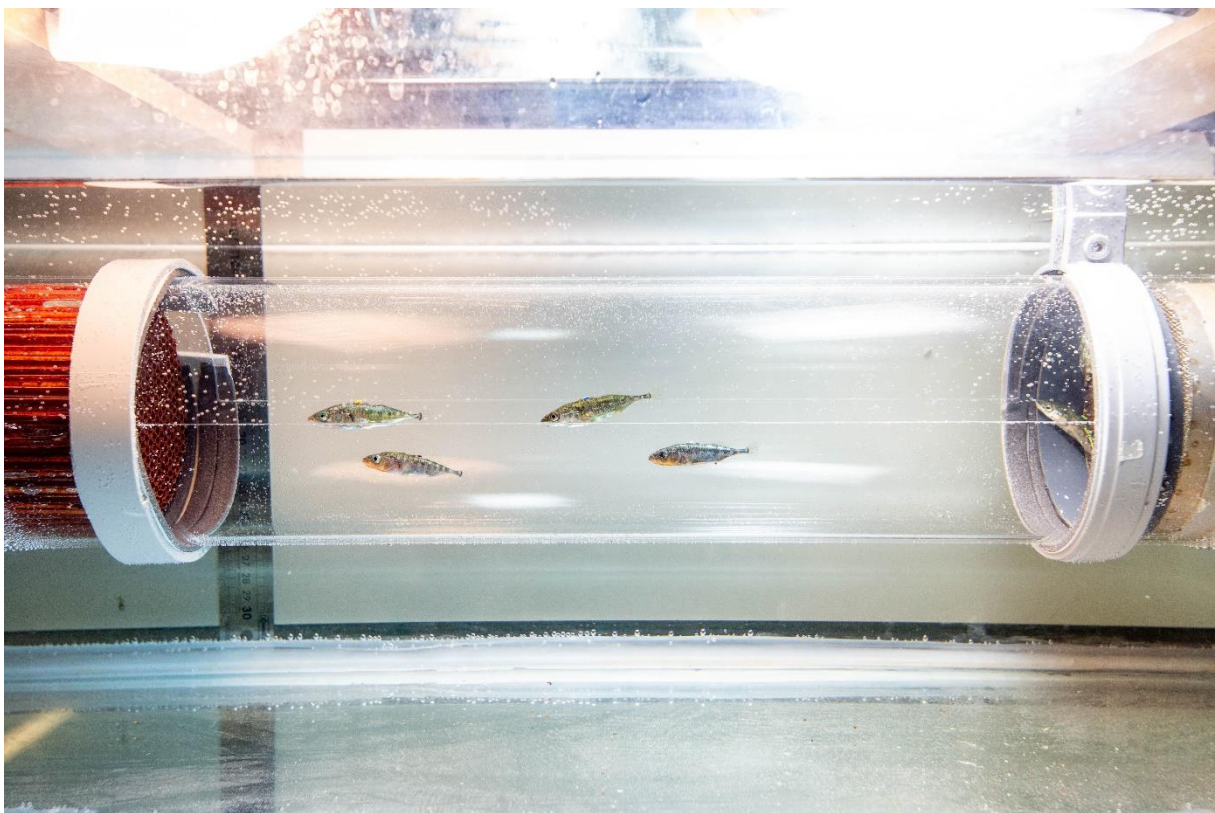# Origin-dependent schooling behaviour in three-spined sticklebacks (*Gasterosteus aculeatus*).

Do lab-bred marine, freshwater and hybrid populations differ consistently in their schooling behaviour?

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Abstract

Water management in the Netherlands can be detrimental to anadromous fish species. Human-induced barriers built on migration routes of fish have split off migratory populations. Land-locked populations lose the ability to migrate, while marine populations may need to find new migration routes. Previously, we have shown that land-locked populations exhibit a significantly different behavioural pattern compared to their migratory counterparts. However, in lab-bred crosses of pure land-locked and migratory fish and a hybrid of the two populations a majority of the behaviour patterns did not show significant differences anymore. An exception on this was the shoaling tendency of individual fish, indicating a genetic basis for this specific behaviour. In this research, we expand on previous results by comparing the schooling behaviour of the three lab-bred populations. This was done by introducing groups of six fish, all from one population, to a flow chamber. By recording their swimming behaviour, we extracted for each population the distance to the centre of the school, the interindividual distance and the orientation. With these measures, we provided new insights into the mechanisms underlying population divergence in schooling behaviour. Our main finding is the significant difference in orientation between the migrants and residents, indicating genetic population differentiation in this aspect of their schooling behaviour. For the other aspects of schooling, the populations showed no significant differences, pointing towards no genetic basis for distance to the centre of the school and interindividual distance. Alternatively, these behaviours may be genetically constrained and not able to evolve in the timeframe of isolation. The interindividual distance of fish swimming relatively close together in a school exhibited high individual repeatability, suggesting that such social behaviour may be heritable. The interindividual distance of fish swimming relatively far apart in a school exhibited high repeatability in different trials, suggesting that school composition is important for the density of a school.

Introduction

Fish migration and schooling behaviour

Migratory behaviour, defined as movements of individuals or populations (or parts of populations) between two well-defined habitats on a temporally predictable basis (Brönmark et al., 2014), is a widespread phenomenon in the animal kingdom (Dingle & Alistair Drake, 2007). For example, many bird species migrate half the world to track the availability of food (Greenberg, 1986). Winters in temperate climates offer very little food compared to other seasons, with some food niches, like fruit and insects, almost completely disappearing. When food availability decreases, competition for the scarce resources becomes very high. Therefore organisms may maximize their performance by adaptively migrating to more suitable wintering areas where the fitness benefits associated with better local conditions will outweigh the costs of the long and energy-costly journeys to the destinations (Dingle & Alistair Drake, 2007). During spring, food becomes more abundant in temperate climates again and the birds will return to occupy their original niche (Greenberg, 1986). The relatively low competition for the rapidly emerging high amount of food in this area is the reason for their return. It gives opportunity to fulfil their own needs, but also to raise offspring as raising offspring requires a large food and energy investment. Through migration bird species can avoid a costly winter and return in time for the food peak that is essential for reproduction. Tracking suitable habitats for migratory birds is therefore key for their survival and reproduction.

With the ability to fly, migration is possible in birds. Flying means fewer obstacles than traveling over land and it is a relatively energy efficient mode of transport. Swimming has similar properties, with a constant medium to travel through and the energy needed per unit of distance is relatively low compared to other modes of transport. This is why migration is also possible in fish and similar benefits are gained (Jensen, Finstad, & Fiske, 2019). Many fish species follow sea currents and food availability for spawning, feeding or reaching nursery grounds, thereby maximizing their fitness (Brönmark et al., 2014). However, there are still significant costs to the migratory behaviour (Jensen et al., 2019). The fish need to swim long distances and against currents, which requires a large energy investment. Also, moving from point A to B with a large population can attract predators on this route. The chokepoints in estuaries can funnel migrating fish during a short period, which in turn can increases the attraction of predators in these areas (Roby, Lyons, Craig, Collis, & Visser, 2003). Swimming in a school reduces both the energy cost because of its hydrodynamic efficiency (Hemelrijk, Reid, Hildenbrandt, & Padding, 2015; Johansen, Vaknin, Steffensen, & Domenici, 2010) and predation risk because of safety in numbers, its confusion effects and higher predator detection rates (Magurran, 1990; Morgan & Godin, 1985). Schooling behaviour is defined as a combination of behaviours. Fish must gather socially (i.e. shoaling), but also precisely synchronize their position, speed, and angle of motion with the behaviour of their neighbours to form a school (Wark, Greenwood, Taylor, Yoshida, & Peichel, 2011). Because costs of migration are mitigated by schooling, it is highly prevalent in anadromous fish. Anadromous fish will live most of their lives in the open ocean, except during the breeding season. They typically travel back in schools to freshwater rivers in order to reach their spawning grounds, being relatively safe with enough food to successfully spawn (Jensen et al., 2019).

