Review

Adult vertebrate behavioural aquatic toxicology: reliability and validity

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Keywords: toxicology; aquatic vertebrates; anxiety; behaviour; reliability; validity

Abstract

Current advances the ability to assay adult aquatic vertebrate behaviour are potentially very useful to aquatic toxicologists wishing to characterise the effects of pollutants on behaviour, cognition or neurodevelopment. This review considers two specific challenges faced by researchers wishing to exploit these technologies: maximising reliability and validity. It will suggest two behavioural procedures, with the potential for automation and high-throughput implementation, which can be used to measure social cohesion and anxiety, two areas of interest in behavioural aquatic toxicology. In addition, the review will make recommendations about how these procedures (and others) could be carried out to maximise reliability and validity.

34 1. Introduction

35

36 There have been a number of recent technological and intellectual advances in
37 understanding the behaviour of aquatic vertebrates. This raises some exciting
38 possibilities for research in aquatic toxicology, particularly in terms of the critical need
39 to link behaviour to physiological and biochemical processes (Sloman and McNeil,
40 2012). First, it increases the potential for understanding how environmental pollutants
41 in aquatic ecosystems may affect subtle behaviours of aquatic populations (and the
42 wider connotations of this). Second, there may be increased potential for expanding
43 translational toxicology (Kalueff et al., 2014a; Mattes and Walker, 2009). For example,
44 there is potential to replace classical mammalian models, or indeed, refine existing
45 aquatic models, in particular in the light of increasing potential for non-invasive
46 physiological measurement in aquatic vertebrates (Scott et al., 2008). Third, it presents
47 the opportunity for increasing throughput in an age of 'big data', thus increasing the
48 potential for scientific breakthrough. However, extensive experience with rodent
49 models, coupled with increasing evidence from laboratory-based aquatic vertebrate
50 research, have demonstrated that there are a number of challenges that accompany the
51 developmental of large-scale laboratory animal behavioural testing. The goal of this
52 review is to consider what are the main challenges faced by researchers when
53 implementing behavioural tests. Finally, the review will suggest behavioural tests that
54 could be used to overcome these challenges.

55

56 2. What are the main challenges faced by researchers when implementing behavioural
57 tests?

58

59 When attempting to carry out work of a translational nature (i.e., work that uses
60 observaions in model systems to inform human condition; Mattes and Walker, 2009), a

challenge faced by all involved in preclinical research is confirming validity to ensure the usefulness of data derived from the model (Nestler and Hyman, 2010). There are three types of validity associated with translational models (as first discussed by Willner, 1997): (i) face validity; the phenomenological and subjective similarity between the model and the intended translational target (e.g., superficial similarities between the effects of anxiolytic drugs on marine vertebrates/invertebrates and humans; Brodin et al., 2013; Guler and Ford, 2010; Olsén et al., 2014); (ii) construct validity; a solid theoretical basis for the model (e.g., endocrine disruptors such as bisphenol A have developmental effects in mammals and marine vertebrates/invertebrates through mimicking the sex hormone oestradiol; Howdeshell et al., 1999; Kang et al., 2007); and (iii) predictive validity; the ability of the model make accurate predictions (e.g., if an animal was exposed to an endocrine disrupting compound during early development, and this increases stress reactivity in later life, is this rescued by anxiolytic drugs?). A fourth type of validity, ecological validity, describes the degree to which work carried out within a laboratory can be generalised outside to the 'real world', and this is discussed in detail below.

Other major challenges faced in behavioural aquatic toxicology involve maximising the critical components of good experimental design: 1) within- and between-laboratory reproducibility of experiments to ensure reliability; and 2) high external validity (e.g., ecological validity) to ensure biological relevance of the findings/ observations/ measurements. Generally, the most significant challenge is to reach equilibrium, with high reliability often leading to low external validity, and highly reliable experiments often being low in generalisability to situations outside of the laboratory (Carter et al., 2013). These considerations are particularly important during the increasing momentum towards high-throughput testing. Below, each of these challenges will be considered in turn with respect to aquatic toxicology.

