Measuring behavioral and endocrine responses to novelty stress in adult zebrafish

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Several behavioral assays are currently used for high-throughput neurophenotyping and screening of genetic mutations and psychotropic drugs in zebrafish (*Danio rerio*). In this protocol, we describe a battery of two assays to characterize anxiety-related behavioral and endocrine phenotypes in adult zebrafish. Here, we detail how to use the 'novel tank' test to assess behavioral indices of anxiety (including reduced exploration, increased freezing behavior and erratic movement), which are quantifiable using manual registration and computer-aided video-tracking analyses. In addition, we describe how to analyze whole-body zebrafish cortisol concentrations that correspond to their behavior in the novel tank test. This protocol is an easy, inexpensive and effective alternative to other methods of measuring stress responses in zebrafish, thus enabling the rapid acquisition and analysis of large amounts of data. As will be shown here, fish anxiety-like behavior can be either attenuated or exaggerated depending on stress or drug exposure, with cortisol levels generally expected to parallel anxiety behaviors. This protocol can be completed over the course of 2 d, with a variable testing duration depending on the number of fish used.

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INTRODUCTION

Zebrafish (*Danio rerio*) are commonly used as an experimental animal model for developmental, genetic and drug discovery research[1–4]. They are also becoming increasingly popular in neuroscience research[2,5–8], including phenotyping of various zebrafish strains and screening psychotropic drugs[9–12]. Their active behavior, ease of acclimation to new environments, low maintenance cost, rapid reproductive cycle and large number of offspring further emphasize the utility of zebrafish as a model species[13,14].

In the novel tank test presented here (**Fig. 1a**), zebrafish exhibit robust behavioral responses to anxiety evoked by novelty[9,10,15]. This test is based on the animal's natural instinct to seek protection in an unfamiliar environment by diving, freezing and reducing exploration. As the fish gradually acclimates to the new environment, an increase in exploration (e.g., increased locomotion, decreased freezing and more entries to the top half of the tank; see **Table 1**) usually occurs[16,17]. Importantly, these endpoints (exploration, freezing and top entries) can be applied to zebrafish models of anxiety[10,12,15,16] in a way similar to the rodent open-field test, in which mice or rats show anxiety-like behavior by staying close to the walls (thigmotaxis), but increase exploration of the center as they become less stressed[18–20].

Our group has successfully applied this novelty-based paradigm in a wide array of experiments[12,16,21]. Several groups use a three-compartment novel tank, with top, bottom and intermediate virtual zones[10,15,22]. We have modified this test by using only two (top and bottom) zones (**Fig. 1a**), to enable a more efficient quantification of zebrafish behavior. The novelty-based tank paradigm is an excellent assay for drug screening, as anxiety-like behavior can be modulated by anxiogenic and anxiolytic agents[12,16]. For example, the effects of drugs such as ethanol[14], nicotine[10], morphine[23], amphetamine[24], benzodiazepines[15] and cocaine[11] have been previously tested in zebrafish. Although providing a variety of endpoints (**Table 1**) and showing an extensive history of demonstrated applications[17], this model is not applicable for all behavioral domains (such as drug reward properties) or all anxiety behaviors (such as scototaxis or dark/light preference; see ref. 25 for details). Therefore, in addition to that described here, other novelty-based paradigms can be used. These include zebrafish open field[10], light/dark box[25,26], Y- or T-maze[22] and shoaling[27] tests, all of which can be used to complement the novel tank within a battery of tests (e.g., see ref. 28), to enable a comprehensive characterization of zebrafish neurobehavioral phenotypes.

To quantify zebrafish behavior, experimenters have traditionally relied on manual observation, which is prone to various human errors[17,29,30]. The growing availability of computerized video-tracking technology has enabled researchers to more accurately and objectively assess zebrafish behavioral endpoints, as well as to perform swim path reconstruction and motor-posture pattern analyses (**Table 1**)[9,12,29]. Moreover, this method has been shown to reflect manual recording data with a high degree of accuracy and precision[9,12] (**Fig. 1b**). However, one of the downsides of video tracking is that, if testing conditions are not optimal, it may lead to aberrant data. To avoid this, troubleshooting advice is provided. As is shown here, a combination of manual analysis and properly set-up video-tracking tools enables the comprehensive characterization of zebrafish behaviors (**Figs. 1–3**). This protocol is suitable for studying behavioral phenotypes of adult zebrafish. Larval zebrafish models, also widely used in neurobehavioral research[6,31–35], require other behavioral tests and are not discussed here.

Another relevant tool in zebrafish neuroscience research is the analysis of their physiological (endocrine) responses to stress[36,37]. As zebrafish have a developed hypothalamus-pituitary-interrenal axis and use cortisol as their primary stress hormone, they represent a valuable model to study cortisol-mediated stress responses[38,39]. Here, we provide a simple protocol for the analysis of whole-body zebrafish cortisol concentration (as a physiological marker of stress and anxiety).

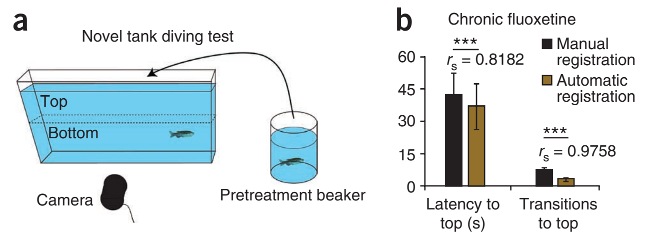

Figure 1 | Novel tank test for behavioral testing in adult zebrafish. (**a**) Experimental setup (zebrafish are exposed to the experimental challenge in a pretreatment beaker before being transferred into the novel tank for behavioral observation and phenotyping; control groups undergo same procedures without challenge in pretreatment beaker; see refs. 12,79 for details). (**b**) Comparison of behavioral data generated by manual and CleverSys video-tracking analyses in the 6-min novel tank test. In this experiment, zebrafish were treated daily with fluoxetine (100 µg per liter) for 2 weeks before testing[12]. Spearman's rank correlation was used to compare each method of quantification. Note significant strong correlation (***$P<0.005$) for different types of behavioral endpoints, such as time and frequency measures. Data are presented as mean ± s.e.m.

Paralleling reports showing the effects of stressors or drugs on corticosterone levels in rodents[40–42], this technique has been successfully applied to zebrafish in our laboratory[12,21]. The use of cortisol assays represents a logical addition to the novel tank test-based behavioral testing[12,21,43]. The whole-body cortisol analysis protocol described here for zebrafish is a sensitive method based on human salivary enzyme-linked immunosorbent assays (ELISA)[12,44]. Alternative approaches to analyzing zebrafish cortisol may include the use of human serum cortisol ELISA[36] or radioimmunoassay[43,45]. Limitations of some of these methods, however, include the need to combine several adult fish body samples to obtain detectable levels of cortisol. This precludes correlational analysis between behavioral and endocrine markers of anxiety, and limits application of this method in small fish.

