Supplementary Data

Supplementary Templates and Stock Videos

S1: Boldness assay

A *.zip* folder containing a collection of five predator models in *.png* and animated *.gif* format, a *.ppt* motion animation template, and five motion animated videos.

To utilize the motion animation template, open the *.ppt* file. To substitute predator models, right click on the predator model and select *Change Picture*. Save as a Windows Media Video (*.wmv*).

S2: Neophobia assay

A *.zip* folder containing a collection of 11 novel objects in *.png* format, a *.ppt* motion animation template, and 22 motion animated videos.

To utilize the motion animation template, open the *.ppt* file. To substitute novel objects, right click on the novel object and select *Change Picture*. Save as a Windows Media Video (*.wmv*).

S3: Aggression assay

A *.zip* folder containing a collection of ten 5-min video clips of an aggressive stimulus (an individual adult zebrafish performing aggressive behaviors).

S4: Sociability assay

A *.zip* folder containing a collection of thirteen 5-min video clips of a social stimulus (a shoal of five adult zebrafish).

S5: Stimulus video composition

A *.zip* folder containing a Windows Live Movie Maker template, a Photoshop CS6 template, and accompanying video tutorials demonstrating their use.

S6: Stimulus videos

A *.zip* folder containing 24 compressed stimulus videos, as used in this experiment.

S7: Trial data collection

An *.xls* template for preliminary data collection.

S8: EthoVision template

This template includes arena settings, trial control settings, data profiles and analysis profiles utilized in this experiment. Also included are sample videos, a sample trial list, and descriptions of the data and analysis profiles used. Additional, more detailed settings that were not analyzed in this experiment have also been included (additional arena zones, data profiles, and measured parameters) for exploratory purposes. This template should serve as a starting point to navigate EthoVision according to the associated written protocol provided (see S12), and should be adapted according to specific experimental needs.

Supplementary Protocols

Protocols have been written for use in this study and may include details specific to our laboratory and equipment. Protocols have been supplied as a guide only, and may be adapted as required.

S9: How to: Film aggression and sociability stimulus videos

A written protocol detailing the methods used to select and film zebrafish for aggression and sociability contexts.

S10: How to: Zebrafish personality trials

A written protocol detailing the methods used to perform zebrafish personality trials. These methods outline setting up the experimental tanks, setting up the video camcorders and computer tablets, and trial execution. In these methods, Camera 1 was used to film tank group 1 (tanks 1–4), and Camera 2 was used to film tank group 2 (tanks 5–8).

S11: Video tutorial: Zebrafish personality trials

A video tutorial demonstrating how zebrafish personality trials were conducted for this experiment. This includes setting up the computer tablets and trial execution. To be viewed in conjunction with the written protocol (see S10).

S12: How to: EthoVision

A written protocol detailing the use and functionality of EthoVision as used in this experiment. This protocol outlines all steps from creating a new experiment to data acquisition for this study. Included are instructional figures and troubleshooting tips to maximize successful data acquisition. To be used in conjunction with the supplied EthoVision template (see S8).

