

A standardized method for quantifying consistent individual differences in schooling behaviour

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A method for quantifying consistent individual differences in schooling behaviour is presented. This method, which utilizes a school of models, improves on previous methods by removing the unwanted variation that is introduced by live stimulus fish while still providing the physiological experience of schooling to the focal fish. Three-spined stickleback *Gasterosteus aculeatus* observed in the model school assay exhibited consistent individual differences in schooling behaviour.

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Growing interest in consistent individual differences in behaviour within populations has led researchers to ask questions about the causes (Biro & Stamps, 2008, 2010; Stamps & Groothuis, 2010*a, b*) and consequences (Dingemanse & Réale, 2005; Smith & Blumstein, 2008; Bolnick *et al.*, 2011, 2003; Sih *et al.*, 2012; Wolf & Weissing, 2012) associated with animal personality traits. A first step in addressing questions about consistent individual differences in behaviour within populations is accurately quantifying among-individual variation in the trait in question (Dingemanse & Dochtermann, 2013).

Individual variation in behaviour is typically estimated by repeatedly measuring focal individuals during standardized exposures to an ecologically relevant stimulus. This methodology is appropriate when the stimulus can be standardized across all individuals. Of the animal personality traits that have received the most attention (Réale *et al.*, 2007), sociability, where conspecifics are the relevant stimuli, presents the most difficulty for standardization. Live animals are likely to behave differently from one behavioural observation to the next, either in response to changes in internal states or in response to external stimuli. At best, the behaviour of stimulus animals differs randomly from trial to trial such that the variation they introduce is random noise.

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It is possible, however, that stimulus animals react to the focal animal that is being observed and *vice versa* creating feedback that can obscure the underlying tendency of the focal animal (Kodric-Brown & Nicoletto, 1997; Rands *et al.*, 2003; Conrads & Roper, 2005). For example, Jolles *et al.* (2015) found that the social attraction of individual three-spined stickleback *Gasterosteus aculeatus* L. 1758 was affected not only by the boldness of the focal fish, but also by the boldness of their social partner (boldness measured prior to social partner introduction).

A number of techniques have been developed to minimize unwanted variation from stimulus animals. For example, Wright & Krause (2006) presented a protocol for repeated measures of fish social behaviour that utilized one-way glass to prevent stimulus fish from seeing the focal fish. Researchers have also used mirror images (Adriaenssens & Johnsson, 2013) or video (Evans & Marler, 1991; Kodric-Brown & Nicoletto, 1997; Rowland, 1999) to replace stimulus animals altogether. The drawback of the aforementioned assays is that they are not well designed for measuring what is perhaps the most social behaviour exhibited by fishes, schooling, because they eliminate the experience of swimming with conspecifics.

Consistent individual differences in schooling behaviour are of special interest to animal personality researchers because they epitomize the problem of limited behavioural plasticity (Sih *et al.*, 2004); individuals differ from one another in how they behave in schools (Harcourt *et al.*, 2009) despite pressure from predators to conform (Landeau & Terborgh, 1986; Ioannou *et al.*, 2012). Assays that replace stimulus animals with images or block physical interactions with partitions eliminate the potential for stimulation of the lateral line system that normally occurs when fishes school. Partridge & Pitcher (1980) showed that lateral line stimulation is essential to normal schooling behaviour. In schooling species, assays that allow the stimulation of the lateral line system should be employed to get an accurate representation of variation in sociability. Faria *et al.* (2010) developed a robotic fish stimulus that can provide adequate physiological stimulation, standardization and can be programmed to do complex behaviours. The design and construction of a robotic fish system, however, is technologically challenging.

Wark *et al.* (2011) introduced an experimental protocol to quantify schooling behaviour that effectively controls for interactions between the focal and stimulus fishes while providing physiological stimulation to the lateral line system. Rather than live fish, they used a model school consisting of size-matched models as the stimulus. Models were arranged in the formation of a school and moved in a circle to simulate schooling behaviour. Using this model school assay, the authors showed that populations of *G. aculeatus* differ in schooling behaviour (Wark *et al.*, 2011). This research group went on to show that within-species differences in schooling tendency might be genetically linked to variation in the lateral line system (Greenwood *et al.*, 2013, 2015), further demonstrating the importance of physiological stimulation in assays of schooling behaviour. The goal of the present study was to test whether the model school assay developed by Wark *et al.* (2011) can be utilized to quantify consistent individual differences in schooling behaviour within a population.