Overall, behavioral and physiological assays have become instrumental for the establishment of zebrafish as a promising model of stress, anxiety and other neurobehavioral disorders. For instance, the various mutant zebrafish strains exhibit marked behavioral differences[12,46–48], and more research is needed to correlate the genotypes and phenotypes of these strains. Further studies may also enable a better understanding of brain-endocrine interactions. For example, drug withdrawal modulates zebrafish cortisol levels, resembling glucocorticoid dysregulations in human and rodent withdrawal syndrome[21,40,41,49,50]. Applying the protocol described

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TABLE 1 | Summary of commonly used behavioral endpoints measured in the novel tank test.

Endpoint (units)	Method of behavior registration	Manual recording of observation	Increasing value indicates
Number of entries to the top	m, a	The fish swims to the upper half of the tank (crossing the horizontal dividing line described in EQUIPMENT SETUP)[a]	Lower anxiety levels
Entries top/bottom ratio	d	NA	Lower anxiety levels
Time spent in top (s)	m, a	The amount of time the fish spends in the upper half of the tank	Lower anxiety levels
Time spent top/bottom ratio	d	NA	Lower anxiety levels
Average entry duration (s) (time spent in the top divided by the number of entries to the top)	d	NA	Lower anxiety levels
Distance traveled in the top (m)	a	NA	Lower anxiety levels
Distance traveled top/bottom ratio (m)	d	NA	Lower anxiety levels
Latency to enter the top (s)	m, a	The amount of time it takes the fish to cross into the upper half of the tank[a]	Higher anxiety levels
The number of erratic movements	m, a	A sharp, rapid change in direction or movement, or repeated darting	Higher anxiety levels
Freezing bouts (frequency)	m, a	A period of immobility at the floor of the tank for at least 1 s, characterized by movement of only the eyes and gills	Higher anxiety levels
Freezing duration (s)	m, a	The amount of time spent frozen (immobile)	Higher anxiety levels
Distance traveled (m)	a	NA	Hyperactivity (low levels may reflect abnormal motor/ neurological function)
Velocity (m s^{-1})	a	NA	Hyperactivity (low levels may reflect abnormal motor/ neurological function)

a, automatic observation; m, manual observation; d, endpoint can be derived from manually or automatically recorded data; NA, not applicable.
[a]Count only those instances in which the entire fish crosses the dividing line as a top entry. (Fish will sometimes poke only their head above this line and quickly retreat to the bottom of the tank.) For endpoints capable of being recorded manually, a detailed description has been included. Interpretations are with regard to anxiety level, as expressed by the level of exploration and activity[30].

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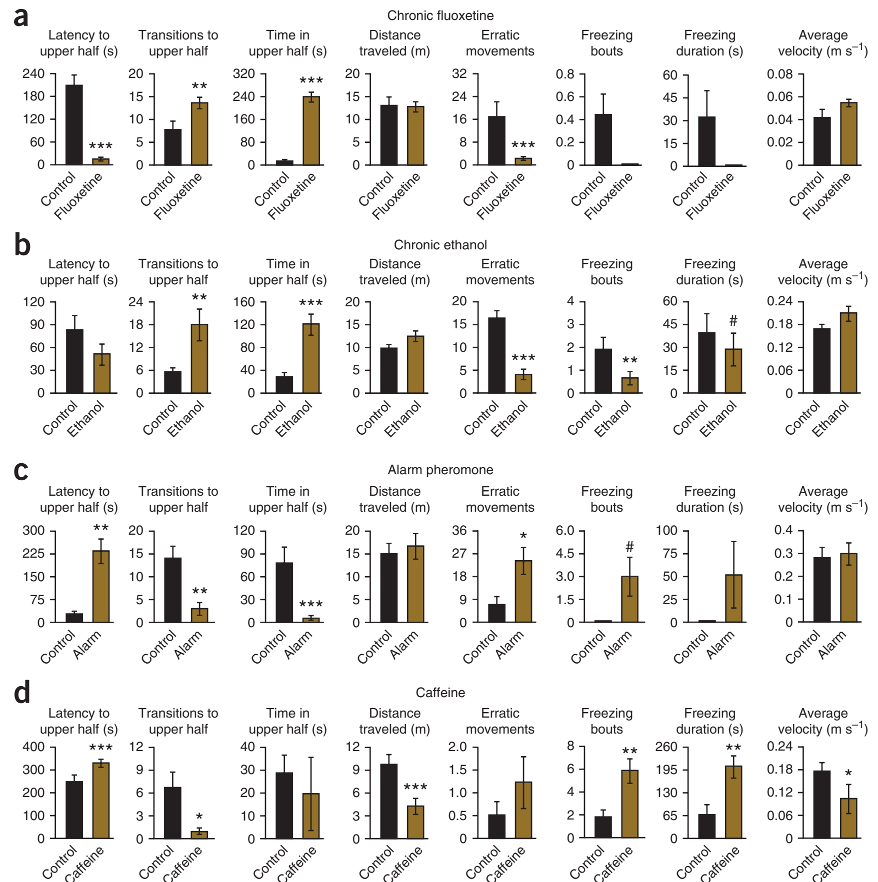

Figure 2 | Behavioral effects of anxiolytic and anxiogenic manipulations in adult zebrafish tested in the 6-min novel tank test. Anxiolytic treatments included (**a**) chronic fluoxetine (100 µg per liter for 2 weeks) analyzed manually and by using CleverSys[12] and (**b**) chronic ethanol (0.3% (vol/vol) for 1 week) analyzed manually and by using Noldus EthoVision XT7 video-tracking system. Anxiogenic manipulations included (**c**) acute alarm pheromone (7 ml added to the novel tank; see **Supplementary Video 1**) and (**d**) acute caffeine pretreatment (250 mg per liter for 20 min), analyzed manually and by using Noldus EthoVision XT7 video-tracking system. Data are presented as mean ± s.e.m. *P<0.05, **P<0.01, ***P<0.005, #P = 0.05–0.1, trend (U-test). Note that although anxiety-sensitive endpoints are predictably affected by different manipulations, neurological phenotypes reflecting the swimming ability of the fish (distance traveled and velocity) remain mostly unaltered. Panels are based on our own unpublished observations or data previously published by our group[12,30,79].

below, we have also shown marked behavioral and endocrine responses evoked in zebrafish by hallucinogenic drugs such as lysergic acid diethylamide[28]. In addition to modeling drug abuse, zebrafish have also provided clinically relevant data on many other neurobehavioral conditions such as neurodegenerative[6,51] and developmental disorders[52]. Therefore, current behavioral and endocrine analyses in adult zebrafish models may be expanded to other fields of experimental neuroscience research.