High within- and between-laboratory reproducibility of experiments is essential in ecotoxicology research, not only in terms of ensuring good science and reducing redundant duplication of experiments, but also to ensure that reliable advice is given with respect to risk-assessment to user-groups (Klimisch et al., 1997). In order to ensure high levels of reproducibility, the assumed intelligence in laboratory animal science was to reduce within-animal variation, referred to as environmental standardisation (Paylor, 2009). However, some have argued that instead of increasing reliability, standardised environments reduce individual variability, thus increasing the risk of subtle extraneous factors affecting the dependent variable (Richter et al., 2009). Direct evidence for this came from Richter et al. (2011), who carried out a multi-laboratory standardisation vs. heterogenisation procedure, examining strain x environment interactions in a series of commonly used behavioural procedures in mice (including open field test and elevated maze). They demonstrated that systematic heterogenisation (i.e., increasing variation between cages/tanks/group allocation) increased both within- and between-laboratory reliability. This view is not undisputed, however, with some laboratory animal scientists arguing that rigorous standardisation remains essential (Jonker et al., 2013; Josef van der Staay et al., 2010; Örink and Rehbinder, 2000).

As is clear from this debate, the argument surrounding standardisation is not one for which there is a straightforward answer. In aquatic vertebrates, work with zebrafish has demonstrated that environmental enrichment increases exploration (Collymore et al., 2015), but reduces locomotor behaviour, and increases neuronal proliferation and whole-body cortisol (von Krogh et al., 2010). von Krogh et al. (2010) kept fish in social isolation, which theirs, and other studies, have shown reduces whole-body cortisol. For example, we found keeping fish either in isolation or in pairs for two-weeks prior to testing removes potential ceiling effects observed in group housed fish during the novel

115 tank diving test (Parker et al., 2012); effects that may mask the efficacy of some
116 environmental toxins to affect subtle behaviours. However, while chronic social
117 isolation reduces cortisol, acute isolation reliably *increases* cortisol (Kalueff et al.,
118 2014a). It is clear in zebrafish that changes in the environment can have severe effects
119 on behaviour and physiology masking, or in some cases reversing, the effects of
120 treatments.
121
122 Aquatic vertebrates have a number of personality/individual differences factors that
123 must be accounted for in experimental design. Much of the work in this area has
124 concentrated on social hierarchies. For example, Galhardo et al. (2012) observed
125 notable differences in the exploration of novel objects between male and female cichlids
126 (*Oreochromis mossambicus)*, and that these effects were strongly mediated by social
127 context (e.g., social isolation, unfamiliar social groups). In addition, social plasticity has
128 been observed in a number of teleost species (Matessi et al., 2010; Taborsky and
129 Oliveira, 2012), and represents an important function both for survival and evolutionary
130 fitness (Oliveira, 2012).
131
132 [FIGURE 1 ABOUT HERE]
133
134 Furthermore, choosing an testing environment and assay that best mimics conditions
135 experienced in the relevant ecosystem is critical in behavioural aquatic toxicology in
136 order to ensure the external validity (generalisability to subjects outside of the study
137 sample) of the findings (Moore and Robinson, 2004). This relates also to ensuring
138 ecologically relevant concentrations of putative contaminants are used (Brodin et al.,
139 2013), but a detailed discussion of this is beyond the scope of this review (see Carvalho
140 et al., 1995). In terms of behavioural testing, during animal experimentation, the subject
141 is removed from its social group (e.g., shoal), taken to an unfamiliar environment, and

142 exposed to the test while socially isolated. This raises a number of potential problems
143 for social animals, not only in terms of task reliability, but also for ecological validity. For
144 example, if the process of exposing the animal to test substrates is associated with
145 stress, this may have an impact on the response of that animal to the substrate (Figure
146 1). Some stress in testing is unavoidable, and could in fact be beneficial if stress results
147 in a specific measurable behavioural pattern (Parker et al., 2012). The gold standard
148 should perhaps be either to minimise stress (handling, novel environments, isolation) or
149 to utilise procedures that rely on a typical 'escape-avoidance' response.
150
151 In summary, in behavioural tests where the involvement of the experimenter during the
152 testing is necessary, it becomes almost impossible to standardise or to systematically
153 heterogenise, and results may vary considerably between laboratories. As some
154 experimental stress may be unavoidable, it may be prudent to utilise procedures that
155 exploit species-typical stress responses in order to increase external validity. In
156 addition, certain animal-level differences (e.g., social and behavioural plasticity) are
157 crucial factors, and should be considerations in experimental design. Below, this review
158 will propose some suggestions for maximising both within- and between-laboratory
159 repeatability, and external validity.
160
161 3. Which behavioural tests could be employed in adult vertebrate behavioural aquatic
162 toxicology that maximise reliability and validity?
163
164 There is a growing literature in vertebrate aquatic toxicology in which behavioural tests
165 have been adopted to examine responses to a variety of environmental toxins. It is
166 noteworthy at this point to say that many of these test have been adopted for zebrafish:
167 this is symptomatic of this species' growing popularity as an alternative to mammals in
168 behavioural neuroscience over the past decade or so (Kalueff et al., 2014a; Parker et al.,