Preliminary observations revealed that some individuals did not school when tested in the model school assay immediately after handling. Handling-induced stress (Brydges *et al.*, 2009) might contribute to the failure to respond. Furthermore, experience with social partners immediately prior to testing might influence the behaviour of the focal fish (Webster & Laland, 2012; Hotta *et al.*, 2014; Jolles *et al.*, 2016) and time in

isolation can mitigate behavioural differences related to experience (Gómez-Laplaza & Morgan, 2003). Therefore, the effect of isolation time on schooling was examined experimentally.

Young-of-the-year *G. aculeatus* were collected from the Navarro River, CA, in June 2010 and transported to the laboratory in Urbana, IL. Experiments were conducted the following May 2011, when *G. aculeatus* were sexually mature. *Gasterosteus aculeatus* were housed in 9.5 l tanks in groups of four to five. Light regimen was adjusted to approximate natural conditions in the field (39° N). Temperature was constant at 20° C. Clean water was provided *via* a recirculating flow-through system that consisted of a series of particulate, biological and UV filters (Aquaneering; www.aquaneering.com). Ten per cent of the water volume in the tanks was replaced each day. *Gasterosteus aculeatus* were fed a mixed diet consisting of frozen bloodworms *Chironomus* sp., brine shrimp *Artemia* sp. and mysis shrimp *Mysis* sp. in excess each day. Two weeks prior to testing, *G. aculeatus* were individually tagged with a unique combination of coloured marks using fluorescent visual implant elastomer (Northwest Marine Technology, Inc.; www.nmt.us).

The testing arena was similar to that used in Wark *et al.* (2011). A circular plastic pool (1 m diameter, 10 cm water depth) was filled with water from the flow-through system. The model school was composed of seven clay models that were shaped and coloured to match *G. aculeatus* from this population [Fig. 1, but see Wark *et al.* (2011) for an alternative to clay models]. *Gasterosteus aculeatus* prefer to group with similar sized conspecifics (Ranta *et al.*, 1992). To reduce differences in size-based preferences, models that spanned the size range of adult fish in this population (30–45 mm standard length, L_S) were used. Models were arranged in a 5 cm radius hexagon with one model in the centre (*c.* one body length nearest neighbour distance; Fig. 1). The model school was suspended with fishing line from a clear disc, which hung 30 cm above the surface of the water. The disc was connected to a microwave turntable motor (Timetech Inc., Catalogue No. S430.1; www.timetechusa.net). When the motor was activated, the models moved together in a circle at a velocity of 7.5 cm s^{-1} (models were offset 15 cm from centre, rotation direction was random). While still, the models were oriented haphazardly. When set in motion remotely, all models oriented in the forward direction.

The amount of time that *G. aculeatus* were allowed to recover between netting and testing was manipulated to test the hypothesis that isolation time affected the likelihood of schooling. On day one, three tanks were randomly selected for the 24 h isolation treatment. After the daily feeding, individuals from these tanks ($n = 14$) were netted and placed individually into 500 ml opaque white, cylindrical isolation chambers (diameter = 10 cm, height = 12 cm) filled with tank water for 24 h. On day two, *G. aculeatus* from the three remaining tanks ($n = 13$) were moved to isolation chambers 1 h before testing (1 h isolation). This was done prior to the daily feeding on day two to eliminate differences in time since last feeding between isolation time treatments. *Gasterosteus aculeatus* were given random ID numbers when placed in isolation chambers by S.P. Observations were conducted by L.H., who was blind to treatment.