Experimental design

Use of adult fish. Although both larval and adult zebrafish models provide translational data and quantifiable neurobehavioral phenotypes, larvae are currently more frequently used because of their high-throughput capabilities[6,31,32] and relatively simple behavior, with a limited number of endpoints[53]. However, although some drugs may be screened easily in this model, other agents, especially those affecting the emotionality domain, may require more complex organisms with fully developed neural and endocrine systems[17,54]. Although larval behavior is too simple, adult zebrafish are beneficial in the behavioral analysis of complex traits with a wide spectrum of behavioral and physiological responses[10,12,15,55], and are well suited for use in the novel tank test. Furthermore, only adult-sized fish (depending on age, adult zebrafish body size may vary from 2.5 to 4 cm) are suitable for cortisol analysis.

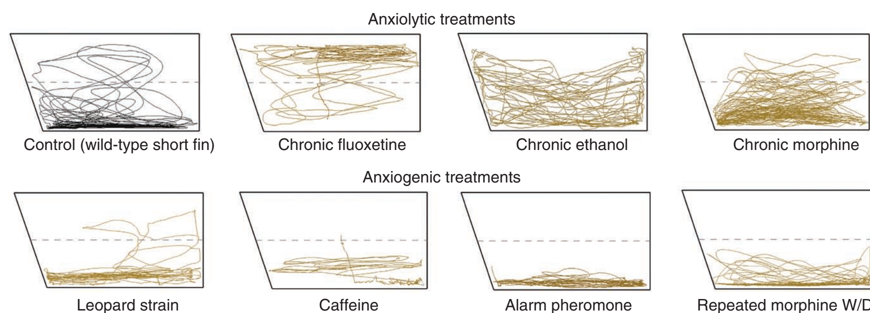

Figure 3 | Typical representative trace images of zebrafish behavior in the 6-min novel tank test, generated by CleverSys or Noldus-based video-tracking systems. Note reduced or increased anxiety-like phenotype after different experimental manipulations, compared with control 'wild-type' short-fin adult zebrafish. Anxiolytic treatments, presented in the upper row, include chronic fluoxetine (100 µg per liter daily for 2 weeks), chronic ethanol (0.3% (vol/vol) for 1 week) and chronic morphine (1.5 mg per liter for 2 weeks). Typical anxiogenic factors, presented in the bottom row, included anxious leopard zebrafish strain, acute caffeine pretreatment (250 mg per liter for 20 min), acute alarm pheromone exposure (7 ml in the novel tank) and repeated withdrawal (W/D) from chronic morphine (1.5 mg per liter for 2 weeks, followed by 1 week of twice-daily 3-h withdrawal periods). Traces were generated in the course of our previously published zebrafish studies[12,21] using current protocols.

Zebrafish strain. Most zebrafish wild-type, transgenic and mutant strains, listed in the Zebrafish International Resource Center at http://www.zebrafish.org, are suitable. Note that strain differences in anxiety and activity may be observed[12,46,56].

Drug exposure. Behavioral and endocrine modulation can be evoked by various psychotropic drugs, including anxiolytic, anxiogenic, antidepressant, psychostimulant, nootropic, hallucinogenic and antipsychotic

agents. The most frequent route of drug administration is by immersion. In some cases (e.g., high drug toxicity, fast hydrolysis, poor solubility in water), intraperitoneal (i.p.) injections of small (5–10 μl) volumes may also be used in adult anesthetized fish; however, this method of drug delivery is not suitable for chronic treatment or for small zebrafish. Following acclimation, zebrafish are pretreated with the drug according to the experimental protocol. Note that the drugs' pharmacokinetics and method of administration should be considered when selecting a pretreatment duration for a particular experiment.

Video tracking. In addition to manual observation, computer-aided analysis may be used. A variety of programs offered commercially (e.g., TopScan or EthoVision XT7) present a simple high-throughput method of quantifying endpoints otherwise not possible with traditional manual recording (e.g., characteristics of swim path, distance traveled, velocity, meandering, heading direction and turning direction[9]). These systems are established, user-friendly programs that facilitate analyses of behavioral endpoints and minimize human error. The Noldus EthoVision XT7 system, extensively used by our group, will be discussed here as an example. We have also successfully used CleverSys TopScan[12], which may similarly be applied to zebrafish behavioral analyses, with minor modifications (see **Supplementary Table 1**).

MATERIALS

REAGENTS

- Adult zebrafish (50 Fathoms; see REAGENT SETUP) ▲ CRITICAL Animal experimentation and care should adhere to institutional and national guidelines and standards.
- Tetramin tropical flakes (Tetra)
- Drug(s) of choice for pretreatment of fish (Sigma-Aldrich)
- PBS buffer (1×; TEKnova, cat. no. P0204) ▲ CRITICAL PBS should be kept on ice during use.
- Ethanol (100% (vol/vol); Sigma-Aldrich, cat. no. 459836)
- Diethyl ether (Sigma-Aldrich, cat. no. 296082) ! CAUTION Handle under ventilated fume hood.
- Tricaine methane sulfonate (Sigma-Aldrich, cat. no. E10521)
- Deionized water
- Ultrapurified deionized water
- Human salivary cortisol assay kit (Salimetrics)

EQUIPMENT

- Novel tank (e.g., a 1.5-liter tank; Aquatic Habitats)
- Fish tank(s) or circulating aquatics system (40 liter; Aquatic Habitats)
- Hamilton syringe (Sigma-Aldrich)
- Light meter (Sper Scientific, model 840006)
- Eppendorf tubes (Sigma-Aldrich)
- Homogenizer (Tissuemiser, Fisher Scientific)
- Vortex (Fisher Scientific)
- Centrifuge (Fisher Scientific)
- Glass test tubes with caps for centrifugation (Fisher Scientific)
- Victor-Wallac plate reader for ELISA analyses with manufacturer's software package (Perkin Elmer)
- Video cameras (webcams) connected to a computer through USB port
- Video-tracking programs, such as TopScan (TopView Animal Behavior Analyzing System) (CleverSys) or EthoVision XT7 (Noldus Information Technology)
- Tools for animal dissection
- Wax markers for tube labeling

REAGENT SETUP

Zebrafish Fish should be 6–8 months old (young adults) or older (e.g., 1- to 2-year-old adults or 3- to 4-year-old aged fish), purchased from a commercial vendor or raised in the institute's animal facility. The fish must be experimentally naïve and should be given at least 14 d to adapt to the laboratory environment before testing. Room and water temperatures and lighting must be maintained at standard levels (e.g., 25–27 °C with 12–12 h or 10–14 h illumination cycles provided by ceiling-mounted fluorescent light tubes; see details of housing in ref. 57, available online). The fish can be separated into groups of 20–30 and housed in 40-liter home tanks filled with deionized water or in a circulating aquatics system. Fish can be fed twice a day with Tetramin tropical flakes.

Tricaine In general, a 500 mg per liter solution (dissolved in water, buffered with sodium bicarbonate to pH = 7.0) will be appropriate for euthanizing fish. For anesthesia, a less-concentrated tricaine solution (e.g., 100–120 mg per liter) should be used. Tricane should be made as needed and can be stored for ~1 week at –20 °C.