169 2013b; Stewart et al., 2014b). Thus, many of the examples of behavioural tests in this
170 review may relate to those designed for zebrafish. Examples of behavioural tests include
171 light/dark preference (Serra et al., 1999), novel tank tests for anxiety (Bencan et al.,
172 2009; Parker et al., 2012), aggression (Basquill and Grant, 1998; Norton et al., 2011;
173 Perreault et al., 2003) and cohesion (Miller and Gerlai, 2007; Parker et al., 2014; Parker
174 et al., 2013a). A full review of these tests is beyond the scope of this review; however, I
175 will discuss two: one test of social cohesion and one test of anxiety. These assays are
176 pertinent for three reasons: 1) they use the animals' natural tendencies either to shoal
177 (social cohesion tests) or to take geotaxic evasive action (anxiety), thus increasing the
178 face and ecological validity of the tests; 2) both of these tests are for simple
179 'unconditioned' behaviours, thus increasing the throughput of the tests and the facility
180 to carry out the analyses in the absence of complex custom-designed testing equipment;
181 3) both of the tests can be carried out in a fully automated manner, thus increasing test-
182 retest reliability.
183
184 *3.1. Social Cohesion*
185
186 The presence of xenoestrogens and other endocrine disrupters (EDs) in aquatic
187 ecosystems is almost ubiquitous (Rotchell and Ostrander, 2003; Segner et al., 2003;
188 Sumpter, 2005). For example, 2,2-bis(4-hydroxyphenyl) propane (Bisphenol A; BPA), is
189 a byproduct of the production of some plastics, is a known ED, has been variously shown
190 to be acutely toxic to aquatic organisms, and is becoming wide spread in aquatic
191 environments owing to increases in production (Kang et al., 2007). Low levels of pre-
192 and perinatal BPA has been variously shown to affect social behaviour in mammals
193 (Wolstenholme et al., 2013; Wolstenholme et al., 2011), and this change is
194 transgenerational, with links to alterations in epigenetic reprogramming (Kundakovic et
195 al., 2013; Wolstenholme et al., 2013). The effects of EDs on social behaviour in shoaling

196 animals in wild ecosystems may be of some considerable interest. For example, reduced
197 shoaling may leave individuals more exposed to predation. Indeed if the behavioural
198 effects were found to be transgenerational, this could have potentially catastrophic long
199 terms effects on exposed ecosystems.
200
201 There are a number of approaches used for quantifying social cohesion. For example,
202 Robert Gerlai's laboratory have adopted a comprehensive social behaviour test in which
203 the relative proximity of all shoal mates is measured every 5-seconds over a 420-second
204 trial, including numerous behavioural measures (nearest neighbour, average inter-fish
205 distance, farthest neighbour, shoal polarisation, percent fish leaving shoal) (Miller and
206 Gerlai, 2007). This method has been shown to have good predictive validity, with
207 developmental ethanol altering social behaviour, as well as good construct validity, with
208 similar neurophysiological systems affected in the fish as in mammals (Buske and Gerlai,
209 2011a). An alternative method using cluster analysis was adopted by Parker et al.
210 (2013a) (adapted from Collins et al., 2011) to measure social cohesion in shoaling fish
211 (see Figure 2). Briefly, a shoal can be placed in an arena and video-recorded over a 10
212 min period. The tanks are then split into equal segments (locations) for analysis. Cluster
213 scores (*Clus*) are calculated for each time point (T) once every 30-s to ascertain shoaling
214 by dividing the maximum number of fish in one location of the tank (Max_T) by the total
215 number of segments occupied ($Total_T$), such that:
216

$$Clus_T = \frac{Max_T}{Total_T}$$

217
218 This method has successfully identified phenotypic differences in social cohesion caused
219 by developmental ethanol exposure (Parker et al., 2014) and early exposure to 1-pheny-
220 2-thiourea (Parker et al., 2013a), thus demonstrating its predictive and construct
221 validity. This method additionally benefits from being simple to implement, high in

222 ecological validity and fully automated, thus increasing the potential for reliability.
223 However, it should be noted that despite good inter-laboratory reliability for general
224 social behaviour (Buske and Gerlai, 2011b; Miller and Gerlai, 2007; Shams et al., 2015)
225 and social cohesion (Parker et al., 2014; Parker et al., 2013a), there is currently little in
226 the way of intra-laboratory reliability for either of these methods, with few independent
227 replications in the literature.