Detailed Behavioral Phenotyping Protocol

Stimulus video composition

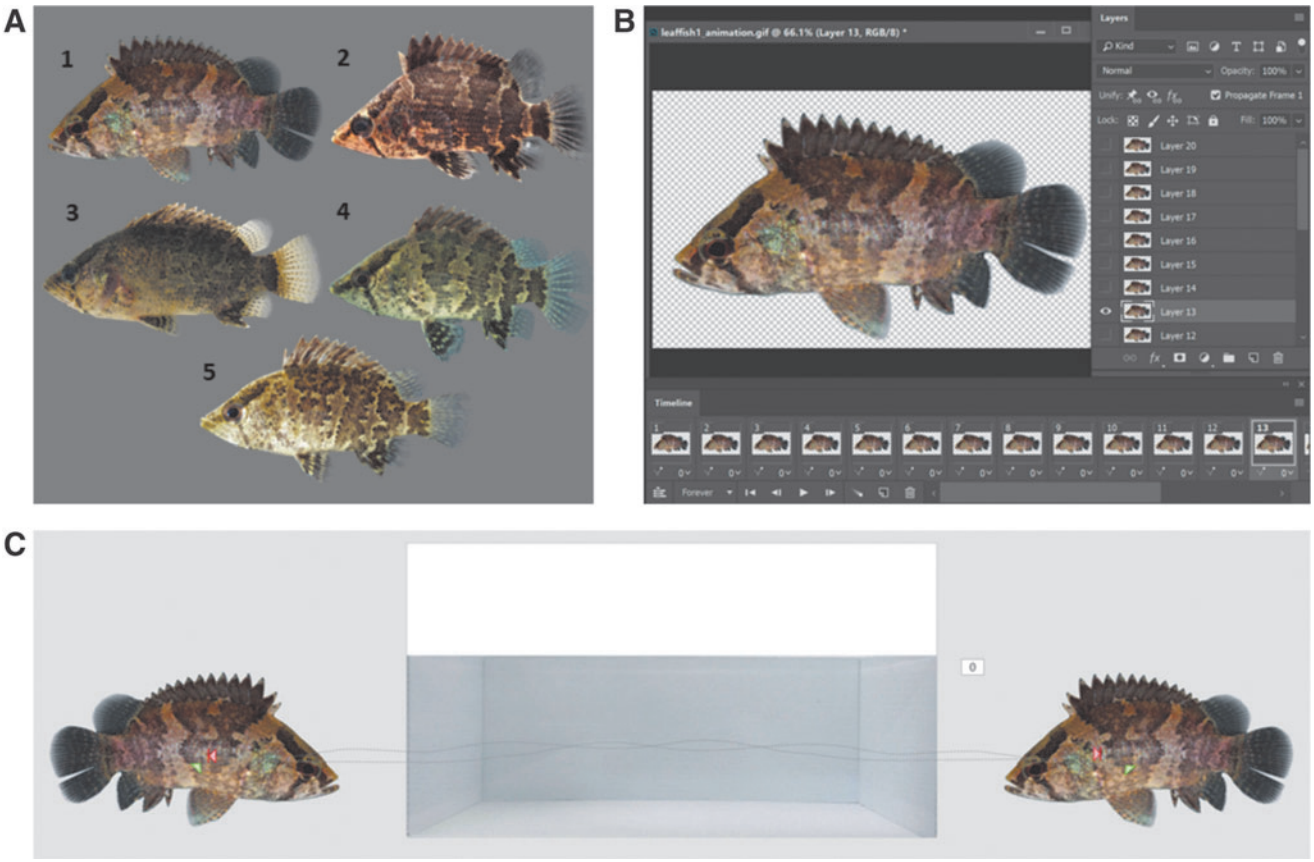
Required windows programs.

(1) Microsoft PowerPoint 2016
(2) Photoshop CS6, or alternative image editing and animating software
(3) Windows Live Movie Maker (Windows Live Essentials, 2012), or alternative video editing software
(4) VLC Media Player, or alternative video compressing software

Animating predator models and novel objects. Predator model species were selected based on their use in previous studies.[1,2] Five different photographs of leaf fish (genus *Nandus*) were sourced and the body of the fish was isolated onto a transparent background using Photoshop CS6 (Supplementary Fig. S1A). Waveform animation was added to simulate a semirealistic swimming effect using the *Filter* > *Distortion* > *Wave* function over a series of at least twenty frames. Frames were compiled using the *Window* > *Timeline* > *Create Frame Animation* function at a frame rate of 0 s until an effective swimming motion was achieved (Supplementary Fig. S1B). Files were exported as *.gif* with matte color selected to blend into the chosen background image. Isolated *.png* files and animated *.gif* files of each of the five predator models are available online (see S1).

Twenty-two novel objects were designed and modified using the *Shapes* tool in Microsoft PowerPoint and saved as transparent *.png* objects. Previous experiments identified red colored objects as the most effective at eliciting a behavioral response, demonstrated by increased distance moved within a 1-min interval (unpublished data). All *.png* objects are available online (see S2).

Predator models and novel object shapes were motion animated in PowerPoint using the *Animation* function. Slide dimensions were 20×11 cm, with a standard background image positioned on the lower edge. Predator models were



SUPPLEMENTARY FIG. S1. **(A)** Five leaf fish (genus *Nandus*) predator models clipped from photographic images: leaf fish 1 (*Nandus nebulosus*, image source), leaf fish 2 (*Nandus oxyrhynchus*, image source), leaf fish 3 (*Nandus oxyrhynchus,* image source), leaf fish 4 (*Nandus oxyrhynchus*, image source), and leaf fish 5 (*Nandus oxyrhynchus,* image source). **(B)** Frame animation clipboard in Photoshop CS6 used to apply waveform animation to each predator model. **(C)** Animation template in PowerPoint used to apply motion animation to each predator model. Predator models were animated to ''swim'' against a neutral (blank) background.

animated with vertical and horizontal movements to simulate a natural swimming motion across the length of the slide, at a speed of ∼0.8 cm/s (Supplementary Fig. S1C). Novel objects were animated with repetitive horizontal movements at ∼0.25 cm/s. Template *.ppt* files were generated to standardize animated motions between the chosen predator models and novel objects, respectively. Template *.ppt* files with additional instructions are accessible online (see S1 and S2). All videos were exported as *.wmv* files.

