Chemosphere 180 (2017) 430–436

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Towards improved behavioural testing in aquatic toxicology: Acclimation and observation times are important factors when designing behavioural tests with fish

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HIGHLIGHTS

- Behavioural toxicology is currently under scrutiny for irreproducibility.
- Baseline behaviours and influencing factors must be understood.
- We identified acclimation and observation time as key factors influencing behaviour in fish.
- Both have the capacity to influence behavioural outcomes and interpretations.
- Review of the literature revealed that both vary substantially between studies.

ARTICLE INFO

Article history:
Received 1 March 2017
Received in revised form
8 April 2017
Accepted 12 April 2017
Available online 14 April 2017

Handling Editor: David volz

Keywords:
Behavioural analysis
Experimental design
Acclimation
Ethovision
Toxicity testing
Data acquisition

ABSTRACT

The quality and reproducibility of science has recently come under scrutiny, with criticisms spanning disciplines. In aquatic toxicology, behavioural tests are currently an area of controversy since inconsistent findings have been highlighted and attributed to poor quality science. The problem likely relates to limitations to our understanding of basic behavioural patterns, which can influence our ability to design statistically robust experiments yielding ecologically relevant data. The present study takes a first step towards understanding baseline behaviours in fish, including how basic choices in experimental design might influence behavioural outcomes and interpretations in aquatic toxicology. Specifically, we explored how fish acclimate to behavioural arenas and how different lengths of observation time impact estimates of basic swimming parameters (i.e., average, maximum and angular velocity). We performed a semi-quantitative literature review to place our findings in the context of the published literature describing behavioural tests with fish. Our results demonstrate that fish fundamentally change their swimming behaviour over time, and that acclimation and observational timeframes may therefore have implications for influencing both the ecological relevance and statistical robustness of behavioural toxicity tests. Our review identified 165 studies describing behavioural responses in fish exposed to various stressors, and revealed that the majority of publications documenting fish behavioural responses report extremely brief acclimation times and observational durations, which helps explain inconsistencies identified across studies. We recommend that researchers applying behavioural tests with fish, and other species, apply a similar framework to better understand baseline behaviours and the implications of design choices for influencing study outcomes.

1. Introduction

Behavioural analysis is being increasingly applied towards contemporary aquatic toxicology research. The growing popularity of behavioural testing largely stems from recent technological advancements (Parker, 2015), which have made commercial and open-source analysis software widely accessible. Additionally, the general consensus is that behavioural tests are rapid and sensitive to a wide range of pollutants (Melvin and Wilson, 2013), and offer a novel approach that may help link sub-lethal physiological effects

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http://dx.doi.org/10.1016/j.chemosphere.2017.04.058
0045-6535/

with population-level outcomes (Pyle and Ford, 2017). However, while there are several perceived benefits to studying behavioural changes in wildlife exposed to environmental pollutants, our understanding of the factors governing animal behaviour is still very limited and caution is therefore necessary when applying such tests (Melvin, 2017; Sumpter et al., 2014). Considering the marked increase in applications of behavioural techniques towards aquatic toxicology testing, it now seems prudent to evaluate how such tests are being applied, including factors that might influence the validity and repeatability of behavioural outcomes amongst studies (McCallum et al., 2017).

One of the most notable subjective aspects of modern-day behavioural toxicity research relates to the wide range of study designs and test methodologies being applied. One the one hand, the flexibility of behavioural testing can be viewed as a positive attribute since this allows diverse ecological processes to be studied (Parker, 2015). On the other hand, a lack of standardisation makes it very difficult to ensure the validity of different experimental designs and may lead to inconsistency in documented response patterns amongst studies (Huerta et al., 2016; Sumpter et al., 2014). The latter holds consequence for the progression of science because variable study outcomes can lead to continued exploration of potentially unimportant stressors, thereby resulting in unnecessary animal usage and resource expenditure. As a starting point, there is a pressing need to establish basic knowledge about baseline behavioural characteristics for species being used in behavioural toxicology (Melvin et al., 2016).