EQUIPMENT SETUP

Novel tank The apparatus comprises a narrow, transparent tank that permits minimal lateral but easy vertical and horizontal movements. A typical novel tank can be a 1.5-liter trapezoidal tank (15.2-cm height × 27.9-cm top × 22.5-cm bottom × 7.1-cm width) (**Fig. 1a**). The tank should rest on a level, stable surface and should be divided into two equal virtual horizontal portions, marked by a dividing line (e.g., with a magic marker) on the outside walls[12,16].

PROCEDURE

Acclimation and pretreatment ● TIMING ~1h for acclimation; pretreatment 10–30 min per fish

1| Transport animals from their holding room to the experimental room for acclimation 1 h before testing; to minimize crowding stress, have no more than four fish per 1 liter of water.

▲ CRITICAL STEP Fish must acclimate to the experimental room, and the water used in the novel tank and the pretreatment beaker must be of similar temperature to the holding room (see zebrafish housing). The use of home tank or holding tank water (with proper temperature and salinity) is required.

▲ CRITICAL STEP Experiments must follow national and institutional guidelines for the care and use of laboratory animals[58].

▲ CRITICAL STEP Note that this step pertains to acute drug treatment. For chronic exposure, the drug is administered to the home tank 1 or 2 weeks before testing (in this case, the fish would be moved directly from the home tank to the novel tank for testing, and the experimenter would omit Step 2).

? TROUBLESHOOTING

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2| Pretreat zebrafish with a drug. Pretreatment time varies depending on the drug and its doses (based on previous literature review or pilot study), but is often 10, 20 or 30 min for single (acute) treatment. Administer the drug by using either option A (immersion) or B (intraperitoneal injection).

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(A) Immersion

(i) Dissolve the drug in system water in opaque plastic beakers. As zebrafish may be stressed by exposure to shallow beakers, use 3–4 liter beakers filled with ~3 liters of exposure solution.

(ii) After the drug is fully dissolved, immerse the fish in beakers (one fish per beaker) for a desired pretreatment time.

(B) Intraperitoneal injection

(i) Anesthetize fish by immersion in tricaine (100–120 mg per liter) for ~30 to 60 s, until only the gills are moving. Slightly tap on the beaker to ensure that the fish cannot move; this will allow you to determine that it is fully anesthetized.

(ii) Remove the fish from the tricaine and lay it down on a sterile surface. Turn the animal so that its ventral side is facing upward and quickly inject 5–10 μl of the drug solution using a Hamilton syringe.

(iii) Move the fish to a 3–4 liter holding beaker filled with ~3 liters of water for the desired pretreatment time and recovery from tricaine anesthesia.

▲ CRITICAL STEP Do not leave the fish in the tricaine longer than necessary. This is a time-sensitive procedure, as death can result if exposure is prolonged by as little as an additional ~20 to 30 s.

▲ CRITICAL STEP Perform all experiments in a blind manner; behavioral observers should be unaware of the experimental drug pretreatments. Proper training, to ensure high inter-rater reliability on behavioral endpoints, is needed before beginning experiments. Regular intra-rater reliability assessment (e.g., by observing the same video several times) is also recommended for research personnel. Inter/intra-rater reliability of 85% or higher (determined by Spearman correlation coefficient) is necessary to ensure high quality and consistency of manual behavioral registration.

Novel tank testing ● TIMING 1–4 h, depending on number of fish and trial duration

3| Position the novel tank in front of the webcam for optimal video recording. Ensure that the background of the tank is light enough to have a proper contrast (for later video-aided analysis). Use a light meter to ensure that all areas of the novel tank apparatus are illuminated with approximately the same intensity (variation of 5–10% is acceptable).

▲ CRITICAL STEP If multiple experimenters are testing more than one fish at a time (i.e., using multiple novel tank setups), ensure that the fish are not visible to each other during testing (e.g., by placing a lightly colored opaque partition between the tanks).

4| After pretreatment, begin video recording and quickly transfer the zebrafish to the novel tank by gently scooping them with a net (one fish per net) and then gently placing the net in the novel tank, maneuvering it to allow the fish to swim out. Record the fish's behavior for the desired length of time (e.g., 6 min), both manually and using a video camera (**Fig. 1a**). To avoid experimenter-induced behavioral alteration in fish, record their behavior for an additional 2 min, which will be excluded from behavioral analyses (e.g., for a 6 min trial, record for 8 min, and only use the first 6 min for analysis). Refer to **Table 1** for the main behavioral endpoints and to **Table 2** for descriptions on how to record them manually (also see **Supplementary Video 1**).

? TROUBLESHOOTING

▲ CRITICAL STEP Use careful handling to reduce net stress, as it can affect fish behavior. Also avoid sudden and rapid movements during testing, as well as loud noises and/or vibrations (e.g., closing doors) in the testing room, as these may startle the fish. Ideally, the experimenter(s) and computer(s) should be 1–2 m away from the novel tanks.

▲ CRITICAL STEP Begin video recording before placing the fish into the novel tank. This will ensure proper tracking of the subject against its background during the software analysis.

TABLE 2 | Example template for the manual recording of zebrafish behavior in a single trial in a 6-min novel tank test.

Minute	No. of top entries	Time in top		No. of erratic movements	No. of freezing bouts	Time frozen	
		Start time	Duration			Start time	Duration
1							
2							
3							

The number of top entries, erratic movements and freezing bouts can be recorded using tick marks under each respective minute. For top and freezing durations that extend beyond 1 min into the next, do not count this as an additional entry or bout. In addition, although the 'Start time' columns are helpful guide for recording duration, they need not be recorded for each top entry or freezing bout. The only start time that must always be recorded is for the first top entry (as it represents the 'latency to enter the top' endpoint mentioned in **Table 1**).

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5| After each trial, assign the experimental subject a subject number (ID), and change the recorded video file name accordingly to correlate to that fish number. Remove the animal from the novel tank and immediately euthanize it by immersing in a 500 mg per liter tricaine solution (complete body immobility and the lack of eye/gill movements for 2 min will indicate that the fish is dead). Place the fish body in an Eppendorf tube on ice, and prepare for the next trial by changing the water in the novel tank and cleaning it with fresh water (to remove olfactory stimuli).
▲ **CRITICAL STEP** The recorded video file must be in the correct format (e.g., AVI, MPEG), required by the video-tracking software to analyze it.

6| When all trials are completed, proceed with cortisol extraction and analysis (Steps 7–27) and video-aided analysis (Steps 28–50).
■ **PAUSE POINT** To obtain endocrine data at a later time point, store the fish in a −80 °C freezer. As cortisol is a relatively stable substance, samples can be kept in a freezer for several months.

Cortisol extraction ● TIMING 150 min per group of 15 samples with an additional 12 h for ether evaporation

7| If fish samples were previously frozen, thaw them before dissection. Remove the head using a razor blade. Ensure that dissection tools are sterilized with 100% (vol/vol) ethanol between each animal.
▲ **CRITICAL STEP** The outcome of these extractions may be affected by temperature. Thus, the entire cortisol extraction procedure should be carried out at a standardized room temperature (within a range of a few degrees e.g., 20–25 °C) to prevent confounding of the results.

