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SHORT COMMUNICATION

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Inter and intra-population variation in shoaling and boldness in the zebrafish (*Danio rerio*)

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Abstract Population differences in anti-predator behaviour have been demonstrated in several species, although less is known about the genetic basis of these traits. To determine the extent of genetic differences in boldness (defined as exploration of a novel object) and shoaling within and between zebrafish (*Danio rerio*) populations, and to examine the genetic basis of shoaling behaviour in general, we carried out a study that involved laboratory-raised fish derived from four wild-caught populations. Controlling for differences in rearing environment, significant inter-population differences were found in boldness but not shoaling. A larger shoaling experiment was also performed using one of the populations as the basis of a North Carolina type II breeding design (174 fish in total) to estimate heritability of shoaling tendency. A narrow-sense heritability estimate of 0.40 was obtained, with no apparent dominance effects.

Introduction

Grouping is an adaptive behavioural trait found in many different species, decreasing predation risk and optimising resource acquisition (Krause and Ruxton 2002). Although grouping behaviour is widespread, relatively little is known about its genetic basis. Most of the work that has been done on the heritability of grouping behaviour was carried out on different species of fish, the principal reasons for this being the strong differences in shoaling behaviour that often exist between fish populations and the fact that many small freshwater species are easily bred in the laboratory and have short generation times (Ruzzante and Doyle 1991). However, most of the studies concerning laboratory-reared populations lack adequate control for the effects of variation in rearing conditions. Using a 'split-family' design, with each population being raised in at least two tanks, inter-population differences can be tested against variation due to uncontrolled differences in rearing conditions. Within populations, no study has yet quantified the heritability of shoaling tendency or estimated the contribution of additive and non-additive components of genetic variation. We report here a set of experiments analysing inter-population differences in shoaling tendency and boldness in laboratory-reared fish as well as an estimate of the heritability of shoaling tendency for the zebrafish (*Danio rerio*).

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Methods

Fish collection sites

Microsatellite analysis of several populations of wild zebrafish (including three of the four populations described below) indicates that genetic differences exist between these populations, with isolation by distance not apparent (D. Wright et al., unpublished data). The zebrafish stocks used in this experiment were collected from a single site in Nepal and three sites in Bangladesh, with brief site descriptions as follows.

Nepal

Collected from shallow ditches and an adjacent pond, numerically superior to the two other species found there, a barb, *Esomus* sp., and an unidentified catfish.

Tangail

Collected from a medium-sized pond, with clear water and large amounts of vegetation. The area suffers from extensive flooding in the monsoon season.

Santal

Taken from small, shallow pools, co-occurred with two other fish species, *Esomus* spp. and *Puntius* spp., with some aquatic vegetation present.

Canal

Taken from an artificial concrete channel. Vegetation was absent and water in the channel was still and extremely turbid. Fish occurred at high densities, together with a species of *Esomus* and freshwater prawns.

Fish maintenance

The fish used in the experiments were first-generation offspring reared in standard conditions from the wild stocks, with two tanks raised per population. Of the Nepalese fish, nine wild-collected individuals were used as the basis for a North Carolina type II experimental cross (Lynch and Walsh 1998), i.e. offspring were obtained from various combinations of the five females and four males (hereafter 'families'). A total of 12 families was raised, with six being reared over two tanks and six in a single tank (see the Figures for cross combinations). Stocking density per tank was 11.9±3.2 individuals for the North Carolina Type II cross and 13.25±2.5 for the standard populations (mean ± SD). Behavioural experiments were carried out between 11.00 and 17.00 hours, when the fish were not reproductively active (the fish require a lengthened light period in the diurnal cycle to spawn). The standard body lengths for the different populations were 25±2.5 mm (mean ± SD) for the within-population shoaling experiment and 27±1.8 mm (mean ± SD) for the inter-population shoaling and boldness experiment (Canal 26.2±1.55, Santal 27.56±1.24, Tangail 26.34±0.86, Nepal 28.27±2.31, stimulus fish 26.9±0.82).