Generating aggressive and social stimuli. Detailed methods are available online (see S9). Briefly, adult zebrafish were video-recorded in a clear acrylic tank lined with white corrugated corflute board. Zebrafish were recorded either individually or in a shoal consisting of five individuals for aggression and social stimulus videos, respectively. Collected videos were imported to Photoshop CS6 for video cleaning. Cleaning consisted of repositioning the captured video within a 20 × 11 cm canvas such that the bottom edge of the tank perfectly aligned with the bottom edge of the canvas. Space above the waterline (7 cm from the bottom of the canvas) was filled in white. Video was exported as *.mp4*, imported into Windows Live Movie Maker, and split into a series of 5-min stimulus clips. Video clips were exported in *.mp4* format.

Exported clips underwent a screening process by three of the authors (M.F., S.N., and R.O.). Aggression stimulus clips were assessed for aggressive behavior (evidence of charging, darting, or biting behavior) directed toward or away from the viewer with minimal freezing behavior. Social stimulus clips were assessed for consistent shoaling behavior of all individuals with minimal freezing by one or more individuals. Videos that did not meet predefined criteria were excluded. In total, 10 aggression and 8 social preference task clips were generated. All usable aggression and sociability stimulus video clips are available online (see S3 and S4).

Compilation of computer-generated stimuli. Stimulus animations (novel object, predator) and video clips (aggression, social) were compiled into one 38-min video using Windows Live Movie Maker (WLMM). This video consisted of a 34-min trial period surrounded by two 2-min buffer (no stimulus) periods. Videos were designed to play on an infinite loop until all subjects had been trialed for that session. Buffer periods were included to allow zebrafish entry and removal from the experimental tanks by a single operator.

The trial period consisted of six phases. Phases 1 and 6, the ''exploration'' phases, consisted of a 3-min neutral background image (no stimulus, control). Phases 2–5, the ''stimulus'' phases, consisted of a 3-min prestimulus (no stimulus, control)

period, a 3-min stimulus period, and a 1-min interval (no stimulus). A series of pilot studies were conducted to determine the optimal period and interval lengths (unpublished data), where the minimum required interval for zebrafish to return to baseline activity measurements was 1 min. Each stimulus phase comprised one of four behavioral contexts: sociability, neophobia, aggression, or boldness (see Fig. 1B in the main text).

Periods without a stimulus were created by inserting a neutral ''blank'' background image (no stimulus) to achieve a seamless transition between each trial phase. This frame was retrieved from video recorded of an empty tank before recording the aggression and social preference stimulus videos. For all video phases, captions were inserted in the part of the screen that would be located above the water line to clearly differentiate between the different video phases.

Phases 2–5 were pseudorandomized to create 24 possible phase combinations. For each combination, different stimulus animations and video clips were selected to increase variability. Inserted video clips were randomly mirrored using *Visual Effects* to further increase stimulus variability and variate the swimming direction of the predator models. A WLMM template was created to standardize the stimulus phases, and is available online with an accompanying video tutorial (see S5). All videos were published in *.mp4* format.

Published videos were imported to Photoshop CS6 for a final rendering step. This step included adjusting video dimensions to match the dimensions of the computer tablets (12×7.5 cm), adding labeling features to clearly differentiate each video at the first frame, and adding a timer to monitor video progress during the experimental trials. A Photoshop template with accompanying video tutorial is available online (see S5). All videos were rendered in *.mp4* format.

Photoshop rendered videos were compressed using VLC Media Player to improve performance when played on computer tablets. All ready-to-use compressed stimulus videos used in this experiment are available online (see S6).

These methods may be modified for use for alternative video editing programs or computer operating systems. It should be noted that the programs used in this study were selected due to accessibility and knowledge of use. However, accessibility to WLMM and Photoshop is limited by download availability and cost, respectively. In addition, time taken to render videos in both WLMM and Photoshop was substantial, and may be more so in older operating systems. An alternative free video editing program for Windows that combines the functionality of WLMM and Photoshop CS6 (for video editing) is VSDC Video Editor (available online, here), which may simplify the video composition process and reduce video rendering time.

Behavioral phenotyping

Materials used.

