non-parametric tests. A.E. subsequently conducted statistical analyses utilizing logistic regression. K.N., T.K. and S.T. contributed interpretation of data. J.P.S. wrote the first draft of the paper. All authors then provided input to improve it. J.P.S. supervised the project from start to finish. All authors have read and approved the final version of the manuscript for publication.

Declaration of interest

Three of the authors (L.M-C., T.J.R. and J.P.S.) have received financial support from a large pharmaceutical company for some of their research. However, that company did not provide any support of any kind for the research reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.02.090.

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