Boldness inter-population experiment

Inter-population boldness was tested using a tank measuring 600×170×170 mm high, filled to a depth of 120 mm. A 'novel' object, in the form of a roughly cylindrical shape, fashioned from black modelling plastic and approximately 140 mm in length, was suspended at one end of the tank, mid-way in the water column. A single focal fish was given 10 min to acclimatize in a beaker before being gently poured into a plastic tube at the opposite end of the tank to the novel object. After a further 5 min of acclimatisation, the tube was remotely raised and the experiment began. Boldness was measured as the total time spent within one-and-a-half body-lengths of the novel object in a 10 min period. The focal fish always went to within two body-lengths of the novel object within the first 30 s of the experiment, allowing the experiment to begin as soon as the tube was raised. Ten fish were tested per tank, with each population being represented by two tanks and with each fish being tested a total of three times (240 trials in total).

Shoaling tendency: inter-population experiment

Shoaling tendency of offspring from each of the four wild-derived populations was measured as the time spent associating with a stimulus shoal, with association defined as swimming within 40 mm of the stimulus. The test tank comprised of a central compartment incorporating one-way glass and two outer compartments. A single focal fish was presented with a shoal of six fish in one compartment, with the other outer compartment identically lit but empty. To ensure that the stimulus shoal had been observed by the test fish, the trial only began when the focal fish first entered the shoaling zone next to the stimulus fish. All experiments were conducted at 25°C. Ten focal fish were tested from each tank and trials were repeated once for each focal fish, and at different times of the day to control for any variation in satiety levels. Between trials, fish were housed in a tank subdivided into individual mesh compartments, enabling them to see, smell and, to a limited degree, touch conspecifics, thereby reducing isolation stress. Prior to the experiment, focal and stimulus fish had no contact with one another, and due to the one-way glass, stimulus fish were unable to see focal fish during the trial. The same single stimulus shoal was used for all trials. The sex of each fish was not determined due to the relatively small degree of sexual dimorphism in the zebrafish, with mixed-sex shoals being the norm. Two tanks were tested per population, with a total of four populations (160 trials in total).

Shoaling tendency intra-population experiment

The within-population experiment used an identical experimental protocol, with the only differences arising from trials being repeated three times rather than twice in order to obtain a more precise measure of individual behaviour. In total 522 trials were performed (ten fish being tested per tank where possible, with a total of 18 tanks and three trials per fish).

Data analysis

A general linear model was used to partition variation in each of these data sets: between-population and within-population shoaling data and between-population boldness data. 'Fish', 'tank' and 'population' (or 'family') were treated as nested random factors and 'trial' as a fixed effect to account for consistent differences between first and subsequent exposures. Due to the non-normal and heteroscedastic nature of the data for boldness (Kolmogorov-Smirnov test P=0.0001, Levene's test P=0.0001), which could not be improved by transformations, bootstrap resampling was used to verify the significance of variance ratio statistics). Shoaling data met the assumptions for parametric analysis (after removal of outliers in the within-population case). The GLMs were performed using SPSS v10.1 and the bootstrap in Genstat v5.3.

Results

Inter-population boldness

Bootstrap analysis revealed significant trial (P=0.006, 1,000 replicates, $F=5.319_{2,158}$), population ($P<0.001$, 1,000 replicates, $F=51.560_{3,3.812}$) and pop(tank(fish)) effect ($P<0.001$, 1,000 replicates, $F=5.819_{72,158}$) (see Fig. 1). The tank effect was non-significant ($P>0.6$, 10,000 replicates, $F=0.130_{4,72}$). The Spearman correlation between boldness score and size was also non-significant ($P>0.9$, $r=-0.005$, n=201). The partitioning of variance was as follows: var(pop)=2,662, var(pop(tank))=−712, var(pop(tank(fish)))=6,699, var(error)=4,170.

Inter-population shoaling experiment

For this analysis the overall model was constructed as 'trial', 'population', 'population(tank)', 'population(tank(fish))'. The effect of body length was tested using a standard Pearson's correlation between time spent shoaling and body size but was found to be non-significant (P=0.253, $r=-0.101$, n=129). The GLM showed no significant effect for trial ($F_{1,77}$=1.297, P=0.258), population ($F_{3,3.992}$=2.33, P=0.216) or population(tank) ($F_{4,72.511}$=1.467, P=0.221). Significant variation among fish was found ($F_{72,77}$=2.735, P=0.0001), indicating that individual fish were different from each other. Removal of either the tank or population term rendered the other significant (removal of population,

