Predictability of food supply, but not ration, increases exploratory behaviour in zebrafish *Danio rerio*

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Individual zebrafish *Danio rerio* were assayed for exploratory tendency in a serial open field test before and after being maintained on one of four diet treatments that differed in ration and in predictability of food delivery. *Danio rerio* became more exploratory after being maintained on a diet with a predictable delivery schedule. There was no effect of ration. Thus, exploratory behaviour is inducible by environmental influences independent of genetic predisposition or social interactions. These results have implications for management of correlated behavioural syndromes of exploratory and boldness of animals reared in captivity for later release into wild populations.

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INTRODUCTION

Correlated behavioural traits comprise major axes of variation in animal behaviour (Sih & Bell, 2008). An important property of correlated traits is that behavioural types are consistent across contexts (Wilson & Godin, 2009; Bell *et al.*, 2010). For example, animals that are bold in the context of foraging also tend to be more aggressive in courtship and engage in riskier decision making in the face of predation risk (Huntingford, 1976). The optimal behavioural phenotype in natural populations is the one that maximizes reproductive success for interactions over multiple contexts (Bell & Stamps, 2004; Brown *et al.*, 2005; Álvarez & Bell, 2007); therefore, an environment with high predation risk will also select for subdued courtship behaviours, reduced activity and more tentative foraging behaviours (Magnhagen, 2006; Bell & Sih, 2007), and *vice versa*.

Fish reared in hatcheries live in an environment that favours a bold and exploratory behavioural phenotype because of the absence of predation risk and the rewards to bold individuals that out-compete their neighbours for food in high-density hatchery conditions (Huntingford, 2004; Sundström *et al.*, 2004; Huntingford & Adams, 2005; Salonen & Peuhkuri, 2006; Conrad *et al.*, 2011). Consequently, individuals in captivity tend to be bolder than their free-living counterparts in the wild (Huntingford & Adams, 2005). When captive-reared fishes are released into the wild their survival is poor, in

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part because the boldness that served them well in captivity becomes a liability in the presence of predation (Huntingford & Garcia de Leaniz, 1997; Biro *et al.*, 2004, 2007; Biro & Post, 2008).

Zebrafish *Danio rerio* (Hamilton 1822) show behavioural syndromes in exploratory and boldness (Dahlbom *et al.*, 2011; Wisenden *et al.*, 2011; Ariyomo & Watt, 2012) and activity and aggression (Moretz *et al.*, 2007) within and across populations and genetic strains (Wright *et al.*, 2003; Robison & Rowland, 2005) including some evidence that increased aggression and boldness is correlated with domestication (Wright *et al.*, 2003, 2006; Robison & Rowland, 2005; Moretz *et al.*, 2007).

Food resources, particularly the timing of food delivery, may influence behavioural phenotype. In neonate guppies *Poecilia reticulate* Peters 1859 an unpredictable feeding schedule leads to increased exploratory behaviour (Chapman *et al.*, 2010). In salmonids, *D. rerio* and others (Huntingford & Adams, 2005; Conrad *et al.*, 2011) the opposite effect occurs: Hatchery conditions, where the feeding schedule is highly predictable, consistently shift fishes towards a bolder behavioural phenotype (Sneddon, 2003; Sundström *et al.*, 2004; Wright *et al.*, 2006). This may be a consequence of heightened competitive environment created when food arrives on a predictable schedule into a densely stocked raceway, or because the absence of predation risk removes the selective advantage for the shy phenotype. To explicitly test the effect of predictability of food delivery on fish behaviour the influence of social interactions was eliminated. *Danio rerio* were used as the model organism (Robison & Rowland, 2005) to test the effect of predictability of food delivery on behavioural phenotype. In this experiment, ration was experimentally manipulated (high and low) over two feeding schedules (constant and variable) to test for experiential influence on behavioural phenotype, as measured by exploratory tendency in an open field test.