Fish migration and habitat fragmentation

Habitat fragmentation means that discontinuities appear in a habitat which can be detrimental to a species if the habitat becomes too small or if specific important areas are split off (Tuomainen & Candolin, 2011). The latter can happen to anadromous fish when a barrier appears between rivers and open sea. The barrier blocks the migration between the sea and spawning grounds, therefore interfering with the lifecycle and reproduction chances (van Puijenbroek, Buijse, Kraak, & Verdonschot, 2019).

The Dutch coast has many rivers ending in the North Sea or Wadden Sea, which creates suitable breeding environments for anadromous fish species. However, because of water management, barriers like pumping stations or sluices are prevalent in the Netherlands and represent uncrossable barriers between sea and rivers. As a result, many fish populations have been permanently split up in a landlocked group without a possibility for migration and a group in sea that had to search for a different migration route (de Groot, 2002). Our study system, the Three-spined stickleback (*Gasterosteus aculeatus*), has been split into free migrating and several land-locked populations for at least 50 years. Because of the isolation and difference in lifestyle of the two groups, an adaptation to their respective environment is expected (Ramesh, Domingues, et al., 2021; Ramesh, Groothuis, Weissing, & Nicolaus, 2021). Three-spined sticklebacks are widely used as subjects to scientific study, because of their large variation in morphology and great adaptability (M. A. Bell & Foster, 1994), which showed to be advantageous for this research as well.

Previous research has shown behavioural and morphological differences between landlocked and migratory wild populations of Three-spined sticklebacks after about 50 generations of separation (Ramesh, Groothuis, et al., 2021). After conducting several behavioural trials and morphological measures, this study concluded that impassable barriers in migration routes have major consequences for the phenotype and potentially life-histories and population dynamics. A follow-up study focussed on the question if there is genetic basis for the behavioural and morphological differences observed in wild migratory and land-locked populations (Ramesh, Domingues, et al., 2021). To reach that aim, F1 populations of pure and crosses of the migratory and landlocked populations were raised in the lab and exposed to similar behavioural assays. This study found that lab-raised resident sticklebacks exhibited lower shoaling and migratory tendencies as compared to lab-raised migrants, while other traits showed no significant differences. Therefore, the conclusion was that population differences for these traits were underpinned by genetic differentiation. However, whether the populations are genetically differentiated for schooling behaviour is still unknown, as the trials only investigated shoaling and migration and not the schooling behaviour as described above. The current study aims to fill this gap.

Migration/dispersal and Animal personality

It is well established that individuals within populations of animals differ consistently in their behaviour across time and contexts (Webster & Ward, 2011). This can be attributed to animal personality. Personality variation in individuals is found to be repeatable, heritable and often covary with other personalities on population or species level to form a behavioural syndrome (A. M. Bell, Hankison, & Laskowski, 2009; Sih, Bell, Johnson, & Ziemba, 2004). Individual dispersal tendencies appear to covary with personality traits in animals (Magnhagen, 2012). For example, typically highly dispersive individuals in fish behave less social and more aggressive than less dispersive individuals, with both of these personality traits contributing to increased dispersion levels. Variation in dispersal tendencies and level of sociability are expected to relate to schooling tendencies, because fish must gather socially to form a school as described above.

Research question and hypotheses

The goal of this thesis is to quantify consistent differences in schooling behaviour in three different F1 lab-raised populations of three-spined sticklebacks: migratory, hybrid and land-locked populations.

We expect populations to maintain different levels of schooling behaviour if this behaviour has a genetic basis. Specifically, we expect landlocked populations ('residents') to have lost its ability to migrate and therefore exhibit lower social behaviour tendencies ('schooling behaviour') compared to the migratory population ('migrants'). Because the hybrid population ('hybrids') are a genetic cross

between the residents and migrants, we expect an intermediate result (compared to the residents and migrants) in their schooling behaviour. In contrast, if schooling behaviour is purely driven by the environment in which the fish grew up, we expect no difference between the populations due to them being raised in the same environment.

Variation in schooling behaviour will be quantified through three commonly used variables: 1) Distance to the centre of the school, 2) interindividual distance and 3) orientation (Masuda & Tsukamoto, 1998; Wark et al., 2011): 1) The distance to the centre of the school is the distance at which each fish is swimming from the geometrical centre of a school (see fig. 6 and 7). It is a measure for the density of a school, where low distances result in a high school density. A higher density is an indication of social behaviour and schooling. Therefore, we expect the migrants to have the lowest average distance to the centre, followed by the hybrids and the resident to have the highest. 2) The interindividual distance is the distance at which each fish is swimming from each other fish in a school (see fig. 8). This is also a measure for the density of a school, where low distances also indicate a high density, but this measure focusses on the individual level instead of the group level. Fish could show preferences or aversions for certain other fish due to for example familiarity or swimming motions. It shows if certain fish consistently swim relatively close to or far away from other fish. In the same reasoning as the distance to the centre, we expect the migrants to have the lowest average interindividual distance to each other fish in the school, followed by the hybrids and the resident to have the highest. 3) The orientation is the angle of motion at which a fish is swimming relative to the average angle of motion of the school, or (and henceforth mentioned as) alignment to the school (see fig. 9). A high alignment to the school is beneficial for the energy efficiency of each fish and is therefore an indication of schooling behaviour. We expect the migrants to exhibit the highest alignment followed by the hybrids and the residents to have the lowest.

Material and methods

Wild populations

For this study, the catching, breeding and raising of the sticklebacks were done in a previous study (Ramesh, Domingues, et al., 2021). Sticklebacks of both origins ('migrants' and 'residents') were caught over a period of four weeks between March and April in 2019 in the Eems Dollard estuary and along the Westerwoldse Aa River in the province of Groningen, the Netherlands. The inland water system, apart from the main river and canals are comprised of polder systems, which are land-locked water networks, usually in the form of ditches (<1m deep in most cases) that are all connected to the main canal with or without pumping stations. Incoming migratory sticklebacks were caught at two sea locks ("TER" (53018'7.24'', 702'17.11'')) and "NSTZ" (53013'54.49'', 7012'30.99'')) whereas resident sticklebacks were caught in two land-locked polders ("LL-A" (53017'56.14'', 702'1.28'') and "LL-B" (53017'16.52'', 702'26.46'')). Lift-, hand- and fyke nets were used to prevent sampling biases by personality in land-locked populations whereas only lift nets were sufficient for migrants. All individuals were transported to the laboratory within 2 hours of capture in aerated bags. Individuals were housed in groups of 5 in 5 litre aerated tanks filled with freshwater. They were housed outdoors, exposed to the natural day-light cycles and temperatures. They were fed brine shrimps and blood worms (3F Frozen Fish Food bv.) ad libitum every day. Males were separated once they reached breeding colours and females were checked daily for the presence of gravid females.

Lab-bred populations

Lab-bred F1 juveniles of resident, migrant and hybrid sticklebacks arose from partial-full sib crosses (see fig. 1). Each family of crosses consisted of all combination of crosses between a male and female migrant and male and female resident, leading to F1 juveniles of pure migrant (MM) or resident (RR) background and hybrids with migrant father and resident mother (MR) and vice versa (RM). The previous study (Ramesh, Domingues, et al., 2021) showed no significant differences in social behaviour between the MR and RM hybrid crosses, therefore we pooled them together into one hybrid group during the experiment and made no distinction in the results.
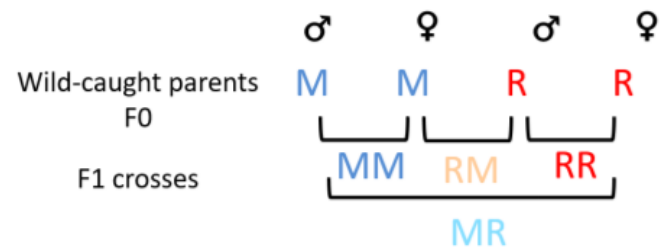

Figure 1: Partial-full sib crosses of the wild-caught parents to produce the lab-bred F1 juveniles (Ramesh, Domingues, et al., 2021).