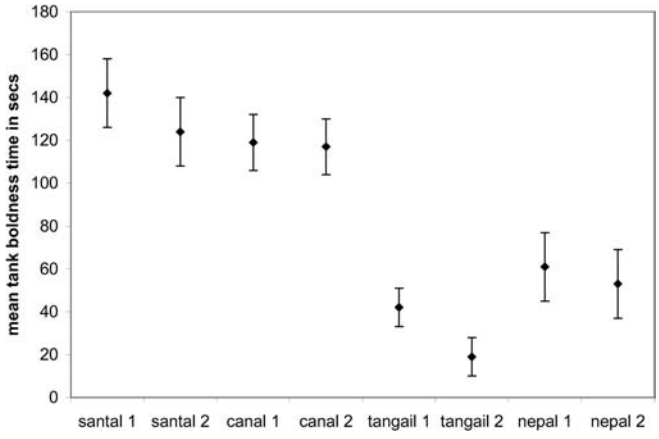

Fig. 1 Inter-population boldness experiment. Mean time spent exploring novel object by fish in each of the four populations (10 fish per tank, mean of 2 trials per fish), subdivided into tank 1 and tank 2 [time in seconds of a possible 600 s maximum (± standard error) spent within 40 mm of a novel object]

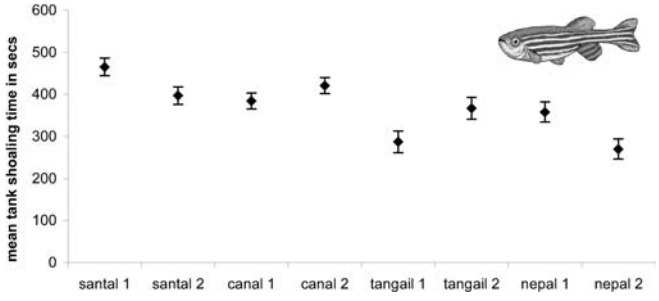

Fig. 2 Inter-population shoaling experiment. Mean shoaling time of populations (10 fish per tank, mean of 2 trials per fish), subdivided into tank 1 and tank 2 [time in seconds of a possible 600 s maximum (± standard error) spent within 40 mm of the stimulus shoal compartment]

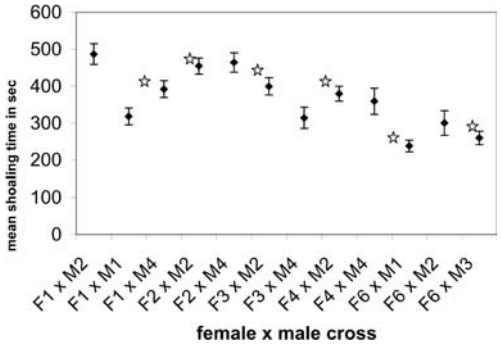

Fig. 3 Intra-population shoaling experiment. Mean shoaling time of families [time in seconds of a possible 600 s maximum (± standard error) spent within 40 mm of the stimulus shoal compartment, mean of 10 fish, each an average of 3 trials)]. *Asterisks* indicate families reared over two tanks

tank significance, $F_{7,72.536}$=2.628, P=0.045; removal of tank, population significance $F_{3,76.605}$=3.067, P=0.033) (see Fig. 2).

Intra-population shoaling experiment

Normality tests for this data set indicated 31 extreme residual values (12 high, 19 low). After removal of these observations the distribution of residuals did not deviate significantly from normal (Kolmogorov-Smirnov test, P>0.20). The variance of individual fish mean shoaling tendency was consistent across tanks and between families (Levene's test, P>0.05). All analyses were carried out with both the full and trimmed data sets. Since there was no qualitative difference between the two sets of results, only test results for the full data set are given below. Shoaling tendency varied significantly among trials ($F_{2,340}$ = 3.76, P=0.011), families ($F_{11,5.97}$=4.75, P=0.035) and individual fish within families and tanks ($F_{155,340}$=3.90, P<0.001) (see Fig. 3), whilst the family(tank) effect was found to be non-significant ($F_{6,154.69}$=1.22, P=0.301). When body length was included as a covariate it did not improve the fit of the model ($F_{1,387}$=0.081, P>0.5).