MATERIALS AND METHODS

Wild-strain *D. rerio* were purchased from EkkWill Waterlife Resources (www.ekkwill.com). Fish were initially held in 75 l stock tanks of *c.* 50 fish per tank, maintained on a diet of standard flake food at 24° C fed twice per day and a 12L:12D light cycle. To sort *D. rerio* along an exploratory–boldness behavioural axis, a serial maze was used based on the design described by Sailer *et al.* (2012) (Fig. 1). Mazes were long, narrow tanks (76 cm × 16 cm × 16 cm deep) partitioned into five sections. An opaque black barrier separated each section. In the centre of each barrier was a horizontal PVC tube that allowed fish to move between sections of the maze. The first connecting tube was a straight section of PVC tube with an inner diameter of 26 mm. The second connector was a straight tube that began with an inner diameter of 18 mm and expanded to 26 mm. The third connector was similar to the second connector except that it had a 90° bend in it. The fourth and final connector was a straight tube with an inner diameter of 15 mm. Each connector was *c.* 60 mm in length. Twelve identical mazes were used to run four batches of trials, two in March 2013 and two in July 2013, for a total of 48 trials.

The experimental protocol proceeded in three steps (Table I). In the initial step, the exploratory tendency of each fish was scored using the maze described above. Each fish was run twice so that repeatability could be calculated. In the second step, individual fish were transferred to isolated aquaria and maintained on one of four diet treatments. In the third step, each fish was assessed for exploratory tendency using the mazes. As for the initial step, the final measure of exploratory behaviour was repeated twice to calculate repeatability.

To assess exploratory behaviour, individual fish were placed in the end section of each maze. The position of the fish was recorded after 48 h. In preliminary trials, 48 h was determined to be long enough for the boldest fish to reach the end of the maze but not so long that fish began
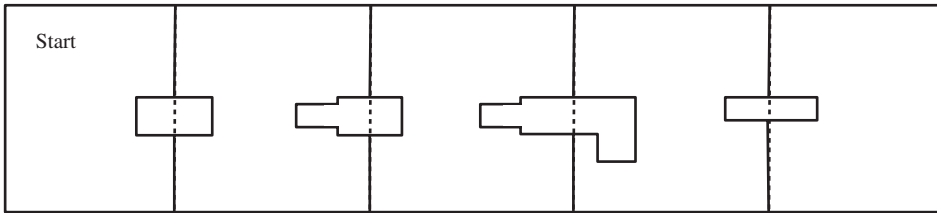
FIG. 1. Overhead view of the serial maze used to sort *Danio rerio* along the exploratory–boldness behavioural axis.

to double back. A score of 1 indicated that the fish remained in the starting chamber. A score of 5 indicated that the fish navigated to the end (fifth) chamber of the maze. After a 24 h rest period in a separate 38 l aquarium, each fish was run through the maze a second time to assess repeatability.

Each fish was randomly assigned to one of four diet treatments. The diet treatments were conducted in 24 separate holding chambers created by subdividing each of 12, 38 l aquaria for 3 weeks. Two variables were manipulated: ration richness (high and low) and feeding schedule (variable and non-variable) in a 2×2 experimental design. Fish on the non-variable diet were fed twice daily with the good diet and once every other day with the poor diet. Fish on the variable diet were fed on a random schedule. A random number generator was used to produce numbers ranging from zero to three that represented the number of feedings the fish would receive per day. For example, one day the fish on variable diets may have been fed twice, the next day fed once, the next day not at all and the next day three times, over the 3 week period. Despite the variability, all fish on variable diets were fed the same total number of feedings as fish on non-variable diets (either 42 or 11 feedings with the good and poor diets, respectively). Each feeding was 0·25 ml of freshly hatched brine shrimp *Artemia* sp. nauplii. Therefore, fish on the good diet received a total of 10·5 ml of brine shrimp over the 3 week period, whereas fish on the poor diet received a total of 2·75 ml of brine shrimp over the 3 week period. Presenting all four treatments simultaneously was logistically difficult; consequently, four batches of 12 trials were conducted ($n = 48$). In the first and third batches, high ration on variable or non-variable schedules were tested. In the second and fourth batches of the trials, low ration on variable or non-variable schedules were tested.