The juveniles resulted from a common-garden experiment by artificially insemination, without parental care. Females which were gravid were pressed gently in anterior to posterior direction on their stomach to induce release of eggs. If this was unsuccessful, the female was placed back in its "home tank". Females were measured before and after release of eggs. In addition, egg masses were measured and split into two equal halves for insemination with sperms extracted from a migrant and resident father. The eggs were placed in Hank's solution in a petri dish. The male was then selected and euthanised with an overdose of buffered solution of MS-222 (0.5 mg/L) for 10 minutes. The males were then removed and cleaned in freshwater to remove residue of MS-222. The male was then dissected along the lower abdomen, starting from anus suing a scalpel and the testes were removed. The testes were placed in a mortar filled with a few drops of Hank's solution and crushed using a pestle until it turned milky. Using a pipette, equal amounts were added to the petri dish containing eggs of same or different origin females. The petri dish was gently swirled, making sure that the eggs did not form clumps but a layer of single eggs and incubated for 20 minutes. The fertilized eggs were then placed in clean aerated tanks filled with freshwater, in stand-alone tanks to prevent potential infection.

The fish larvae hatched 5-7 days after fertilization and started maintaining buoyance and independent feeding after a week of hatching. The fish larvae were fed a mixture of frozen cyclops, freshly hatched Artemia nauplii and zebrafish diet (GEMMA Micro 75, Skretting, Tooele, Utah) daily. The densities never exceeded 40 fish larvae in 5 litre home tanks (30 x 16 x 18 cm (L x W x H)). Once fish reached ~2 cm, they were isolated randomly, assigning 10 individuals from the same family into separate tanks. After this, the individuals were fed ad libitum with brine shrimps and blood worms (3F Frozen Fish Food bv.) and tanks were connected to the same water system at 16.0 °C. The photoperiod was set at 16:8 (L:D), mimicking summer conditions during juvenile growth. When the fish reached a length of ~4 cm, they received a unique identification (see below). Autumn conditions were induced when the fish were ~12-13 months old, characterized by 12:12 (L:D) photoperiod and temperatures being lowered to ~15.0 °C. This is the autumn condition, which kept all fish in non-breeding state. This remained the same during the period of experimentation, which was performed when fish were ~15-16 months old.

Individual identification

When the juveniles reached 4 cm length (~12 months), clipped spines or injection of an 8 mm Passive Integrated Transponder (PIT tag; Trovan, Ltd., Santa Barbara, California) were used for unique individual identification. Since there was a limited sample size of juveniles, PIT tag injection was used for only half of the fish to be tested (20 fish X 4 groups = 80 fish) and the rest were tagged using spine clipping of a combination of dorsal and pelvic spines (20 fish X 4 groups = 80 fish). PIT tags were injected in the abdominal cavity and under anaesthesia following the standard protocol (following Cousin et al., 2012). During tagging/clipping, weight and standard length (the length from the tip of the snout to the base of the tail) were also measured as proxy for size. After individual tagging, juveniles from different families were mixed to be housed together in group size of 10 in their new home tanks while keeping them together with the same background (MM, RR, MR or RM).

Schooling assays

To quantify variation in schooling behaviour of the migrants, residents and hybrids, the fish were put in a flow-chamber (see Fig. 2). The flow-chamber setup consists of a glass rectangular aquarium with an open plexiglass tube inside. After the fish were placed inside, a lid was put on the tube and sealed off with plastic rings. This tube was connected to PVC tubes and a motor to pump the water in a loop. To induce the schooling behaviour a laminar flow was created in the tubes. This flow was kept laminar by honeycomb structures at the point of entrance for the water in the area where the fish resided. A metal grid stopped the fish from swimming further in the tube. The area where the fish could swim had a diameter of 11.9 cm and was 44.5 cm long (from honeycomb to metal grid).
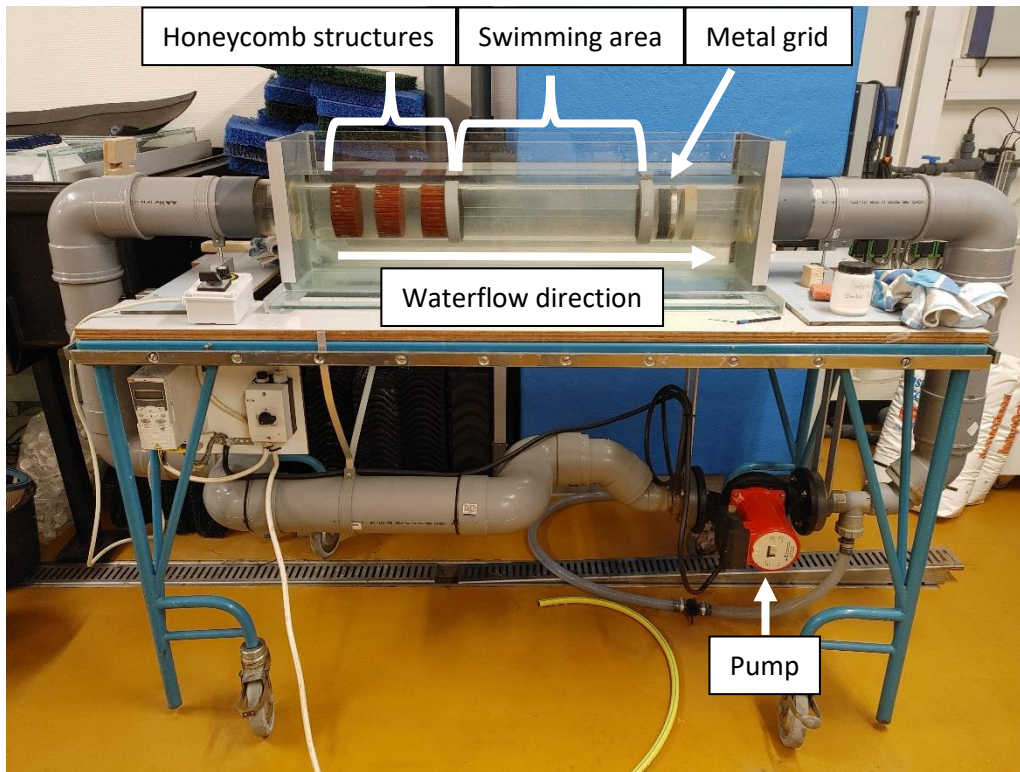
Figure 2: The flow-chamber setup.

This process was filmed from a side view and a top view via a mirror with a GoPro HERO 8 (GoPro, Inc.) to determine the position of each fish in a three-dimensional space. For each trial, the GoPro was positioned at approximately the same position at ~20 cm from the glass aquarium plus 6.8 cm to the plexiglass tube. To prevent shadows from interfere with visibility, a single light source was used to light up the area where the fish would swim and light from other directions were covered up. Blinds were placed around the setup to prevent disturbance during the trials.

A trial consisted of six randomly chosen fish from the three different origins: migrants (M), residents (R) and hybrids (H). The 6 tested fish were made of three pairs that originated from the same home tank. Each origin had one exception where a trial consisted of two pairs from the same home tank and two individuals from two different home tanks. For M and R six to ten different fish from all four home tanks were used. For H eight or nine fish were used from three home tanks of the MR cross (migrant father and resident mother). From the fourth MR home tank, three fish were used for two trials and from one home tank of the RM cross (migrant mother and resident father) two fish were used for two trials (see table 1).

Each origin was tested in 10 trials, of which one of each origin had to be redone due to a technical problem, resulting in a total of 13 trials for each origin. The 3 trials that had to be redone, were done 19 and 20 days after the last regular test day. The rest of the trials were done in a span of 12 days. In total 96 individual fish were used (32 M, 33 R and 31 H). Most fish were used twice with a minimal of 3 days between the two trials. The M, R and H fish from the redone trials were used an average of respectively 3.5, 3.7 and 3 times (see table 1).