228 [FIGURE 2 ABOUT HERE]

229

230 *3.2. Anxiety*

231

232 Since the first report of widespread pharmaceutical contamination of streams in the
233 U.S.A. (Heberer, 2002; Kolpin et al., 2002), in particular by the widely used
234 antidepressant fluoxetine, studies of the endocrine and reproductive effects of this
235 environmental contamination in Japanese *Medaka* (Brooks et al., 2003), among other
236 vertebrate (Gaworecki and Klaine, 2008; Olsén et al., 2014; Stewart et al., 2014a) and
237 invertebrate (De Lange et al., 2006; Guler and Ford, 2010; Nentwig, 2007) species, have
238 emerged. The behavioural effects of fluoxetine contamination, and the wider
239 implications of this for the affected ecosystems, are becoming clear (Brodin et al., 2014).
240 Fluoxetine, a selective serotonin reuptake inhibitor (SSRI), is known to have efficacy
241 both as an antidepressant and as an anxiolytic agent (Dulawa et al., 2004). Historically,
242 although the pharmacological mechanisms of action of fluoxetine were well understood,
243 the way that this drug affects gene expression was less clear. Evidence has emerged,
244 however, that fluoxetine may induce chromatin alterations, specifically by suppressing
245 protein kinase calmodulin-dependent protein kinase II (CaMKII) transcription in the
246 brain's reward centres (Robison et al., 2014). Given this, and given the proven potential
247 for transgenerational epigenetic inheritance (Heard and Martienssen, 2014), the long
248 term effects on affected ecosystems of prolonged exposure could be far-reaching.

There are a number of approaches for measuring anxiety in zebrafish, and this may be extended to other aquatic vertebrates. Many of these assays have the added value of having been pharmacologically validated with anxiolytic drugs (see Kalueff et al., 2014b for review), suggesting good predictive validity. Examples include scototaxis (light/dark preference) (Maximino et al., 2010a; Maximino et al., 2010b), black/white preference tests (Blaser and Rosemberg, 2012), thigmotaxis (Shams et al., 2015) and open-field (open-tank) tests (Rosemberg et al., 2011). One of the most widely used approaches is a measure of geotaxis, using the novel tank test (Figure 3). During this test, the fish is introduced into a (typically trapezoid) novel tank and the amount of time spent in the bottom third of the tank is recorded (Figure 3a). This procedure exploits the fish's natural tendency to dive to the bottom of a new environment when first introduced. This procedure has the benefit of having been validated as a measure of anxiety in a number of ways, with the anxiolytic drugs buspirone and diazepam reducing (Bencan et al., 2009) and the anxiogenic compound alarm pheromone increasing (Egan et al., 2009), time spent on the bottom of the novel tank.

There has also been a parametric analysis of the novel tank test, manipulating a number of housing-related variables (Parker et al., 2012). This study demonstrated that the change in time spent in the bottom part of the tank over the course of the five-minute exposure to the novel tank decreased according to a second-order polynomial curve (Figure 3b):

$$y = ax^2 - bx + c$$

where y represents the time spent on the bottom of the tank, and the independent variable, x represents the time (i.e., minutes 1–5). Variables a, b and c were free parameters, which were found to be related to size of pre-test groups and other housing conditions (Parker et al., 2012).

[FIGURE 3 ABOUT HERE]

A variety of other factors have been found to affect performance on the novel tank test, including handling during the test period, the day and time of testing, testing batch, and the order in which the fish were run. For example, Stewart et al. (2015) observed subtle differences in performance between batches and between fish tested on different days, suggesting potential difficulties in establishing test-retest reliability. Both Stewart et al. (2015), and Parker et al.'s (2012) studies highlight the challenges faced by researchers wishing to carry out studies of anxiety in aquatic vertebrates, emphasising the importance of careful consideration of environmental and general laboratory/experimental conditions and of carrying out both internal (i.e, inter-laboratory) and independent (i.e., intra-laboratory) replications.