At the start of each schooling trial, a focal individual was gently poured from the isolation chamber into the pool and allowed to acclimate for 5 min. This method of transfer from the isolation chamber to the testing pool differed from Wark *et al.* (2011), where *G. aculeatus* were transferred *via* netting. After acclimation, L.H. set the model school in motion and recorded whether the *G. aculeatus* swam within one body length

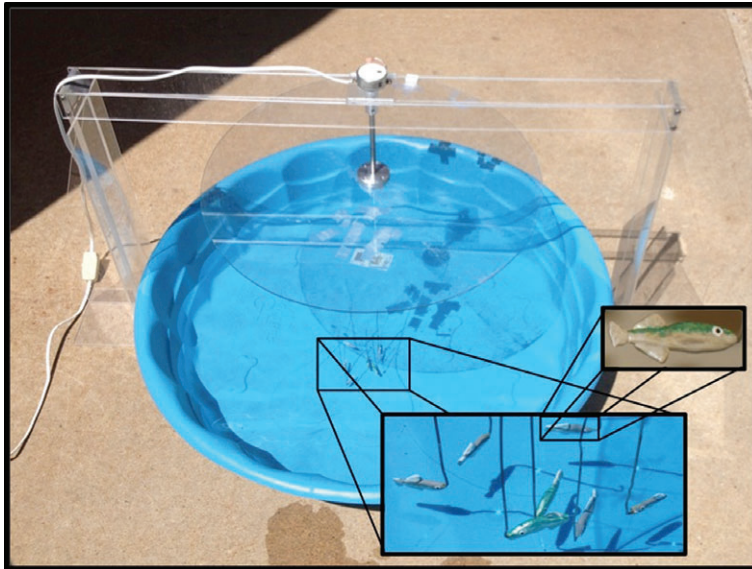


FIG. 1. Testing pool containing model school. The first inset shows the model school at rest. When set in motion, all models orient in the same direction. The second inset shows a clay model *Gasterosteus aculeatus*. Models ranged from 30 to 45 mm in standard length (L_S) to match the range of L_S of adult *G. aculeatus* in the Navarro River population.

of the model school (success or failure to school) for 10 min. The proportion of *G. aculeatus* that schooled in the 1 h v. 24 h isolation treatments was compared using a Fisher's exact test.

Isolation time had a significant effect on the likelihood on schooling (Fisher's exact test, $n=27$; $P<0.05$). All 14 of the *G. aculeatus* in the 24 h isolation treatment schooled while only eight of the 13 *G. aculeatus* in the 1 h isolation time treatment schooled. Therefore, in the subsequent experiment described below, *G. aculeatus* were isolated for 24 h before testing.

A different set of individuals was used to test for consistent individual differences in schooling. The schooling behaviour of 35 individuals (L_S range: 30–45.5 mm, mean \pm s.e. = 39.2 ± 0.7 mm) was observed on 10–12 May 2011 (trial 1). A randomly chosen focal individual was gently poured from the isolation container into the pool and allowed to acclimate for 5 min. After this, the researcher set the model school in motion for 10 min and recorded how much time the focal *G. aculeatus* spent moving within one body length of the models (time schooling). Observations were made from a distance of c. 0.5 m and distance between focal *G. aculeatus* and models was judged by observer. At the end of the trial, *G. aculeatus* were returned to their home tanks after nuptial colouration (indicator of sex), ID (using elastomer tags) and L_S were recorded. Observations of time schooling were repeated with the same individuals on 26 and 27 May 2011 (trial 2).

A linear mixed model was used to estimate the fixed effects of presence of nuptial colouration, L_S and trial on time schooling with ID included as a random effect. The normality of time schooling was determined through visual inspection of a QQ plot of the residuals. The 95% c.i. of fixed effects indicated whether they contributed to

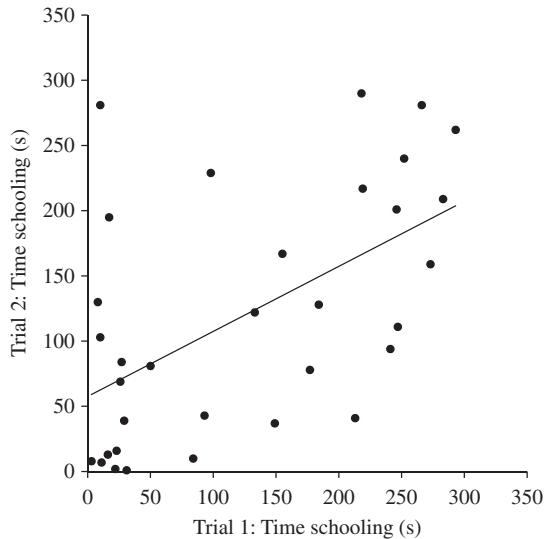


FIG. 2. Individuals consistently differed in time schooling across 2 weeks. ●, the amount of time individuals spent schooling with models over 600 s observations. The curve was fitted by $y = 57.56 + 0.50x$ ($n = 35$).

the variation and therefore should be considered when calculating repeatability. To test whether individuals consistently differed from one another in time schooling, repeatability and 95% C.I. were calculated using the R package ICC (Wolak *et al.*, 2012). Statistical significance was inferred if the 95% C.I. surrounding a repeatability estimate did not overlap zero (Nakagawa & Schielzeth, 2010; Wolak *et al.*, 2012). R was used for all statistical calculations (www.R-project.org).