Since the increased prevalence of behavioural tests in aquatic toxicology seems to largely correspond with the wide accessibility of computational software tools (Bae and Park, 2014), basic approaches for using these technologies must be carefully evaluated. The most straightforward application of specialised behavioural analysis software involves measurement of basic swimming characteristics, such as velocity and other aspects of animal movement. As such, the most obvious areas where subjectivity in study design might be introduced are in the timeframes for acclimation, exposure, and data collection. Kane et al. (2005) identified 1) the timeframe for acclimation to experimental arenas and 2) the duration of observation as key factors that require consideration when designing behavioural toxicity tests with fish. This was reinforced by a recent study demonstrating how different observational timeframes can influence overall conclusions of behavioural analysis (Melvin, 2017). However, despite the identified importance of these factors, there have been no studies explicitly focused on understanding how choices in experimental methodology, and specifically acclimation time and the duration of observation, might influence fish behaviour and subsequent study outcomes in aquatic toxicology.

The present study explores swimming performance and temporal behavioural variability of adult mosquitofish (*Gambusia holbrooki*) using commercially available behavioural analysis software, to investigate the importance of adequately acclimating fish to observational arenas for testing. We calculated the theoretical statistical power achievable with different acclimation times, and observational durations, to explore how these factors might influence the quality of behavioural toxicity tests. Finally, we performed a literature review to document acclimation timeframes and observational durations reported in published behavioural toxicity tests using fish.

2. Materials and methods

2.1. Experimental fish

Adult mosquitofish (*Gambusia holbrooki*) were used for the experiment, due to their wide geographical distribution (Pyke, 2008, 2005) and recent interest into the use of this species for behavioural testing (Jakka et al., 2008; Magellan et al., 2014; Melvin, 2017; Melvin et al., 2016; Saaristo et al., 2014; Sismeiro-Vivas et al., 2007). Fish were collected from a local woodland pond near Griffith University's Gold Coast campus and transported in water from the collection site to the laboratory, where they were separated by sex and size and acclimated to experimental conditions for three months prior to experimentation. Moderately hard testing water was used for holding and experimentation (USEPA, 1994) and temperature and photoperiod were maintained at 22.2 ± 0.8 °C and 12: 12-h light: dark, respectively. Housing aquaria, which were artificially enriched with terra cotta pots and an artificial plant, were stocked at density of approximately one fish per litre, and water changes were performed (80% tank volume) twice weekly during holding. Experiments were approved by the Griffith University Animal Ethics Committee (Protocol No. ENV/03/16/AEC), and performed in accordance with the guidelines of the Australian Code for the Care and Use of Animals for Scientific Purposes.

2.2. Video recording fish swimming behaviour

Our experimental setup consisted of 20 square glass dishes (21 × 21 × 6 cm; Pyrex®) arranged in a 4 × 5 array. Dishes were placed on a large LED panel providing dim backlighting to increase contrast and achieve optimal tracking of the fish. We fed the fish staple flaked food *ad libitum* in their holding aquaria first thing in the morning on the day of testing, while setting up the behavioural arenas and software. Approximately 30 min after feeding, we transferred twenty sexually mature females weighting 730.65 ± 105.82 mg and measuring 32.43 ± 1.38 mm (standard length), to behavioural arenas filled with 800 mL control water using a fine mesh dip-net. Mosquitofish are well known to prefer shallow, calm waters where risk from predation is low (Casterlin and Reynolds, 1977; Pyke, 2008). We therefore chose this volume to offer a semblance of ecological relevance and provided ample depth for free movement (3 cm), while also limiting vertical movement and thereby focussing the study to two-dimensional behaviour to simplify the analysis. Barriers were in place to prevent fish from observing and interacting with each other during recording. Video recording commenced immediately after the fish were placed into their respective test arenas at 9:00am and continued for a period of 8 h. Recordings were made using Ethovision XT 9.0 (Noldus Technologies, Inc) connected to an acA1300-30gc GigE camera (Basler AG, Germany) mounted above the test arenas. The experiment was performed in an empty laboratory behind closed doors, and no one entered the room during filming.

Following video recording, we analysed data over both 5min and 2hr intervals and exported the results as excel files. Standard behavioural endpoints generated by the software were chosen for our assessment, including average and maximum swimming velocities (mm/s), and angular velocity (°/s). Since these endpoints are automatically produced, they are commonly reported in behavioural studies. However, such parameters also provide useful information including assessment of basic swimming performance, and when combined indicate behavioural complexity and occurrences of erratic movements (Benhaïm et al., 2012).

2.3. Acclimation characteristics and statistical power analysis

We plotted behavioural data over time to visualise temporal patterns in how fish acclimate to experimental arenas, and to facilitate comparison of short (5min) and longer (2 h) observational timeframes. Basic statistical comparisons of differences in each behavioural endpoint over time were assessed via non-parametric