3.3. Summary and recommendations

This section has outlined two specific, validated, unconditioned behavioural tests that can be employed in vertebrate aquatic toxicology research. These tests benefit from being high in construct, predictive and face validity, but also from the potential for high-throughput implementation, being fully automated. There is potential for using such tests in aquatic toxicology to help characterise behavioural and physiological parameters linked to environmental toxins in a high-throughput, internally reliable, externally valid manner. However, as we have seen, there is a critical need for laboratories to replicate and publish data from these tests in a systematic manner to ensure reliability is maximised. In addition, it is crucial for laboratories to ensure internal reliability of tests prior to using them, particularly in the light of the relatively early stages of development of many assays. Again, the use of automated systems will

help to an extent, but other factors such as batch number, test order (Stewart et al., 2015) and housing conditions prior to testing (Parker et al., 2012) may also have effects on outcome measures.

[FIGURE 4 ABOUT HERE]

As outlined in the work of Richter and colleagues (Richter et al., 2010; Richter et al., 2009; Richter et al., 2011), it may be prudent to employ systematic heterogenisation of environments in order to improve reliability. Figure 4 displays a worked example of a fictitious dose range-finding experiment examining the effects on anxiety (behavioural and physiological) of a putative environmental toxin using the novel tank test, followed by whole-body cortisol extraction (see Cachat et al., 2010 for detailed protocols). Here, the experiment begins with a large population of wild-type (outbred) fish, preferably of various ages and equally male/female. These fish are randomly allocated to one of three exposure groups: Control, low dose of compound (referred to as 'Drug Lo', for simplicity), high dose of compound (Drug Hi). Within this, there are three enrichment options, enrichment with plants, enrichment with stones, and enrichment with plants + stones. In addition, there is one entire replicate of this included (i.e., Batch 1 and Batch 2). After the treatment period, half of the fish are removed from the groups for behavioural testing (novel tank test) and the other half for whole-body cortisol analysis. The fish designated for behavioural testing should then be housed in pairs if possible (if a washout is required) or individually if they must remain exposed continuously prior to assay. This should be organised such that fish from different treatments are housed together in a random manner. The fish should then be individually exposed to the novel tank test, preferably with half of the fish tested in the morning, and half in the afternoon, balanced according to group allocation. This experimental design benefits from being systematically heterogenised , thus decreasing uncontrolled between-group factors and

329 minimising the effects of individual variability.

330

331 A number of other procedures can be carried out in this manner in order to attempt to
332 move towards a more systematic heterogenisation during experiments. It should be
333 noted that this particular design also lends itself to mixed model statistical analysis, with
334 both fixed and random effects. For example, the model that could be used to analyse the
335 data generated in an experiment similar to that represented in Figure 4 could be
336 represented in simple form as:

337 $$Y_{ij} = \beta_{ij} + U_i + W_{ij},$$

338 where (for example) the time spent on the bottom of the tank (Y) for the *j*th fish in the
339 *i*th tank. In this model, β_{ij} is a fixed effect (e.g., dose of the compound), U_i represents a
340 tank-specific random effect, W_{ij} is the individual-specific error. These random effects
341 control for sources of random variation that might affect the outcome variable, allowing
342 the researcher to be clear on the extent of the variance that is accounted for by the fixed
343 effect (in this case, the dose of compound). Finally, in the light of variability at the
344 individual animal level, using this approach will ensure that this is accounted for in any
345 statistical model.

346

347 4. Conclusion

348

349 This short review has considered some of the challenges faced by researchers carrying
350 out behavioural tests with adult aquatic vertebrates in toxicology. The main challenge
351 remains to maximise both reliability and ecological validity. Based on the evidence
352 presented, it is prudent to concentrate on validated assays that minimise stress (e.g.,
353 handling stress, changing of social groups, novel environments), seek to exploit the
354 species' natural behavioural tendencies and, where possible, seek to automate tests.

Two such assays may be social cohesion and tank diving, both of which are validated, exploit species-typical behaviours and are fully automatable, minimising experimenter interference. We are at a crucial juncture as vertebrate behavioural toxicology begins to gain momentum. If we get it right from the start, this will be hugely beneficial for the field, ensuring that the data being produced are of exemplary quality.

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