TABLE I. Summary of experimental design

Step in protocol	Details of protocol for each step
Pre-diet exploratory scores	Recorded progress in maze after 48 h
	Rested in a neutral aquarium for 24 h
	Re-recorded progress in maze after 48 h
Diet treatments	Batch 1: high ration on variable or non-variable schedule ($n = 12$)
	Batch 2: low ration on variable or non-variable schedule ($n = 12$)
	Batch 3: high ration on variable or non-variable schedule ($n = 12$)
	Batch 4: low ration on variable or non-variable schedule ($n = 12$)
Post-diet exploratory scores	Recorded progress in maze after 48 h
	Rested in a neutral aquarium for 24 h
	Re-recorded progress in maze after 48 h

Exploratory scores were integers and limited to values of $1-5$ and therefore could not be supposed to be normally distributed. Consequently, repeatability analysis was performed on ranked exploratory scores, which is analogous to a non-parametric Kruskal–Wallis ANOVA except that variance components could be extracted. Separate repeatability analyses were performed on three subgroups: (1) pre-diet fish, (2) post-diet fish after receiving the variable diet and (3) post-diet fish after receiving the constant diet.

To test the effects of diet richness and delivery schedule, pre-diet and post-diet exploratory–boldness scores were compared by summing the scores from the two pre-diet treatments separately from the two post-diet treatments and considering the difference in these sums (post-diet minus pre-diet) as the response variable. Four different response variables were tested with a single predictive model constructed with a split-plot formulation with the two different feeding schedules (constant or variable) split within food ration (high or low). This split plot was nested within a repeated measures block (trial batches $1 + 2$ and trial batches $3 + 4$). If the post-diet minus pre-diet difference was positive, the fish was scored as 'became bold'. If the difference was negative, the fish was scored as 'became shy'. If the difference was zero, the fish was considered to show no change in exploratory behaviour (*i.e.* 'neutral'). These designations were then converted to a binomial variable in three different ways: (1) bold *v.* shy and neutral, (2) shy *v.* bold and neutral and (3) bold and shy *v.* neutral. These three different responses were used in four separate models: (1) ANOVA using the ranked scores for the overall magnitude of the change in number of maze sections to compare between pre- and post-diet tests, (2) logistic regression on whether fish became bold rather than shy or neutral, (3) logistic regression on whether fish became shy (*i.e.* less bold) rather than bold or neutral and (4) logistic regression on whether there was any change (*i.e.* bold or shy) relative to no post-pre change (*i.e.* neutral). All statistic tests used $\alpha = 0.05$.

All protocols used in this study were pre-approved by the Minnesota State University Moorhead Institutional and Animal Care and Use Committee protocol number 10-R/T-Biol-018-N-Y-C.

RESULTS

When repeatability was tested within the two pre-diet and between the two post-diet measures of exploratory behaviour, individual fish did not show repeatable behaviour in the pre-feeding trials ($r = 0.016$, $F_{44,48} = 0.859$, $P > 0.05$) or the post-trial low-constant-diet treatment ($r = 0.412$, $F_{11,12} = 2.40$, $P > 0.05$) but showed high repeatability in post-feeding trials for the high-constant diet ($r = 0.510$, $F_{11,12} = 3.08$, $P < 0.05$), the low-variable diet ($r = 0.797$, $F_{10,11} = 8.866$, $P < 0.001$) and the high-variable diet ($r = 0.641$, $F_{11,12} = 4.568$, $P < 0.01$), where r is the measure of repeatability given as the proportion of variance in rank-transformed scores explained by individuals.

Fish on predictable diets became more exploratory than did fish on variable diets, regardless of richness of ration (Figs 2 and 3). Only the logistic regression testing whether the overall post-pre comparison led to a change in a fish becoming more exploratory yielded a significant result ($P < 0.05$) and then only for the main effect for predictability of feeding schedule. Fish that were fed a predicable amount each day were over seven times as likely to increase in exploratory tendency than were fish fed the same total amount of food but delivered on a variable schedule [odds ratio (OR) $= 7.05$, $P < 0.05$]. The overall ration level (high *v.* low) or the interaction between ration and predictability of delivery schedule did not have an effect on this exploratory score. A mean $\pm$ S.E. $= 50.0 \pm 10.0\%$ of fish became more exploratory when fed on a predictable schedule *v.* $26.0 \pm 9.2\%$ that became more exploratory when feeding schedule was unpredictable, or nearly twice as many (Fig. 3). While not
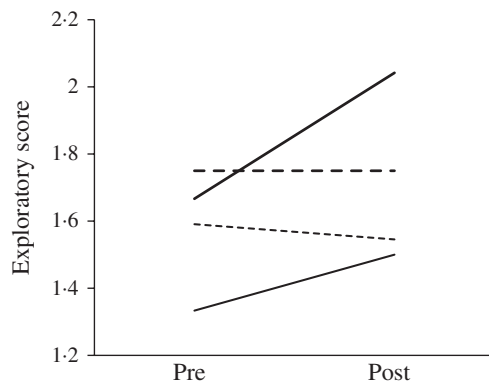
FIG. 2. Mean exploratory score in open field mazes for fish on each of the four diet treatments: high ration (——), poor ration (----), predictable food delivery (——) and unpredictable food delivery (– –). S.E. bars omitted for clarity.