(1) Experimental tanks (composed of 6 mm acrylic; $200 \times 400 \times 120$ mm w×l×d, with a white base, white long walls, and transparent short walls, Australian Plastic Fabricators Pty Ltd)
(2) Dark green acrylic panels (composed of 3 mm acrylic; $3 \times 299 \times 125$ mm w×l×d); two for every experimental tank
(3) Heat mat (URS Ultimate Heat Mat, 40×60 cm, iPetz); one for every two experimental tanks
(4) Thermostat (Ringder Digital Aquarium Reptile Dimming Thermostat, DTC-120, 230 V, 50HZ, 0–50°C); one for every two heat mats
(5) Video camcorder (Panasonic HC-V770 M camcorder; MP4/720p recording mode); one for every four experimental tanks
(6) Corrugated white corflute board
(7) Metal book ends; one for every experimental tank
(8) Digital thermometer (Digital Thermometer for Fridge or Freezer IC7209, Instrument Choice); one for every experimental tank
(9) Computer tablet (Ematic EWT826BK 8-inch Tablet, 1GB Ram, 32GB Storage, Windows 10) connected to OneDrive; two for every experimental tank
(10) Computer tablet or iOS device with the Panasonic Image App installed; one for every video camcorder
(11) False-bottom zebrafish breeding containers

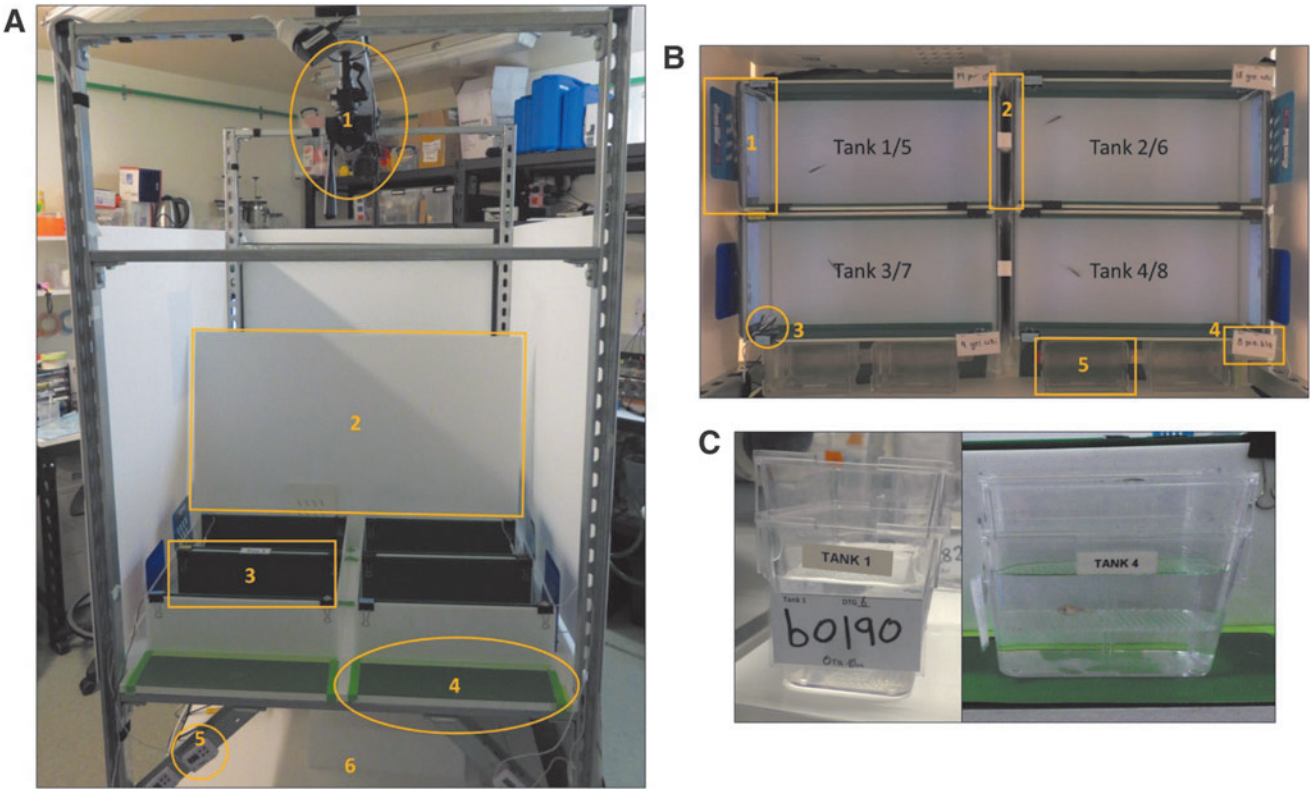
Used Windows programs and applications.