Tank	Total times fish were picked from this tank	Total individual fish used (multiple times or once)	Amount of fish used one time	Amount of fish used two times	Amount of fish used three times	Amount of fish used four times	Amount of fish used five times
M tank 1	17	8	1	5	0	0	2
M tank 2	15	7	1	4	0	1	1
M tank 3	16	8	1	6	0	1	0
M tank 4	18	9	1	7	0	1	0
Total M	66	32	4	22	0	3	3
R tank 1	13	6	1	3	0	0	2
R tank 2	19	9	1	6	0	1	1
R tank 3	13	8	3	5	0	0	0
R tank 4	21	10	1	7	0	1	1
Total R	66	33	6	21	0	2	4
MR tank 1	20	9	0	7	2	0	0
MR tank 2	20	9	0	7	2	0	0
MR tank 3	18	8	0	6	2	0	0
MR tank 4	4	3	2	1	0	0	0
RM tank 1	4	2	0	2	0	0	0
Total H	66	31	2	23	6	0	0
Total	198	96	12	66	6	5	7

To reduce handling stress, fish were given two acclimatisation phases. First, the six focal fish were moved from their home tank to a similar tank filled with tap water mixed with water from their home tank. They were given 10 minutes of acclimatization. Then, for individual recognition, each fish was fitted with a unique coloured "tag" (electric wire insulator) on their spine (see fig. 3). After this handling, which lasted about 20 seconds per fish, the fish were introduced into the flow-chamber with a water temperature between 13.0 and 15.3 °C (similar to the temperature of their home tank). A flow speed of approximately 4,0 cm/s was started and after the handling with the tags and introduction to the flow, they had an acclimatization period of 20 minutes. Then we started the recording with the GoPro, mostly remotely and sometimes as calm as possible not remotely. Films were set at 1440p and 60 frames per second, which changed to 1080p and 30 frames per second after editing and the flow speed was raised to approximately 6.2 cm/s. After 11 minutes of filming the trial was done. The fish placed back in the mixed water tank for 20 minutes to acclimatize back to the water from their home tank and then placed back in their home tank.


Figure 3: Stickleback (right) with a visible black tag.

Data extraction

From each video, a frame was extracted each minute (7 photos per trial, 210 in total), using VLC media player 3.0.14 (VideoLAN). Because of disruption of turning on the GoPro and the change of flow speed, the first 4 frames (3 minutes) were seen as acclimatization and thus excluded. Also, the last frame was excluded because of disruption while turning the GoPro off.

By analysing a 3D situation in a 2D picture, the perception of depth was lost. Therefore, the scale of a video was corrected by measuring the length of the lid of the tube, which was 400 mm in reality. For the side view, the length of the lid was measured in pixels in the front and in the back of the picture. For the top view this was done by measuring the top of the lid and a similar length on the bottom (see fig. 4). For both views, these measurements were averaged and divided by 400 mm resulting in the scale for the video.
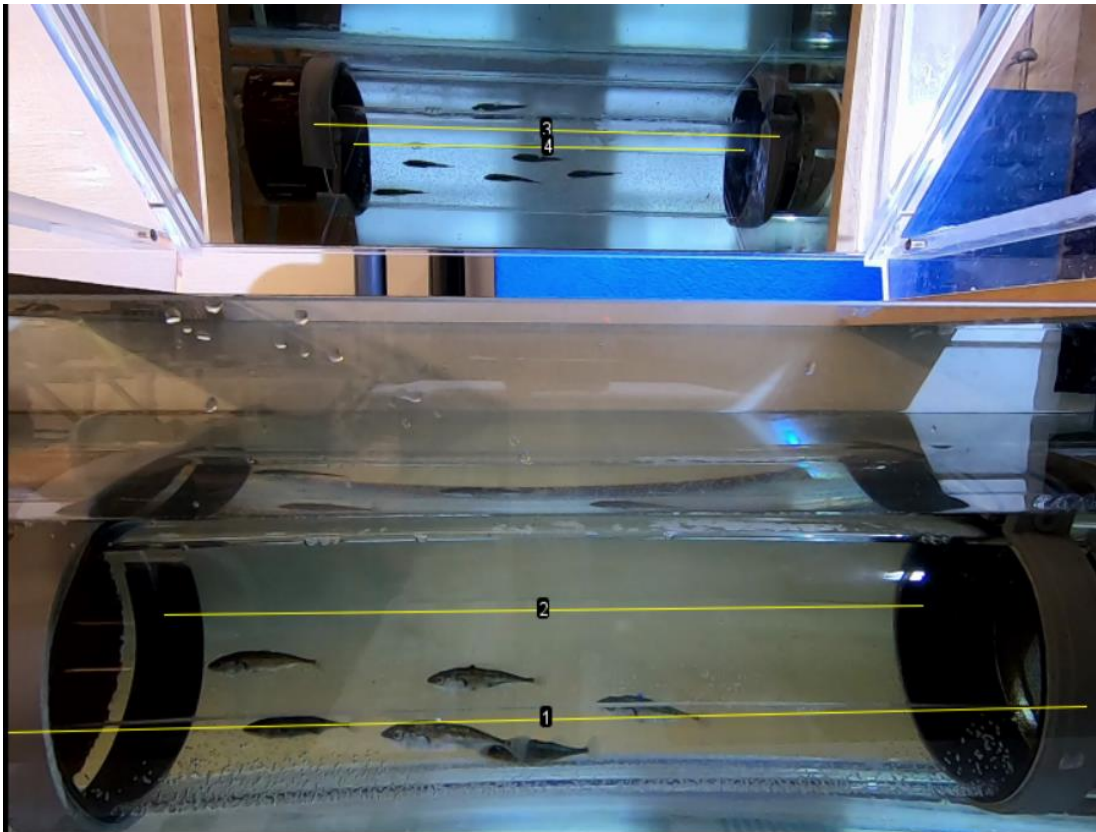
Figure 4: Measurement of the length in pixels of the lid: front, back, top and bottom in side and top view.

To find the three-dimensional position of a fish, a fish was identified by its colour tag. X and Y coordinates were found by pinpointing the middle of the standard length of the fish in the side view. The Z coordinate was found again by pinpointing the middle of the standard length of the corresponding fish in the top view, but only extracting the Y value of this point (see fig. 5). All six positions in all seven frames per video got extracted in this manner.
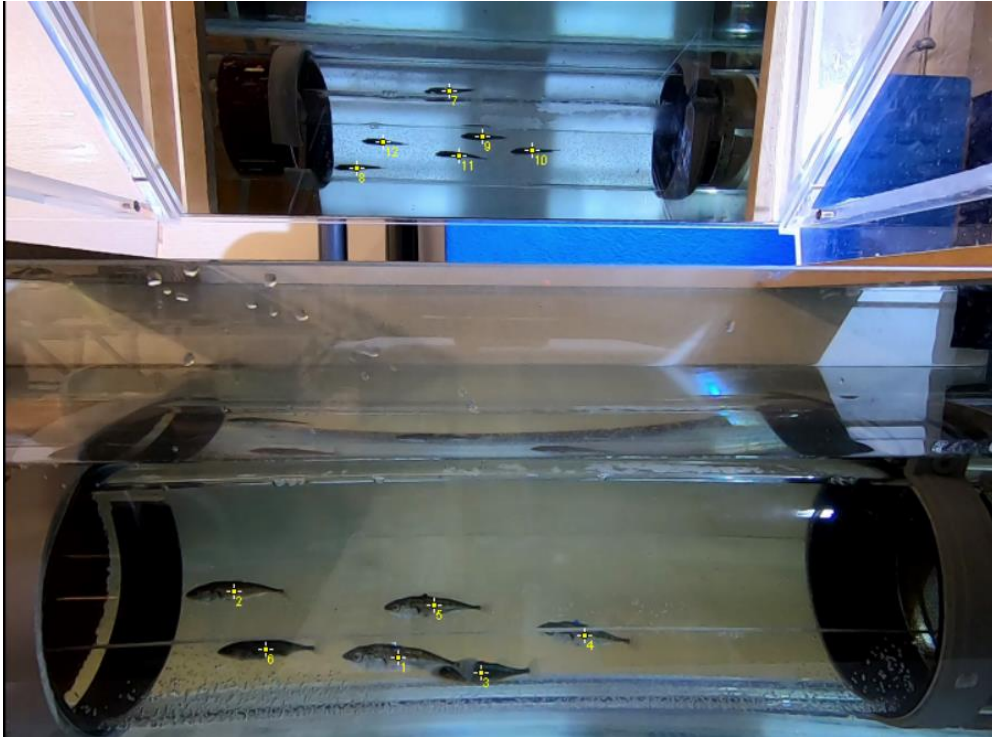
Figure 5: Points on half of SL to find the three-dimensional position of fish.