significantly demonstrated, the data indicated that 37% became less exploratory after being fed on a variable schedule compared with 17% becoming more exploratory on the variable schedule. Overall, 34% of fish tested did not change in exploratory score post- and pre-treatment.

DISCUSSION

The data indicate that predictability of food delivery can induce individual *D. rerio* to become more exploratory. This effect was independent of ration level, social interactions or underlying genetic predisposition for exploratory behaviour. Not only did fish fed on a predictable schedule become more exploratory, the effect was also highly repeatable. Fish on the predictable food delivery schedule were reliably exploratory, whereas fish assigned to the variable delivery schedule were reliably non-exploratory. The post-diet effect on exploratory tendency was highly repeatable despite non-significant repeatability before the diet treatments. Given previous work
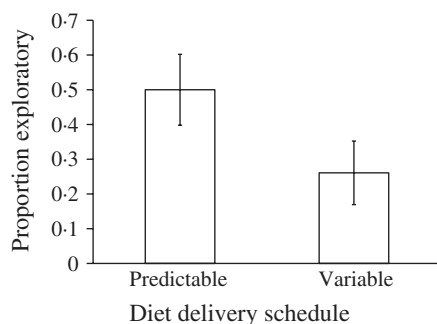

FIG. 3. Mean ± S.E. proportion of fish that increased in exploratory tendency after being reared on predictable *v.* variable diet regimes.

showing that exploratory tendency and boldness in the face of predation risk are correlated (Wisenden *et al.*, 2011), these data indicate that predictability of food supply probably manifests correlated boldness if released into the wild in other contexts such as foraging (Dingemanse *et al.*, 2002) exposure to predation risk (Biro *et al.*, 2004).

Although there is ample evidence that boldness tendencies in *D. rerio* are derived, at least in part, from genetic factors (Wright *et al.*, 2003; Robison & Rowland, 2005; Oswald *et al.*, 2013), there is also evidence that individual differences in behaviour may derive entirely from epigenetic factors (Watters & Meehan, 2007; Wisenden *et al.*, 2011). In this study, fish arbitrarily assigned to different feeding schedules were induced to conform to one particular behavioural phenotype or another independent of genetic substrate.

The results from this study have implications for fish reared in captivity for release into the wild for either fisheries enhancement or for species conservation. Captive environments, such as hatcheries, differ from natural environments in that there is no risk of predation and competition for food is heightened by high fish densities and delivery of food on a set schedule dispensed from feeders fixed in space. This environment favours bold individuals that can out-compete others for access to food. The effect of captivity on behavioural phenotype can result from differential survival (genetic selection) Fraser *et al.*, 2001; Brown *et al.*, 2007; Yokota *et al.*, 2007; Wilson & Godin, 2009) or from experiential (environmental) effects (Ruzzante, 1994; Einum & Fleming, 1997; Watters *et al.*, 2003; Bell & Sih, 2007; Biro *et al.*, 2010). Indeed, manipulating feeding schedules is a management tool that can alter the diversity of behavioural phenotypes in captive fishes (Sneddon, 2003; Yoshida *et al.*, 2005; Watters & Meehan, 2007).

An unanticipated outcome of this experiment was the effect of feeding regime on the repeatability of individual differences in exploratory tendency. It is not clear if inducement of repeatability is independent from inducement of exploratory tendency or if repeatability is a by-product of selection for divergent behavioural phenotypes. The ecological and evolutionary implications of selection for repeatability are interesting directions for future research.

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