(1) Microsoft OneDrive
(2) Films & TV App (Windows App Store)
(3) Panasonic Image App (Windows App Store)
(4) MPEG Streamclip (Squared 5; available online here)

Setup. Experimental tanks and filming equipment were set up according to Supplementary Figure S2. Briefly, eight experimental tanks were used in this experiment. Each tank was set up with two dark green acrylic panels along the long walls, secured in place with fold-back clips. Tanks were designed with a white floor to heighten subject contrast for data extraction purposes, transparent acrylic short walls for stimulus video presentation, and dark green long walls to minimize anxiety induced by white surroundings.[3] Two tanks were positioned on one heat mat whose temperature was controlled by a thermostat. Four tanks were set up in a quadrant formation, with a video camcorder positioned centrally overhead, 104 cm from lens to the base of the tanks. This setup was duplicated to improve high-throughput efficiency, and separated using a sheet of white corflute board. The entire experimental setup was positioned on a custom-made filming rig and surrounded by white corflute board to achieve uniform lighting and minimize visual disturbances during the filming process.

Each experimental tank was filled with water to 7 cm depth, with water temperature maintained at ~28°C throughout the trial. The water temperature of each tank was monitored using a digital thermometer with temperature probe. Water temperature for each tank was recorded at the start of each trial, and the tanks were stirred between trials to minimize temperature gradients.

Stimulus videos were presented to the experimental fish by a series of sixteen computer tablets. Tablets were synchronized using OneDrive such that all tablets had access to all stimulus videos. When in use, computer tablets were inserted into plastic sleeves (to protect from water damage) and positioned flat against the short walls of the experimental tanks, on the outside of the tank (see Fig. 1A in the main text). Each tank was set up with two computer tablets: one ''blank'' tablet that continuously presented a neutral background image (no stimulus, control) and one ''stimulus'' tablet that



SUPPLEMENTARY FIG. S2. **(A)** Experimental tanks were set up on a custom-built filming rig. Tanks were filmed overhead (1) using a video camcorder. Two tank groups of four were divided by one sheet of *white* corflute board (2) with the remaining walls of the rig also surrounded by *white* corflute board. Dark *green* acrylic panels were clipped to the long walls of the experimental tanks using metal clips (3). Each two heat mats were positioned on a temperature-controlled heat mat (4). Digital thermometers were used to monitor temperature of the experimental tanks during the trials (5). All electrical sources and equipment, including thermostats, were stored on the lower shelf of the filming rig (6). **(B)** For each tank group, experimental tanks were set up in a quadrant formation (tank group 1 consisted of tanks 1–4, tank group 2 consisted of tanks 5–8). Blank (no stimulus, control) tablets were positioned against the outer ends of the tanks and were supported with metal book ends (1). Stimulus tablets were positioned against the inner ends of the tanks and were supported with rubber wedges (2). Temperature probes from digital thermometers were secured to the outer corners of the experimental tanks using tape (3) with the temperature probes from the thermostat secured similarly in tank 3 (tank group 1) and tank 7 (tank group 2). Experimental tanks were labeled according to the subject ID for each trial (4). Subjects were held in false bottom breeding containers in front of the experimental tanks before trialing (5). **(C)** The false bottom breeding containers were used to house study subjects before trialing, labeled with their unique subject ID and tank number.

presented one of the 24 stimulus videos. Blank tablets were always positioned on the outer ends of the experimental tanks (Supplementary Fig. S2).