With the extracted coordinates the distance to the centre of the school ('distance to centre') was determined. For this measurement the central point of the group was first found by averaging the highest and lowest values for X, Y and Z of the group (see fig. 6). Then, for each fish, their distance to this point was found (see fig. 7), using the following formula:

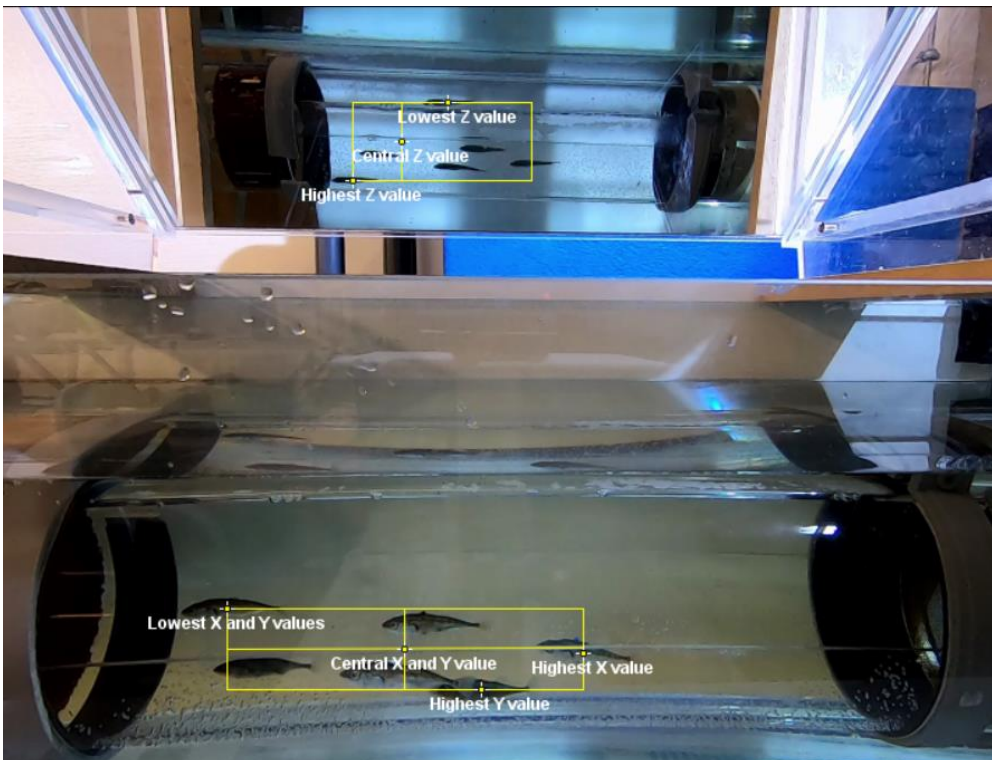((central X-focal fish X)^2+(central Y-focal fish Y)^2+(central Z-focal fish Z)^2)^0.5

Figure 6: Determination of the central point of the school in three dimensions.
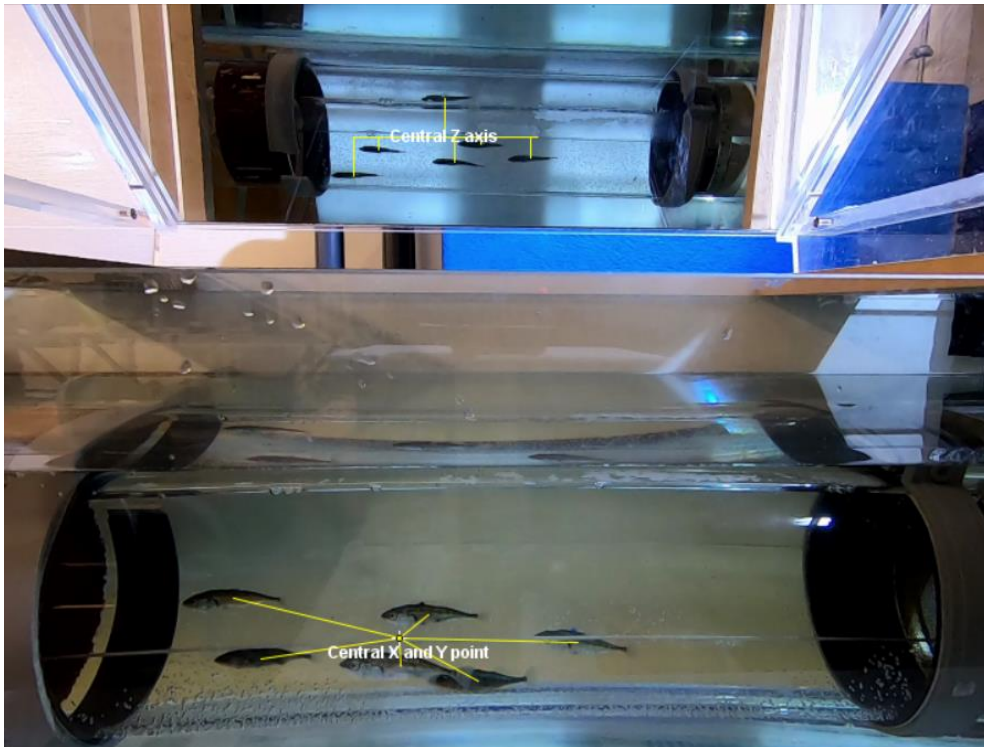
Figure 7: Distance per fish to the three-dimensional central point.

To find the interindividual distances ('nearest to fifth nearest neighbour') a similar method was used. For each fish, the distance to all other fish was was found, using the following formula:

((any other fish X-focal fish X)^2+(any other fish Y-focal fish Y)^2+(any other fish Z-focal fish Z)^2)^0.5 With all interindividual distances, for each focal fish, the other fish were sorted from smallest to largest distance (see fig. 8) to find the nearest to fifth nearest neighbour.
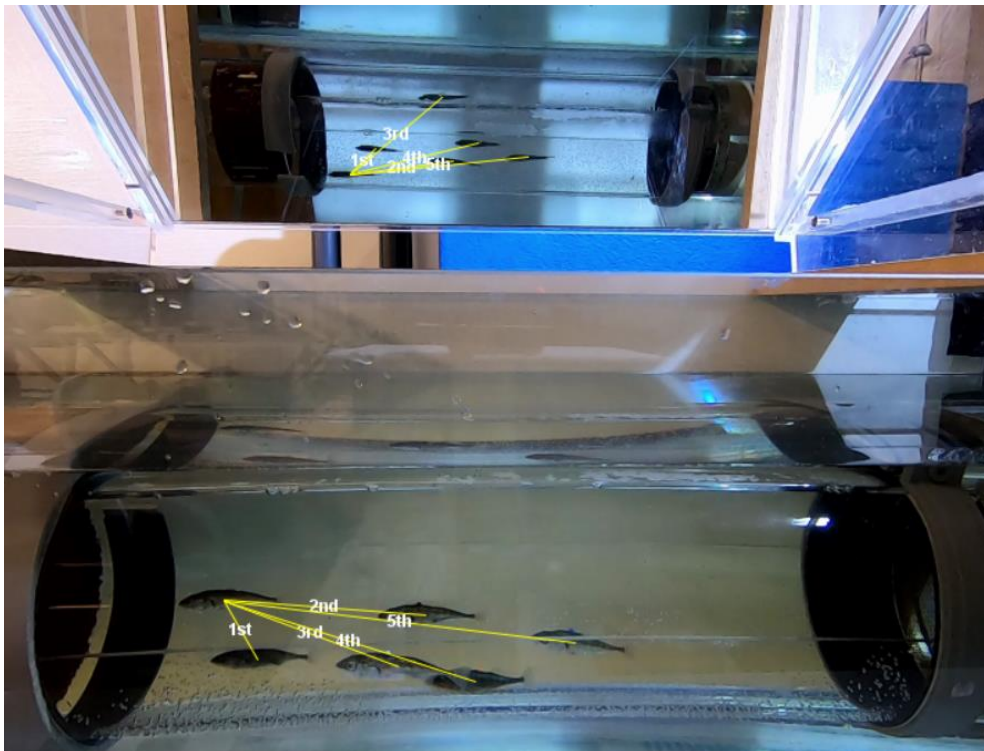

Figure 8: Distance of each fish to a focal fish. These measurements were repeated for each fish.

The orientation of a fish was found by determining its angle, relative to the average angle of the school, in the top view. First, the angle of the tube in the top view was extracted as a baseline angle, which was subtracted from all fish angles. The fish angles were determined by drawing a straight line from the tip of the snout over the middlepoint of the standard length and extracting the angle of this line. The posterior half is excluded due to its movement while swimming (see fig. 9). After all individual fish angles in a frame were extracted, they were averaged and the deviation of each individual fish angle from this average was extracted to find the level of alignment to the school for each fish.
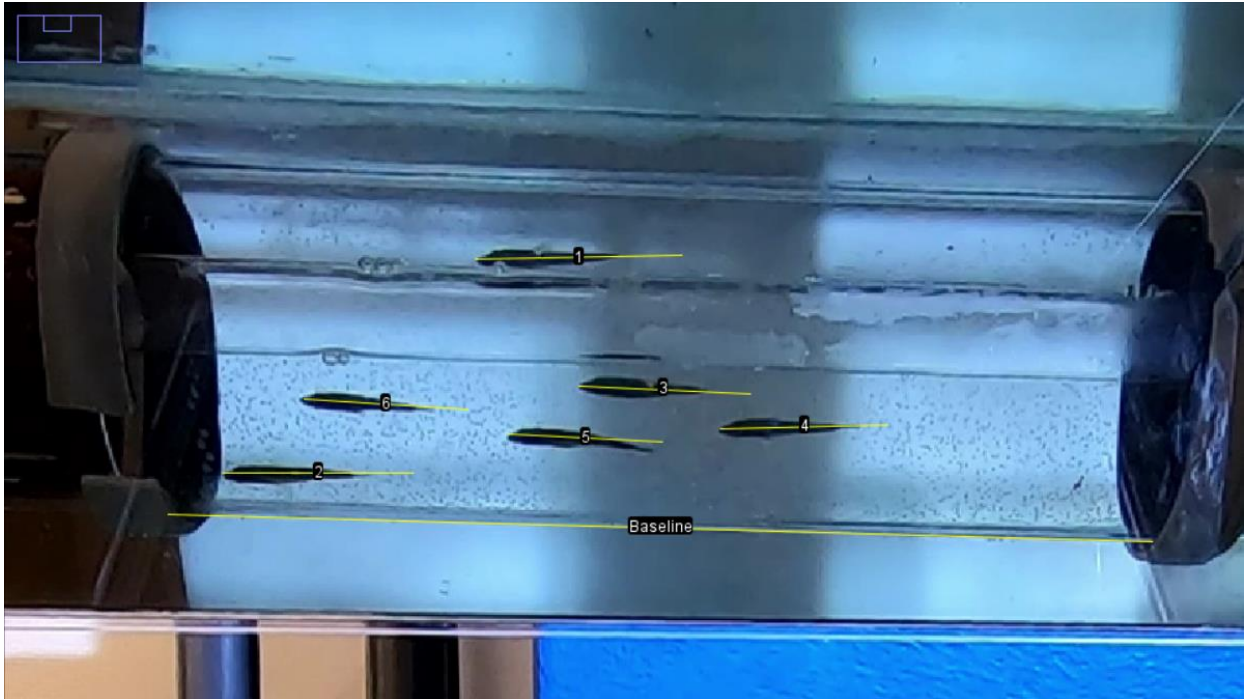

Figure 9: Zoom in of top view with a baseline and lines over the anterior half of fish to extract the alignment to the school for each fish.

Determining the scale, coordinates of the points and angles of interest in the images was done by using ImageJ (NIH and LOCI, University of Wisconsin).

Statistics

Variation in distance to centre, all interindividual distances and orientation was analysed in general linear mixed effect models (GLMM) with Gaussian errors. The fixed effects were origin of the fish (M, H and R with M used as reference category), water temperature, starting time of the trial, waterflow during the trial and the standard length of the fish (the weight of the fish was not included to prevent collinearity). The random effects were the home tank ID, fish ID and trial ID (frame ID was excluded, because it did not explain variance in any of the studied traits).

In total we used 1050 observations in 25 trials with 86 unique fish from 12 different home tanks. These numbers do not match the numbers in table 1, because we were unable to extract data from 5 trials with the H origin due to the fish hiding out of vision.

GLMMs and graphs were performed in R (version 1.4.1717) using the following packages: Matrix (Bates & Maechler, 2021), sjPlot (Lüdecke, 2021), lme4 (Bates, Maechler, Bolker & Walker, 2015), Tidyverse (Wickham et al., 2019), ggplot2 (Wickham, 2016) and ggpubr (Kassambara, 2020).

Results

Repeatability

The results show that Fish ID explained 18% to 46% of the variances in the nearest to third nearest neighbour distance (R=0.18-0.46) whereas Trial ID explained 9% to 12% of the variances in these traits (R=0.09-0.12) (table 2). 16% to 51% of the variance in distance to centre, the fourth and fifth nearest neighbour and the orientation was explained by Trial ID (R=0.16-0.51) but 0% to 3% by Fish ID (R=0.00-0.03) (table 2), implying that these traits did not exhibit consistent among-individual differences (i.e., they were not repeatable at the individual level).

Effect of origin, temperature, time, waterflow and size

The main goal of this study was to test if migrants, residents and hybrids differ in their schooling behaviour. We found that the populations differ significantly in their orientation with migrants swimming overall more aligned: they showed the highest alignment to the school with a deviation of 11.40° (0.51°-22.29°) (table 2, fig. 10B). The hybrid group showed a trend of a lower alignment to the school with a deviation of 17.58° (7.82°-27.35°) (table 2, fig. 10B) and the resident group showed an even lower alignment to the school with a deviation of 21.15° (12.59°-29.72°) (table 2, fig. 10B), which was a significantly lower alignment to the school than the migrant group and therefore a significant difference in schooling behaviour.

In most other variables, however, groups did not differ. The distance to centre and each interindividual distance showed no significant differences between the three origins (table 2, fig. 10A and 11). However, out of those two variables, in distance to centre and second to fifth nearest neighbour distance a trend is visible, where the migrants had the lowest average distance of the three origins (table 2, fig. 10A and 11B to 11E). Only in the nearest neighbour distance, the hybrids had the lowest average distance, while the migrants had the highest average (table 2, fig. 11A). In the distance to the centre and the fifth nearest neighbour distance, the residents had the highest average (table 2, fig. 10A and 11E), while the hybrids had the highest average distance to their second to fourth nearest neighbours (table 2, fig. 11B to 11D).