8| Weigh each zebrafish sample and record the body weights (in g). Measure the weight of the whole-body sample before homogenization, which is necessary for the determination of cortisol concentration after extraction and ELISA (note that absolute cortisol concentrations will not suffice, as data should be normalized and expressed in ng of cortisol per g of fish body weight (e.g., see **Fig. 4** (refs. 12,21)).

9| Cut the whole-body samples on ice into smaller pieces to facilitate homogenization.

10| Transfer the samples to labeled glass tubes and add 500 ml of ice-cold 1× PBS to each sample.

11| Homogenize the samples for 1 min (individual fish should be homogenized separately) while washing the homogenizing rotor blade with an additional 500 µl of ice-cold 1× PBS. Collect the wash in a 2-ml glass tube containing the homogenate and then place the sample on ice. Wash the homogenizing rotor blade and probe with ethanol (100% (vol/vol)) and deionized water between each sample, changing pipette tips after use to avoid cross-contamination.
? TROUBLESHOOTING

12| Add 5 ml diethyl ether to each sample. Vortex for 1 min and then centrifuge at 7,000*g* for 15 min. After centrifugation, collect the top organic layer containing cortisol from each sample and place it in a separate test tube.
▲ **CRITICAL STEP** Each glass tube must be labeled properly using wax markers that are resistant to removal by any spilled ether.
! CAUTION Always handle hazardous materials (such as ether) with care and according to the institutional and laboratory guidelines. Ether emits toxic fumes, and thus must be handled in a fume hood.
? TROUBLESHOOTING

13| Repeat Step 12 three times throughout the experiment, to ensure maximal cortisol extraction. Note that the cortisol-containing layer is usually yellowish in color. The pooled extract from each fish is kept in a separate test tube.

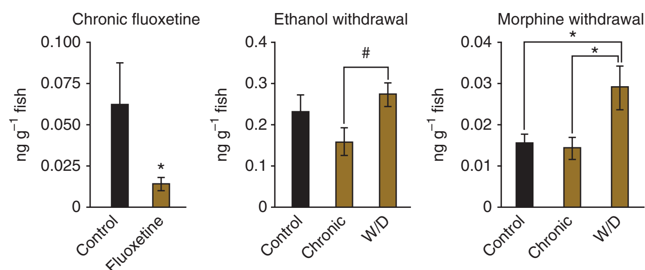

Figure 4 | Endocrine responses (whole-body cortisol levels, assessed by ELISA assay) to various experimental manipulations in adult zebrafish. Treatments include anxiolytic chronic fluoxetine treatment (100 µg per liter for 2 weeks) and anxiogenic acute 12-h withdrawal (W/D) from chronic 0.3% (vol/vol) ethanol (for 1 week) and acute 24-h withdrawal from chronic morphine (1.5 mg per liter for 2 weeks). Data are presented as mean ± s.e.m. $^*P < 0.05$, $^\#P = 0.05$–0.1, trend (*U*-test). Note that zebrafish cohorts previously showing reduced behavioral symptoms of anxiety (see **Figs. 2** and **3** for details) generally exhibit lower cortisol levels. These original diagrams are based on data previously published by our group[12,21].

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14| Keep the extracted cortisol samples (from one individual per glass tube) overnight in the fume hood to allow the ether to evaporate. Other methods of drying the organic solvent can be used, such as a speed vacuum centrifuge equipped with a cryotrap or evaporation under nitrogen sparge.
■ **PAUSE POINT** As cortisol is relatively stable, the extraction procedure may be interrupted for 1–2 d at this stage, if necessary. After ether evaporation, cortisol samples must be covered with foil (to avoid contamination) and stored at room temperature. However, Steps 15–27 are time sensitive, and must be performed without interruption.

Cortisol ELISA assay ● TIMING 18–19 h, including ~12 h for incubation
15| Once the ether has evaporated, reconstitute the cortisol in 1 ml of 1× PBS and incubate overnight in a refrigerator at 4 °C.
▲ **CRITICAL STEP** Before performing the cortisol ELISA, graph the plate layout and the position of each sample (to assist in locating the samples for future quantification).

16| After incubation, vortex thoroughly, bring all reagents and the ELISA (Salimetrics kit; see REAGENTS section) plate to room temperature and proceed with the ELISA to quantify cortisol concentrations. Begin by placing the desired number of strips (containing ELISA wells) in the strip holder.

17| Prepare 1× wash buffer (included in the cortisol assay kit). To ensure accuracy of cortisol activity in developing wells, use deionized water free of organic contaminants to prepare buffers (e.g., purify water by using a carbon filter, or purchase ultrapurified water from vendors).

18| Pipette 24 ml of assay diluent into a disposable tube and set aside for Step 19.
▲ **CRITICAL STEP** To avoid cross-contamination, use a new pipette for each unique solution pipetted.

19| Pipette 25 µl of standards, controls and unknowns into appropriate wells (standards, controls and unknowns should be assayed in duplicate), 25 µl of assay diluent into two wells to serve as the zero value, and 25 µl of assay diluent into each nonspecific binding (NSB) well.

20| Make a final 1:1,600 dilution of the conjugate (15 µl into 24 ml of assay diluent), mix and immediately pipette 200 µl into each well.

21| Mix the plate for 5 min at 500*g* and then incubate for an additional 55 min at room temperature.

22| Wash the plate four times with 1× wash buffer and then blot. Repeat this step three times.

23| Add 200 µl of TMB solution (included in the Salimetrics kit; see REAGENTS section) to each well and then mix the plate for 5 min at 500*g*. Next, incubate the plate in the dark at room temperature for 25 additional minutes.

24| Add 50 µl of stop solution (included in the Salimetrics kit; see REAGENTS section) to each well and mix for 3 min at 500*g*.

25| Wipe the plate bottom clean and read it within 10 min of adding stop solution.

26| Measure ELISA color or reaction intensity in a plate reader using the manufacturer's software package. Compute the average optical density (OD) for the zero (B_0) and NSB wells. Then, subtract the average OD for the NSB wells from the average OD of the zero, standards, controls and unknowns. Lastly, calculate the percent bound (B/B_0) for each standard, control and unknown by dividing the average OD (B) by the average OD for the zero (B_0).