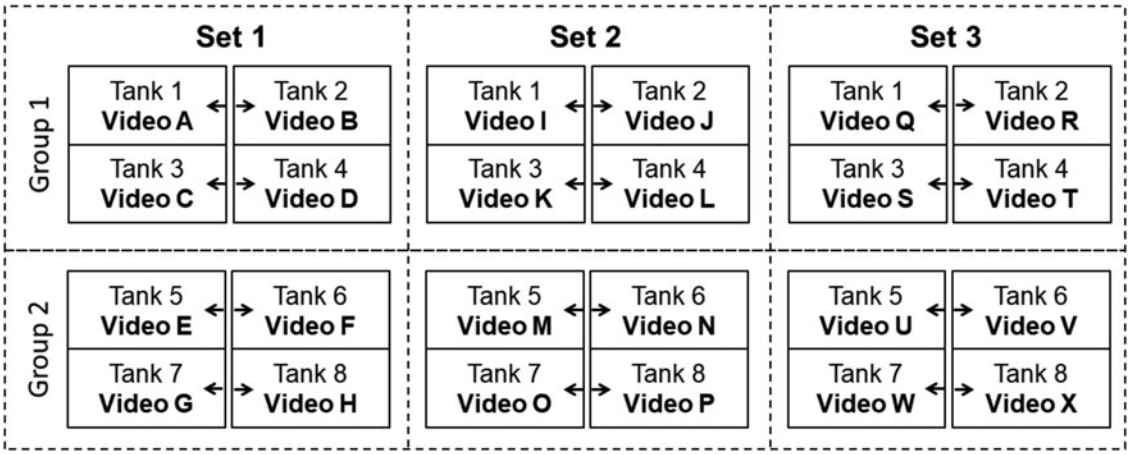
Behavioral phenotyping trials. Twenty-four adult zebrafish (male = 12; female = 12, approximately 6 months old) were trialed in this experiment. All subjects had been marked with uniquely colored elastomer markings for identification, using previously described methods.[4] Behavioral trials occurred between 1 and 4 pm over 4 days, at weekly intervals, measuring each individual four times over the course of the study.

The experiment was designed such that each individual was trialed in four unique tanks, and exposed to four unique stimulus videos. Briefly, zebrafish were randomly assigned to one experimental tank on the first trial day. On the following days, zebrafish were moved between experimental tanks in a systematic order, specifically, $1>8>3>6>5>4>7>2$. That is, if an individual was randomly assigned to tank 8 on day 1, they would be trialed in tank 3 on day 2, and so on. Tank order was designed such that each individual was exposed to both tank groups (tanks 1–4, and tanks 5–8; Supplementary Fig. S3) on either end of the filming rig and received stimulus presentation on both the left- and right-hand sides of the tank in an alternated manner.

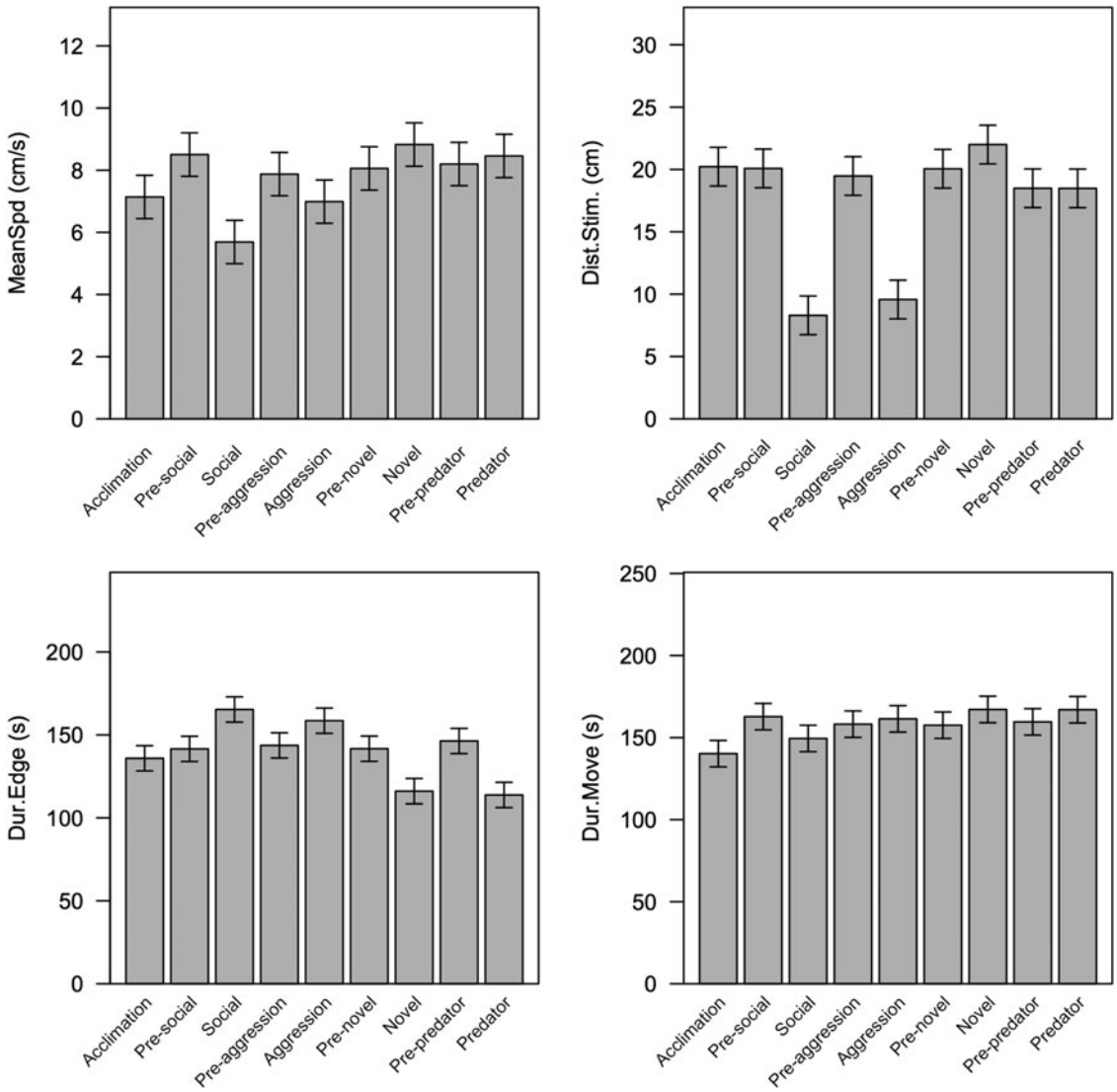
In addition, stimulus videos were grouped into three sets (Supplementary Fig. S3) and assigned to one experimental tank for the duration of the experiment. If an individual was presented with a video from set 1 on the first trial day, they would be presented with a video from set 2 on the second trial day, and so on. In this manner, an individual could be exposed to up to 24 unique stimulus videos without repeated exposure. Thus, this experiment was designed to be expandable to larger sample sizes and repetitions.