The other fixed effects also did not explain variation in any of the schooling measures (table 2). Noteworthy is the consistent effect (nonsignificant) of standard length on all schooling measures, indicating a trend of decreased schooling behaviour with increased fish sizes (table 2).

Table 2: Summary of linear mixed models on schooling behaviour. The effects (with migrants as intercept) of each origin, temperature, time, waterflow and size are denoted in estimates with their 95% confidence interval (CI) computed by bootstrapping method and variance components of the random effects (fish ID, trial ID home tank ID and residuals) are given with their standard deviation. Significant fixed effects are denoted in bold and with asterisk. Sample size (N) represents number of individuals. The repeatability of fish ID and trial ID is given in the fraction of the total variance it explains for each variable. Distances are given in mm and orientation is given in degrees of deviation from alignment to the school.

|	Distance to centre (N=1050)	Interindividual distance; nearest (N=1050)	Interindividual distance; 2nd nearest (N=1050)	Interindividual distance; 3rd nearest (N=1050)	Interindividual distance; 4th nearest (N=1050)	Interindividual distance; 5th nearest (N=1050)	Orientation (N=1050)
Fixed effects	*Estimates (CI=95%)*	*Estimates (CI=95%)*	*Estimates (CI=95%)*	*Estimates (CI=95%)*	*Estimates (CI=95%)*	*Estimates (CI=95%)*	*Estimates (CI=95%)*
Intercept (origin M)	70.00 (38.55 – 101.44)	54.23 (22.77 – 85.69)	72.05 (1.78 – 142.31)	98.69 (25.22 – 172.16)	124.50 (73.22 – 175.79)	171.36 (116.73 – 225.99)	11.40 (0.51 – 22.29)
Origin H	10.22 (-22.70 – 43.15)	-5.20 (-22.67 – 12.28)	14.74 (-15.58 – 45.06)	8.85 (-24.43 – 42.13)	31.64 (-30.41 – 93.68)	18.33 (-55.31 – 91.97)	6.18 (-3.58 – 15.95)
Origin R	12.99 (-15.92 – 41.91)	-0.92 (-16.46 – 14.62)	2.84 (-24.26 – 29.93)	1.31 (-28.37 – 30.98)	18.64 (-35.85 – 73.13)	24.50 (-40.08 – 89.09)	**9.75** [*] **(1.19 – 18.32)**
Water temperature	14.54 (-9.00 – 38.08)	-3.77 (-14.85 – 7.31)	-6.39 (-21.79 – 9.01)	-12.56 (-30.86 – 5.74)	13.10 (-31.36 – 57.57)	24.57 (-28.55 – 77.70)	1.74 (-5.31 – 8.78)
Starting time	0.00 (-0.12 – 0.11)	0.00 (-0.05 – 0.06)	0.00 (-0.07 – 0.08)	0.00 (-0.09 – 0.09)	0.01 (-0.22 – 0.23)	0.01 (-0.25 – 0.28)	-0.02 (-0.05 – 0.02)
Waterflow	8.64 (-265.16 – 282.45)	8.95 (-121.42 – 139.33)	66.76 (-115.42 – 248.94)	-6.65 (-222.95 – 209.64)	-20.94 (-537.72 – 495.85)	29.06 (-587.90 – 646.01)	2.09 (-79.74 – 83.91)
Standard length	0.17 (-0.34 – 0.68)	0.04 (-0.58 – 0.66)	0.19 (-1.22 – 1.61)	0.21 (-1.27 – 1.68)	0.18 (-0.54 – 0.91)	0.23 (-0.43 – 0.89)	0.04 (-0.15 – 0.24)
Random effects	*Variance (st. dev.)*	*Variance (st. dev.)*	*Variance (st. dev.)*	*Variance (st. dev.)*	*Variance (st. dev.)*	*Variance (st. dev.)*	*Variance (st. dev.)*
Residuals	1548.54 (39.35)	937.39 (30.62)	1735.80 (41.66)	2499.90 (50.00)	3653.30 (60.44)	4227.00 (65.02)	358.44 (18.93)
Fish ID	80.21 (8.96)	248.72 (15.77)	1725.50 (41.54)	1774.70 (42.13)	117.08 (10.82)	0.00 (0.00)	0.00 (0.00)
Trial ID	840.95 (29.00)	159.57 (12.63)	294.70 (17.17)	419.10 (20.47)	3062.28 (55.34)	4413.00 (66.43)	70.82 (8.42)
Home tank ID	1.73 (1.32)	5.29 (2.30)	0.00 (0.00)	0.00 (0.00)	14.03 (3.75)	0.00 (0.00)	0.00 (0.00)
Repeatability of fish ID	0.03	0.18	0.46	0.38	0.02	0.00	0.00
Repeatability of trial ID	0.34	0.12	0.08	0.09	0.45	0.51	0.16

* p<0.05
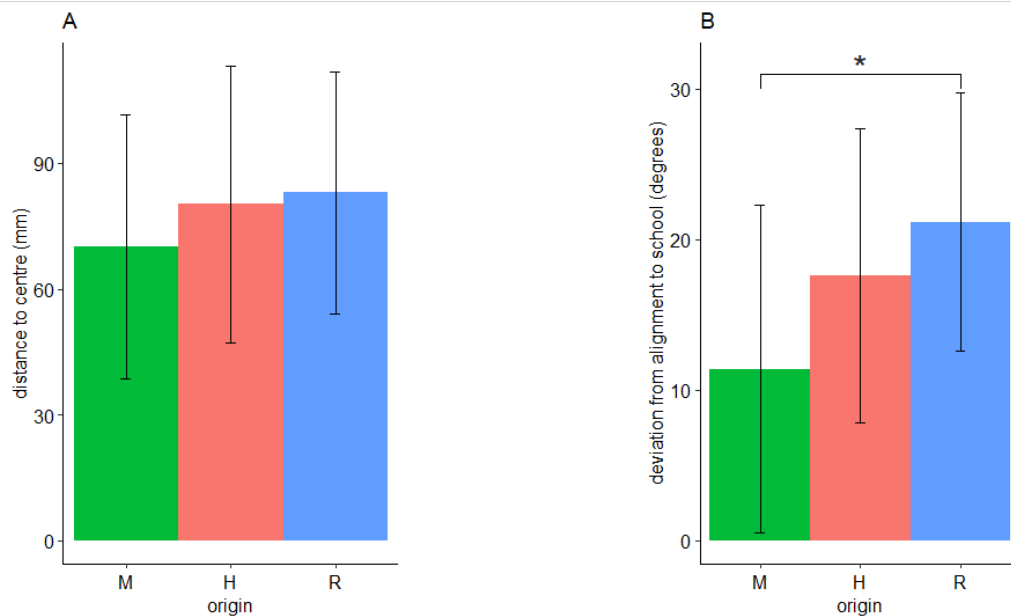
Figure 10: distance to the centre of the school (A) and the orientation in the school (B) for the three populations (M = migrants, H = hybrids, R = residents). Significant difference found between the migrants and residents. Means are shown with their 95% confidence interval.
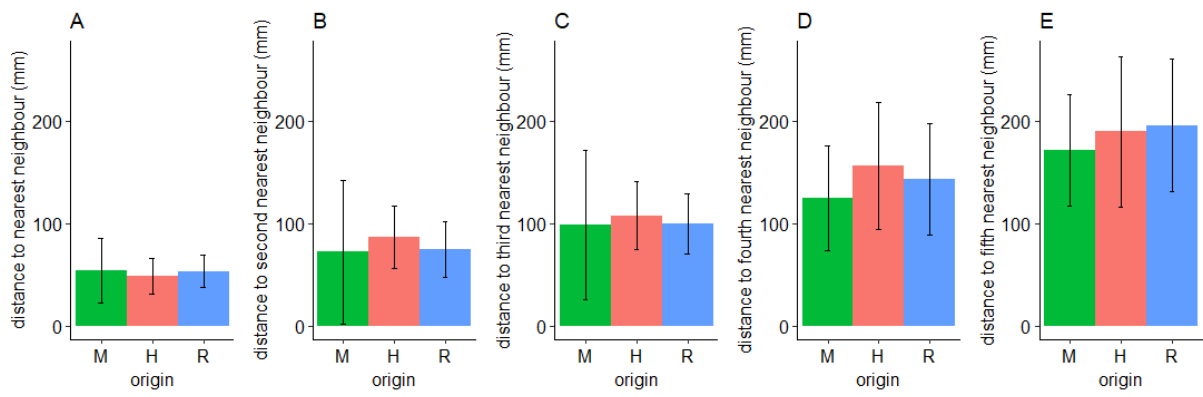
Figure 11: distance to each neighbour, from nearest to fifth nearest for the three populations (M = migrants, H = hybrids, R = residents). Means are shown with their 95% confidence interval.