27| To determine the concentrations of the controls and unknowns by interpolation, use a four-parameter sigmoid curve minus curve fit, based on optical densities of standardized concentrations versus those observed in the samples (the sigmoid curve and detailed instructions are provided by the manufacturer). Normalize the cortisol levels based on the weight of the whole-body sample and report them as relative cortisol concentrations (ng g^{-1} body weight). To aid in calculating cortisol concentrations, we have devised a custom-made template that can be downloaded from our laboratory's website at http://www.kaluefflab.com/science.html.
? TROUBLESHOOTING

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Video-tracking fish behaviors ● TIMING 2–6 h, depending on the number of fish and trial duration

28| Transfer the recorded videos onto a computer containing the video-tracking software for analysis. We have used both TopScan and EthoVision (**Fig. 2a–d**) in our experiments. TopScan is a program that allows the user to set their own parameters and event rules for tracking and quantifying behaviors (see **Supplementary Table 1**); however, EthoVision XT7 is used in Steps 28–47 of this protocol as an example. Other video-recording software may also be used for zebrafish behavioral analyses. Once the analysis is completed, the data can be exported to a Microsoft Excel file for further statistical evaluation.
▲ CRITICAL STEP Proper setup of the video-tracking system is essential for recording the zebrafish movements. Variations in lighting may affect the ability of the software to detect and analyze fish movements. It is therefore very important to ensure a suitable background and appropriate light conditions; these should be determined and standardized for all subjects. In addition, video-tracking systems are less reliable for analyzing erratic movements, and manual observations may therefore be needed for this endpoint.

29| Open EthoVision or other video-tracking software, choose 'New Experiment', and name a folder appropriately.

30| Copy video files into the 'Media Files' folder in the newly created experimental folder. Also make sure that the videos are clearly named; rename them if necessary.

31| Under the 'Experimental Settings' column, write a detailed description for the experiment. Set the 'Detection Features' as 'Center-point, nose-point and tail-base detection', and the 'Units' as m, s and ° (degrees), respectively.

32| Create a 'Trial List' with the following variables: fish group (i.e., control, drug-dose1, drug-dose2 and so on) and fish ID (i.e., C1, C2, C3 and so on). Add trials according to the number of videos in the experiment.

33| The experimenter can also manually score behaviors that EthoVision cannot recognize. If desired, use 'Manual Scoring Settings' to set parameters for manually scoring behaviors (i.e., each time the behavior occurs, pressing the designated key will record the occurrence of the behavior): Click 'Add Behavior' and set up the desired endpoints (refer to **Table 3** for an example of selected endpoints). Add 'Keys', which will be the keys pressed when manually scoring the behavior (**Table 3**).

34| Adjust the 'Arena Settings' as described in Steps 34–38. After being prompted to grab the background image, select the first control video in the 'Media Files' folder, and click 'Grab' quickly (ensuring that the fish is not in the frame).

35| Use the square/rectangle tool to define the novel tank within 'Arena 1'.

36| Click 'Zone Group' on the right side of the screen. Use the line tool to draw a horizontal line across the midline of Arena 1, making sure that it crosses the edges of the rectangle coterminous with Arena 1 within Zone Group 1.

37| Select the Label zone (Z) tool. Click the upper half of the novel tank and label it 'Top'. Click the bottom half and name it 'Bottom.' (Do NOT click the 'Add Zone Group' option on the right; it is unnecessary for this protocol).

38| Click 'Calibrate' on the right side of the screen and then select the calibration/ruler tool. Drag the ruler across the bottom of the novel tank and label it with the correct real-life measurement. Do the same for the top-to-bottom measurement as well.

TABLE 3 | Manual scoring setting for EthoVision XT7.

	Settings			
Behavior name	**Behavior type**	**Behavior group**	**Initially active**	**Keys**
Swimming	Mutually exclusive	Behavior	Checked	S
Drifting	Mutually exclusive	Behavior	Unchecked	D
Erratic movement	Mutually exclusive	Behavior	Unchecked	E
Freezing	Mutually exclusive	Behavior	Unchecked	F

This optional set allows the experimenter to manually score various endpoints that EthoVision software is not equipped to detect. A listing of several key behaviors is provided along with their appropriate settings.

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39| Adjust the 'Trial Control Settings'. Click 'Settings' of the first condition box. Click 'Settings' again. Uncheck 'Arena' and check 'Bottom'. Click 'OK' and ensure that the starting condition is set to 'When center-point is in Bottom' for '< = 1 sec'. Click 'OK' and then move to the 'Settings' of the second condition box, setting the condition to be met after a delay of 360 s.

40| Under 'Detection Settings', make the appropriate adjustments to ensure optimal detection of the subject as described in Steps 40–43. Set 'Method' to 'Dynamic subtraction' and 'Advanced Model-based'.

41| Specify that the subject is 'Darker' than the background for detection.

42| Press play to check that the fish is being tracked with these settings.
? TROUBLESHOOTING

43| Check 'Use scan window'.

44| Analyze the videos under the 'Acquisition' option. Open the first video. Press play to check the detection, and if the settings appear to be working, press the green button once the fish enters the bottom to begin the analysis (if the fish does not enter the bottom, e.g., following antidepressant treatment, adjust the trial control settings accordingly).

45| Watch all the videos, performing the manual registration if desired (the experimenter will be prompted at the end of each trial to select the next video).

46| If you prefer to view the results per min, then move to 'Data Profile' and check 'Use time bins of length:' '+0:01:00' min under the Result 1 box. Under 'Analysis Profile', select the desired endpoints to for which to generate data.

47| Move to the 'Analysis Output' tab and click 'Calculate' to generate the results. Go to the 'Export' menu to transfer the results into an Excel file for further statistical analysis.

Statistical analyses of behavioral data ● TIMING 1–3 h, depending on amount of data collected
48| Several options exist for the data analysis. Use the Mann-Whitney *U*-test for comparing two groups. Student's *t*-test may be used for normally distributed data. Our group has devised a useful template to calculate statistics and generate graphs for zebrafish manual- or video-tracking data, which can be downloaded from our laboratory's website at http://www.kaluefflab.com/science.html. For more than two groups, use analysis of variance (ANOVA), followed by an appropriate *post hoc* test (such as Tukey, Dunn, Newman-Keuls or Dunnet test). In general, *n*-way ANOVA can be applied, with commonly used factors being treatment, dose, sex, strain, time, trial or age.

49| If desired, assess animal habituation responses. For this, compare the first 3 min versus the last 3 min (or min 1 versus min 6) for each behavioral endpoint measured (e.g., see ref. 16). Use longer (30 min) trials in separate cohorts of fish to further assess zebrafish habituation.

50| Once both behavioral and endocrine data are obtained, use the Spearman correlation coefficient to analyze the correlation between behavioral and endocrine endpoints. Consider applying factor, principal component and cluster analyses to more globally assess behavioral and physiological phenomena.
? TROUBLESHOOTING

? TROUBLESHOOTING
Detailed troubleshooting advice can be found below with a summary provided in **Table 4**.

Step 1: Housing can influence cortisol levels
As tank crowding and a lack of adequate food intake have been shown to raise cortisol levels in zebrafish[43], proper housing and care[57] must be maintained to avoid confounding data.

Step 2: Drug is not soluble in water
This situation is not uncommon for different psychotropic drugs; consider i.p. administration instead of immersion. Alternatively, try to dissolve the drug in a small volume of organic solvent (e.g., ethanol or Tween) first before adding the drug to tanks. The control fish should receive the same concentration of solvent/detergent in their tanks. Use very small concentrations of solvents, and only if there are no alternative ways to deliver the drug.