For each trial, stimulus videos were played synchronously using the Films & TV App (Windows 10) for each tank group. Video recording was controlled remotely using Panasonic Image App on a computer tablet. Recording commenced at 00:00 of the stimulus videos and ended at 36:10.

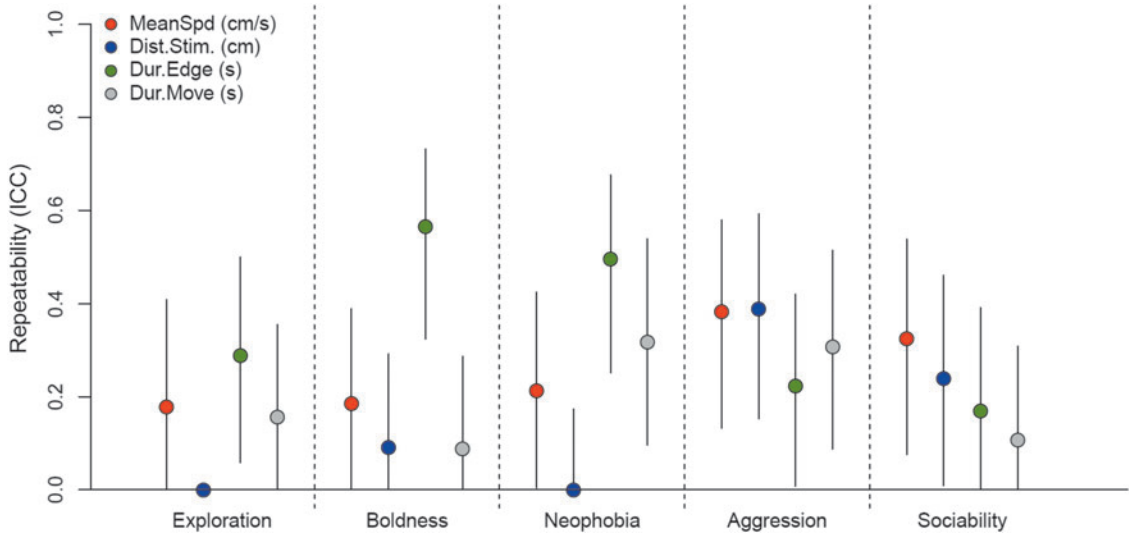
Zebrafish were held in individual holding containers before their trial (Supplementary Fig. S2). For each trial, fish



SUPPLEMENTARY FIG. S3. Eight experimental tanks were grouped into two groups: group 1 (tanks 1–4) and group 2 (tanks 5–8). Stimulus videos were allocated to each experimental tank over a series of three sets: set 1 (videos A–H), set 2 (videos I–P), and set 3 (videos Q–X). *Arrows* indicate the side of stimulus video presentation. Using the methods described herein, an individual randomly assigned to tank 1 set 1 on the first trial day would be assigned to tank 8 set 2 on the second trial day, and so on.