A restricted maximum likelihood method was used to estimate the variance components needed to calculate heritability. The family term was replaced by male parent, female parent and male/female interaction terms. No significant variation was detected among tanks, within families ($-2\Delta L$=0.018), and the male-female interaction component was estimated to be zero. Therefore, these terms were excluded from the model. Female and male terms each significantly improved the fit of the model ($-2\Delta L$=−94.936, P<0.0001 and $-2\Delta L$=−4.342, P<0.05, respectively). Variance component estimates were: female 3,073±2,597, male 1,692±1,904, fish 12,248±1,839, and error variance: 12,489±958 s^2 (estimate ± standard error).

Discussion

The inter-population experiments show that the four populations differ in boldness in the laboratory-reared F_1 offspring. The intra-population shoaling experiment demonstrates a genetic component to shoaling behaviour and provides a preliminary estimate of its heritability.

The inter-population boldness experiment reliably shows that the four populations have genetically based differences in boldness (with the split-tank design controlling for unforeseen variation in rearing conditions) although experience has also been shown to influence this trait (Magurran 1990). Trial number was also significant, with fish growing bolder as the experiment progressed (trial 1: 67.09±10.22, trial 2: 88.80±13.12, trial 3: 99.81±13.53).

No significant differences were found between the four populations for shoaling tendency. Similar proportions of variance were explained at the population and tank(population) levels, emphasising the importance of controlling for subtle effects of variation in rearing conditions and suggesting that a larger experiment might reveal significant effects at both levels. Although the above gives no evidence for genetically based differences in shoaling between populations, the within-population study indicates a genetic component to this behaviour and also enables us to estimate the heritability of this trait. Both broad- and narrow-sense heritability can be estimated using the variances obtained from the experimental design. Using the sire effect, narrow-sense heritability for individual trial shoaling tendency is 0.23±0.25 (0.18±0.22 with the trimmed data, estimate ± standard error). The high standard errors for these estimates (and for the variance component estimates) reflect the small number of fish in the parental generation. The repeatability of shoaling tendency measurements across trials provides an upper limit to the heritability of the trait (Lynch and Walsh 1998). Here the repeatability is 0.58±0.06 (trimmed data 0.75±0.04).

The heritability of 0.23 calculated above refers to the proportion of variation among individual shoaling trials that is due to genetic differences among individuals. However, individuals make many shoaling decisions in their lifetimes. Fitness is most likely to be influenced by their average tendency to join or stay with a shoal and it is likely to be this average tendency that is affected by an individual's genetic makeup. Therefore, the heritability of mean shoaling tendency might be more informative than the heritability of individual decisions. In this case, the within-fish variance in the analysis represents the variability of each fish between trials. By excluding the within-fish variance from the estimate of total phenotypic variance the heritability of mean shoaling tendency can be calculated, giving a value of 0.40±0.41.

Note that systematic effects of trial order have been excluded in both calculations. Trial had a strongly significant effect on shoaling tendency: means for trial 1: (383±13.68 s), trial 2: (355±15.57 s), trial 3: (353±12.72 s) (mean ± SE). It would appear that after the initial trial a degree of habituation occurred, removing the stress of an entirely novel environment. This novel environment apparently had the effect of increasing the shoaling tendency but by the second and third trials this effect had passed. As all fish experienced the stress of a novel environment in the same form, inter-familial differences should be unaffected.

The lack of any significant male–female interaction suggests that dominance and other non-additive genetics effects are negligible. Thus the narrow-sense heritability and broad-sense heritability are equivalent. Maternal effects can be estimated from the difference between male and female variance components. In the case of the zebrafish, these may represent female egg provisioning or mitochondrial genetic input (Lynch and Walsh 1998). Although this study shows that female variance is almost twice that of males, the standard errors greatly overlap, and the relative lack of post-natal female care in this fish leads one to expect relatively weak maternal effects.

In general therefore the analysis indicates heritability with a large additive variance and with no apparent dominance effects. However it should be noted that the standard error of the estimate is as great as the estimate itself, so although the significant family effect provides evidence for a definite genetic element the bounds of the estimate are broad and a larger study is clearly needed. This is by no means uncommon with heritability estimates (Roff and Mousseau 1987).

In conclusion, this study rigorously demonstrates a genetic component to boldness-related novel-object inspection in zebrafish as well as providing a heritability estimate for shoaling tendency. The zebrafish is already an important model organism in developmental biology. This work shows that it can also be valuable in the genetic analysis of behaviour where the many tools available will allow traits to be dissected much more finely than in other species.

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