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TABLE 4 | Troubleshooting table.

Problem (step)	Possible reason	Solution
Housing increases cortisol (Step 1)	Tank crowding; inadequate food intake	Maintain proper housing in care
Drug will not dissolve (Step 2)	Insolubility	Use i.p. injection or solvent
Memory influences results (Step 4)	Conditioned responses developed	Avoid retesting fish for at least 3 weeks
Abnormal locomotion (Step 4)	Low locomotor activity	Extend testing time; environmental enrichment
	Strain differences	Assess strains' viability for testing
Atypical behavior (Step 4)	Nonspecific factors	Reassess phenotypes to be examined
	Thigmotaxis	Use 3D reconstruction (see **Fig. 5** for an example)
	Strong innate stressor	Exclude fish from analysis
High variability of responses (Step 4)	Genetic influences	Assess strains viability for test; increase sample size
	Animals stressed	Improve husbandry; use less stressful challenges before novel tank test
False positives (Step 4)	Anxious or inactive cohorts	Extend testing time; minimize environmental stress
	Behavioral inhibition	Apply additional tests and/or assess cortisol levels
Sample loss (Step 11)	Loss during homogenization	Section sample; limit probe movement; use glassware; adjust ether amount
Emulsified homogenate (Step 12)	Ether emulsification	Add more ether
High nonspecific binding and low maximum binding (Step 27)	Poor washing	Rewash wells and redevelop
	Contamination	Use ultrapurified water and/or change filter
	Dilution	Prepare reagent solutions again
	Underdeveloped plate	Allot more time for development
Software not detecting fish (Step 42)	Glare	Change contrast and/or detection setting; improve test lighting conditions
Behavioral and endocrine data do not correlate (Step 50)	Drug affects behavior but not cortisol	Assess endocrine responses separately
	Handling stress and anesthetics	Use additional caution; apply additional testing to rule out

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Step 4: Role of memory in modulating zebrafish behavior in novel environments
Zebrafish show good learning and memory[13] and show robust intra- and intersession habituation[16]. Because of this, and because conditioning can develop after a single trial[13], avoid retesting zebrafish in the novel tank and other novelty-based paradigms. If retesting cannot be avoided, make sure to wait at least 3 weeks between trials to minimize the test battery effect. Note, however, that the novel tank habituation represents a robust behavioral phenomenon in zebrafish, and it may be used to further dissect the effects of various experimental manipulations on anxiety and spatial memory (see ref. 16 for details).

Step 4: Zebrafish locomotor activity is uncharacteristically high or low
Optimization of environmental/experimental factors may produce less-confounded zebrafish behaviors. For example, consider improved housing and testing conditions, more careful handling during testing, a smaller battery of tests and environmental enrichment[25,59]. Additionally, if zebrafish behavioral activity levels remain abnormally low, minimizing preliminary testing anxiety and/or disinhibiting behavioral activity can be achieved by increasing the testing duration (e.g., up to 30 min). In addition, consider additional environmental enrichment for the experiments with gravel, decorative rocks, castles or other objects. In many laboratory species, environmental enrichment reduces the overall levels of stress and anxiety[60–62], and may also have a role in zebrafish behavioral studies (see refs. 42,59 and 63 for details on zebrafish enrichment). Furthermore, strain differences can also have a role, with some showing strong initial anxiety (**Fig. 3**). For example, the wild-type (short-fin) strain exhibits more exploratory behavior in the novel tank (e.g., more top entries) compared with the albino, leopard and long-finned mutant strains[12]. Abnormal hyperactivity may also be a strain-specific phenomenon confounding other behavioral measures. Therefore,

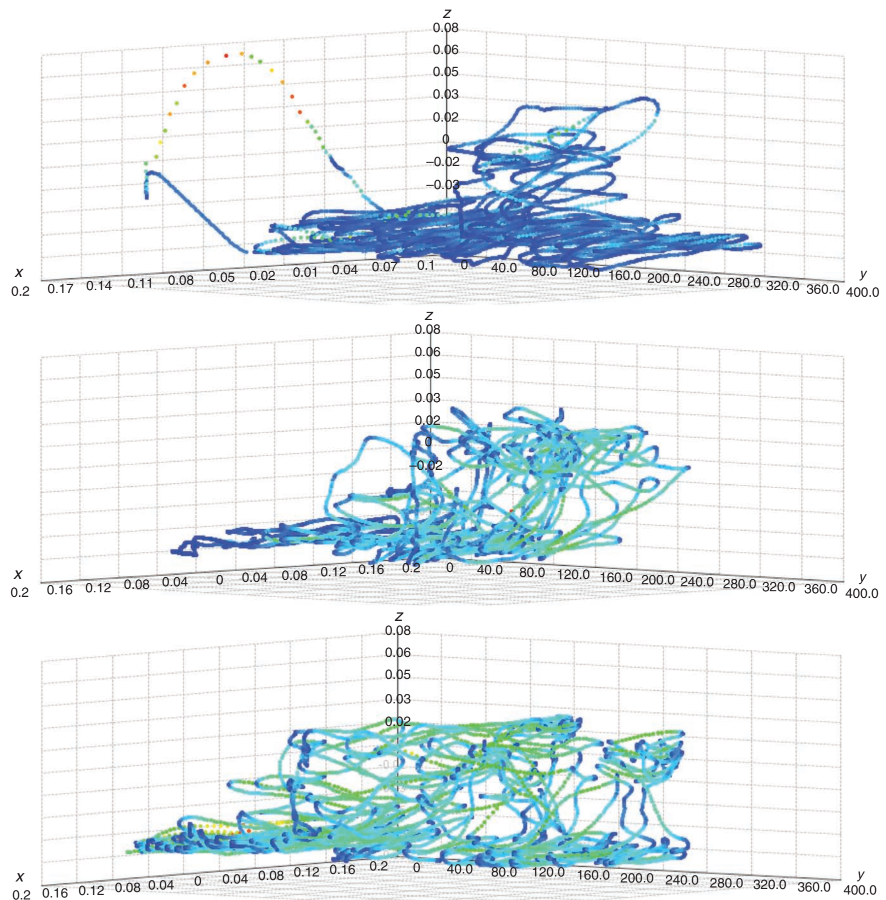

Figure 5 | Temporal three-dimensional (3D) reconstructions of zebrafish traces. In addition to traditional (2D) tracks shown in **Figure 3**, we created the temporal 3D path reconstructions with single-camera recordings in a 3D color scatter plot, in which *X*-center, time and *Y*-center were attributed to the *x*, *y* and *z* axes, respectively. Representative 3D reconstructions of zebrafish novel tank behavior in control zebrafish (top panel) and low-stress fish exposed to either acute (20 min, middle panel) or chronic (2 weeks, bottom panel) anxiolytic 0.2% (vol/vol) ethanol treatment. Color is a function of velocity (increases as the color moves from blue to green). Note that temporal patterning of zebrafish locomotion differs markedly in control versus both 'anxiolytic' ethanol-exposed groups, providing a useful tool for visualizing drug-evoked behavioral alterations in zebrafish (see similar 3D reconstructions used in our lab for characterizing behavioral effects of lysergic acid diethylamide[28]).

it may be necessary to reassess the suitability of the strain for novel tank experiments.