SUPPLEMENTARY FIG. S4. Swimming speed (cm/s), distance to stimulus (cm), time on edge (s), and duration moving (s) for the various experimental periods across individuals. Mean (+/− standard error) are provided.



SUPPLEMENTARY FIG. S5. Adjusted repeatability estimates and their 95% confidence intervals after accounting for sex effects. Significant repeatability is signified by a lack of overlap between 95% CIs and zero.

were placed individually into an experimental tank during an initial 2-min buffer period. Zebrafish were exposed to a 34-min trial period (Fig. 1 in the main text) and then removed from their tanks and returned to their holding containers. Computer tablets were programmed to cycle through a predetermined stimulus video on a loop, allowing subjects to be trialed in succession in an efficient and high-throughput manner. Therefore, for each trial day, the selected video set did not change. Using these methods, up to 64 subjects could be easily trialed in a 6-h period. Detailed methods, including an instructional video tutorial, outlining this process are available online (see S10 and S11).

Preliminary data were collected in an Excel spreadsheet and transferred to EthoVision during data acquisition (available online, S7).

For this experiment, collected video files were renamed according to the trial day, filming session, and camera number (i.e., d1_s1_c1). Due to restrictions with our chosen video camcorder, videos were broken into two clips (one 30-min clip and one 6-min clip). Videos were concatenated using MPEG Streamclip with audio removed for analysis in EthoVision.

EthoVision. Video footage was analyzed using Noldus EthoVision 11.5 XT. An EthoVision template consisting of sample videos, arena settings, trial control settings, detection settings, data profiles, and analysis profiles can be found online (see S8) with detailed methods (S12). Methods may be adapted to utilize alternative, freely available animal tracking software, for example, as in Ref. 5.

Statistical Analyses and Extended Results

To ascertain whether traits show consistent individual differences, we quantified individual repeatability (i.e., intraclass correlation coefficient, ICC) for each behavioral trait of interest using the *rptr* function in the package rtpR[6] in the R statistical environment (version 3.4.1).[7] We choose four independent variables that we deemed, *a priori*, to be biologically relevant measures of behavior in zebrafish and were least correlated with each other [speed of movement (cm/s); distance to stimulus (cm); duration along edge (s); and distance moving (s)]. Distance to stimulus (in Social and Aggression contexts) and duration along edge (in Novel and Predatory contexts) were most clearly affected when the stimulus was conveyed on the tablets (Supplementary Fig. S4). Additional variables that quantify the change in behavior between prestimulus and stimulus periods could also be computed with our design, and repeatability of these traits can also be quantified. Nonetheless, we were interested in understanding, more generally, patterns of repeatability in these traits and so we only utilized the raw behaviors. We calculated unadjusted repeatability (i.e., not accounting for fixed effects) for each variable using a mixed model framework.[6] Confidence intervals were generated by conducting 1000 parametric bootstraps. Sex can be an important source of individual differences, and yet, while accounting for sex did lead to decreased repeatability, many traits remained significantly repeatable across contexts (See ''Adjusted repeatability'' - Supplementary Fig. S5).

References

1. Kalueff AV: The Rights and Wrongs of Zebrafish: Behavioral Phenotyping of Zebrafish. Switzerland: Springer, 2017.
2. Ahmed TS, Fernandes Y, Gerlai R. Effects of animated images of sympatric predators and abstract shapes on fear responses in zebrafish. Behaviour 2014;149:1125–1153.
3. Blaser RE, Chadwick L, McGinnis G. Behavioral measures of anxiety in zebrafish (*Danio rerio*). Behav Brain Res 2010; 208:55–62.
4. Frommen JG, Hanak S, Schmidl CA, Thünken T. Visible implant elastomer tagging influences social preferences of Zebrafish (*Danio Rerio*). Behaviour 2015;152:1765–1777.
5. Rodriquez A, Zhang H, Klaminder J, Brodin T, Andersson PL, Andersson M. ToxTrac: a fast and robust software for tracking organisms. Methods Ecol Evol 2017; [Epub ahead of print]; DOI: 0.1111/2041-210X.12874.
6. Stoffel M, Nakagawa S, Schielzeth H. rptR: repeatability estimation and variance decomposition by generalized linear mixed-effects models. Methods Ecol Evol 2017;8:1639–1644
7. R Core Team. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria https://wwwr-projectorg/. 2017.