Discussion

The main goal of this thesis was to quantify consistent differences in schooling behaviour in migratory, hybrid and land-locked F1 lab-raised populations of three-spined sticklebacks. This was done through distance to the centre of the school, interindividual distance and orientation in the school as measures for schooling behaviour.

Repeatability

We found repeatability between 0.18-0.46 in the interindividual distances of the first, second and third nearest neighbouring fish in the behaviour of individual fish. A meta-analysis of animal behaviour experiments found that in animal populations, repeatability of behaviour is around 0.3 (A. M. Bell et al., 2009). This means that individual consistency across contexts exists in the distances to the three nearest neighbours, i.e., in schooling behaviour. An explanation for this is the importance of sociability in schooling behaviour, where more social fish tend to swim consistently closer to other fish in a school, while more asocial fish swim consistently further away from other fish in a school (Magnhagen, 2012). Because repeatability sets the upper limit to heritability (Dochtermann, Schwab, & Sih, 2015), these results further indicate that these personality traits may have a heritable genetic basis (although populations are not (yet) genetically differentiated).

The repeatability of the fourth and fifth nearest fish for trial ID and of the distance to the centre was relatively high (0.34-0.51) for trial ID. An explanation for this is that the composition of a school is important for its overall spatial distribution. Examples of such compositions are more social or asocial fish or a large influence of key individuals with disruptive behaviour (Jolles, Laskowski, Boogert, & Manica, 2018). To investigate this importance, a new schooling assay should be conducted with a mix of the migrants and residents or test fish on sociability and mix social and asocial fish. The influence of disruptive behaviour could also be tested by excluding those key individuals and comparing the data.

Distance to centre and interindividual distance

Between the different origins, we found no significant differences in the distances to the centre of the school and to other fish in the school. This could be caused by (a combination of) three explanations:

- These behaviours may be socially learned or environmentally determined behaviour, without a genetic basis (Rodriguez-Pinto, Rieucau, Handegard, & Boswell, 2020).
- Schooling behaviour may have high fitness benefits in situations outside of migration, like anti-predator effects, resulting in higher reproductive success and therefore prevalence in a non-migrating population (Magurran, 1990).
- These behaviours may have a genetic basis, but in the wild there might not have been enough genetic variation for selection to act on it after isolation. Alternatively, the underlying genes may be constrained (i.e., not able to respond to selection) due to positioning in a highly conserved area of the DNA or linkage disequilibrium, slowing down the evolution of this part of the genome.

Orientation

Alignment was found to be the highest in migrating fish compared to hybrids and resident fish. Because the groups were raised in a similar environment and the hybrids showed an intermediate mean value, this result implies that the difference in orientation behaviour has a genetic basis. This is in line with a previous study, where three-spined sticklebacks living in different environments were found to significantly differ in their orientation while schooling. After two generations of breeding in a lab, the

weakly schooling population showed a significantly less parallel orientation than the strongly schooling population (Greenwood, Wark, Yoshida, & Peichel, 2013).

A reason for a stronger alignment in migrants could be that such uniform orientation is highly beneficial for the reduction of energetic cost of swimming (Verma, Novati, & Koumoutsakos, 2018) and thus favoured by selection. In the land-locked population selection for alignment may be weaker because fish do not need to travel long distances. Next to that, the behaviour to maintain the alignment to the school while swimming could be too costly for the land-locked population. Therefore, it would lead to a lower reproductive success, assuming no other benefits are lost by losing the behaviour.

Another explanation could be that the fish in the land-locked populations are selected for higher explorative tendencies. These populations live in isolated ditches with potentially high competition levels for access to resources and therefore each fish has to be explorative to find food, while the migratory population could rely more on social information derived from group members to forage (Sih et al., 2004).

A final explanation could be that the land-locked population exhibit higher levels of behavioural plasticity, assuming that this plasticity has a genetic basis, causing them to react more stressed to new environments or danger (Fürtbauer, Pond, Heistermann, & King, 2015). Introducing them to the flow chamber or the handling right before introduction might have caused this stress, resulting in erratic swimming manoeuvres and higher deviation from the alignment to the school. There is evidence of erratic movement as a reaction on high stress levels in fish (Kleinhappel, Pike, & Burman, 2019).

Conclusion and methodological considerations

Using a common garden experiment, we provided new insights into the mechanisms underlying population divergence in schooling behaviour. Our main finding is the significant difference in orientation between the migrants and residents while schooling. This result implies that genetic differentiation in orientation occurred between populations. For the other traits, the populations showed no significant differences, indicating no genetic basis for the behaviour.

A follow up study with mixed groups of migratory and land-locked fish in a single trial could give insight into the importance of composition in a school. It could answer the questions of which population has the largest influences on the density of a school or if there are distinct leader and follower individuals in a trial. We collected these data, but because of time constraints we were not able to analyse it.

The set up of the trials had a few methodological issues and should be considered when continuing with the rest of the data or while setting up a similar experiment. It is also important to consider while interpreting the data of this thesis:

First, the calibration could be improved. The method used to determine the scale does not take into account three dimensional space and breaking of light due to the different materials it had to travel through. It is only a correct scale for the middle the tube. The measurements closer to or farther away from the camera are prone to more error because the scale does not corrected for depth. On the sides of the image, the breaking of light made the scale inaccurate. This could have an effect on the data when certain fish or groups tend to swim in different parts of the flow chamber, such as hiding near the edges where there is less light. The distances in the image would be different than the real life situation. However, all three group were treated the same, therefore it is unlikely to have introduced a bias specific to one origin and in the worst case, the noise caused by the errors might have masked some of the effects.

Second, some fish were used multiple times for trials, which could have led to habituation. To test whether this is the case, the analyses could be run with a dataset consisting of fish tested only once (i.e. by only selecting the first trials).

Third, the schooling assay was done in a tube which was 44.5 cm long and 11.9 cm wide. In the wild schooling behaviour happens in a significantly larger body of water. Also, the watercontents and waterflow are different than the situation in the wild. Because the fish that are used are raised in the lab, they are mostly used to the watercontents and smaller bodies of water. However, if the behaviour is genetic, the differences between the experiment and the situation in the wild could be important. This can be solved by performing the experiment more alike the situation in the wild or in a different setup, such as a commonly used tubulair tank (Greenwood, Mills, Wark, Archambeault, & Peichel, 2016).

Lastly, we noticed outlier behaviour, where some fish did not swim against the flow but let themselves be pushed against the grid in the back (see fig. 12). We assume this is caused by stress or tiredness. We checked the data without fish with coordinates in these areas and did not see a significant difference. However, when looking at schooling behaviour, it should be considered that this is not schooling behaviour, because they do not swim actively and do not benefit or let other fish in the group benefit from the hydrodynamics caused by swimming.

Figure 12: Fish being pushed against the grid by the flow.

Acknowledgments

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