Time spent schooling varied greatly, ranging from 1 to 293 s out of 600 s. The time schooling was 124.6 ± 15.2 s (mean \pm S.E.). Neither presence of nuptial colouration, L_S , nor trial affected time schooling [effect and 95% C.I., sex = 23.8 (-39.6 , 87.0), $L_S = 0.47$ (-7.02 , 8.01), trial = 1.76 (-30.7 , 35.2)]. Therefore, these factors were removed from the model when calculating repeatability. There were consistent individual differences in schooling behaviour over a 2 week period (Fig. 2). Differences among individuals accounted for *c.* 50% of the variation in schooling behaviour [repeatability = 0.57 (0.28 , 0.76), $n = 35$]. To put this in perspective, a meta-analysis of published repeatability estimates found that the average amount of variation explained by differences among individuals was 37% (Bell *et al.*, 2009).

These data suggest that the model school assay can be used to quantify consistent individual differences in schooling behaviour within a population. *Gasterosteus aculeatus* showed consistent individual differences in schooling over 2 weeks and differences in schooling were independent of sex and body size.

The repeatability estimate from this study exceeded estimates from sociability tests in which stimulus fishes were separated from focal fishes by a partition (Ward *et al.*, 2004; Cote *et al.*, 2010; Brown & Irving, 2014). Repeatability estimates can increase by either an increase in variation between individuals or by a decrease in within-individual variation (Nakagawa & Schielzeth, 2010; Dingemanse & Dochtermann, 2013). One of the main purposes of utilizing the model school assay for quantifying consistent individual

differences in behaviour was to decrease unwanted variation introduced by live stimulus animals. Although it is not clear whether the relatively high repeatability estimate in this study is due within- or between-individual variation, an interesting hypothesis for future consideration is that eliminating unwanted variation from stimulus animals actually decreases within-individual variation.

Consistent individual differences in schooling behaviour have important implications for ecology and evolution. For example, whether cooperative behaviour is a fixed individual trait or varies according to the behaviour of others can affect the prevalence of cooperation in nature (Bergmüller *et al.*, 2010). When individuals differ in sociability, the effect of the social environment will differ between individuals. The ecology of some individuals will be shaped by group living, while more solitary individuals might adopt different strategies for finding food or avoiding predators. Preferences for different social environments could affect the maintenance of variation (Ravigné *et al.*, 2004; Holt & Barfield, 2008; Edelaar & Bolnick, 2012) and ultimately promote speciation in a manner analogous to habitat selection (Bush, 1969; Via, 1999).

One potential advantage of the model school assay that was not explored in this study is automated data collection *via* tracking software (Wark *et al.*, 2011; Greenwood *et al.*, 2013, 2015; Jolles *et al.*, 2015, 2016). In the present study, an observer scored behaviour manually in real time. Automated tracking offers two advantages over this method. First, researchers could increase the pace of data collection by making video recordings of trials conducted in multiple model school arenas simultaneously and then outsource data entry to software. Second, tracking software can extract more visual information with a higher level of precision than human observers. For example, Wark *et al.* (2011) were able to extract the distance between the focal and all stimulus models as well as the agreement in the angle of movement between the focal and the models, *i.e.* whether focals were swimming parallel to the models. The additional information provided by tracking software made it possible for Greenwood *et al.* (2015) to discover a genetic association between schooling position and the lateral line system.

In conclusion, the model school assay improves on previous methods used to quantify consistent individual differences in schooling behaviour by avoiding the shortcomings of using live conspecifics as stimuli without sacrificing physiological feedback. *Gasterosteus aculeatus* exhibited consistent individual differences in schooling behaviour that could not be explained by size or sex.

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