Step 4: Zebrafish show atypical behavior

Note that altered pain sensitivity or other physiological dysfunction may nonspecifically influence zebrafish behavior. Pharmacologically induced factors, such as neurotoxicity and seizures, may also contribute to the misinterpretation of the observed phenotype. Other psychoactive agents (e.g., hallucinogens, opioids) can confound conventional behavioral assessment in zebrafish[12,28,64]. A cautious evaluation of zebrafish neurological and sensory characteristics should be undertaken to exclude such nonspecific effects. Thigmotaxic behavior[65] may also represent a problem, as staying close to the walls may interplay with (and eventually confound) the zebrafish natural diving response in the novel tank test. In this case, a three-dimensional reconstruction of zebrafish behavior using two cameras (e.g., side and top view) may better dissect the two phenomena (see **Fig. 5** for details on spatial three-dimensional reconstruction and visualization of zebrafish behavior). Finally, if using strong innate stressors, such as predator or alarm pheromone exposure[12,66,67], zebrafish may show abnormal escape behavior, resulting in complete freezing and/or jumping out of the tank. Although a rare phenomenon, this may affect the validity of behavioral data (exclude such fish from analyses and ensure their proper handling/recovery or euthanasia).

Step 4: High variability of observed responses

High variability in observed responses is not uncommon in behavioral research and, in some cases, can be accounted for by genetic influences or animal facility–induced stress. As mentioned above, improved husbandry techniques can help normalize behavior. It is also important to carefully control conditions (such as temperature, soundproofing and lighting) in the testing room during experiments. Increase the sample size if necessary. On the basis of our experience, significant zebrafish data may be obtained for n = 12–15 per group[12,16,21] for strong effects, although 20–25 animals per group may be needed to detect smaller effects. As many studies currently involve a battery of tests, these could influence novel tank performance. If using a battery of tests, carry out the least stressful tests before using the fish in the novel tank test. Potential confounds should be minimized by acclimatizing fish for a minimum of 7 d before carrying out the tests within a battery.

Step 4: Testing time and false positives/negatives

Previous research has shown that zebrafish behavior is most robustly affected within the first few minutes of novelty exposure[16]. Therefore, a 6-min testing time is usually adequate for assessing zebrafish anxiety-like behavior in the novel tank test. However, some cohorts or strains may exhibit greater anxiety or decreased activity levels, necessitating a longer testing duration (see ref. 16 for details). In addition, as cortisol levels and behavioral activity vary throughout the day, testing and euthanasia should occur at a similar time of the day over the course of experimentation. Owing to the exploratory nature of

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zebrafish swimming, anxiogenic drugs and nonspecific sedative/toxic doses of other drugs may produce similar (anxiety-like) behavioral inhibition. To minimize the risks of false positives, examine erratic movements (as additional indices of anxiety) to separate high anxiety (low locomotor activity + high erratic movements) from behavioral inhibition (low locomotor activity + low erratic movements). Parallel assessment of cortisol levels may further dissect zebrafish anxiety (will be higher in more anxious groups). Finally, additional anxiety tests in zebrafish (e.g., open field, light/dark box, shoaling) may be needed. Repeat experiments several times using different cohorts of fish to ensure good reproducibility of observed responses.

Step 11: Sample loss during homogenization and extraction
To minimize sample loss, slice the body into smaller portions. This will make homogenization easier and more efficient. During homogenization, movement of the probe should be limited. Although a certain amount of vertical movement is needed, excessive movements will cause the sample to froth and bubble out of the tube. As plastic absorbs cortisol, use glassware instead of plastic (e.g., Eppendorf) tubes. A radioactive tracer, such as tritium, can be used to evaluate cortisol recovery (see ref. 12 for details). Additionally, adjusting the volume of ether used (usually a 1:3 to 1:5 solute:solvent ratio), as well as repeating the extraction several times to increase yield, may be recommended. This may be useful for low baseline cortisol levels or small subjects. However, it is important to keep the amount of ether used and the number of extractions performed uniform across all extractions.

Step 12: Accuracy of cortisol detection with emulsified homogenate
Emulsion may form after adding ether to a homogenate. In this case, adding more ether before centrifugation will improve phase separation. However, note that the same amount of ether must be added to all samples for consistency. Additionally, undersized samples may lead to a cortisol concentration that is inadequate for ELISA detection sensitivity. Therefore, using fish of a similar (normal-length) size is required.

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Step 27: High NSB and low maximum binding
High NSB can result from various causes, including poor washing of NSB wells or their exposure to the antibody. If this occurs, rewash and redevelop wells. Low maximum binding can be caused by various errors, including contamination of the water source with organic solvents, dilution error in reagent preparation and inadequate plate-development time. Water contamination can be resolved by using ultrapurified water, or by changing the carbon filter. If dilution error is the cause, prepare reagent solutions again to ensure that correct dilution is achieved. Low maximum binding may also be the result of an underdeveloped plate, which can be rectified by allotting more time for development.

Step 42: Software not detecting fish
Modifying several settings, such as detection, lighting and background, may rectify a lack of object detection. Optimal lighting will ensure proper contrast and prevent glare (which can be mistakenly tracked as the fish). Dim or overly bright lighting may interfere with object tracking as well. Additionally, using a background uniform in color will also help minimize misdetection. It is also important that arenas/zones are properly defined and accurately calibrated. Endpoint conditions and thresholds must also be correctly specified. For example, the mobility threshold must be tailored for an accurate calculation of freezing duration. However, infrequent unexpected errors (e.g., detection lost mid-trial) may still occur during analysis, thereby requiring reanalysis of the recording.

Step 50: Cortisol response does not correlate with observable stress
Generally, behavioral and endocrine markers of stress show high correlation in zebrafish models[12,21]. However, some drugs may affect cortisol levels without affecting anxiety behaviors. For example, naloxone administration in humans and rodents elevates cortisol levels without an exacerbation of anxiety[68–70]. Hallucinogenic drugs, such as lysergic acid diethylamide, may affect zebrafish cortisol levels without causing anxiety[28]. Therefore, for some drugs, zebrafish endocrine responses must be assessed separately from anxiety. In addition, note that both handling stress and anesthetics (including tricaine) may modulate cortisol levels in different fish species[51,71–73]. For example, tricaine anesthesia reduces handling-evoked cortisol levels[74,75] but increases cortisol at high doses[74,76–78]. To examine the effect of tricaine on zebrafish cortisol levels after novel tank testing, we used a 6-min novel tank test followed by a 6-min exposure to 500 mg per liter tricaine. Compared with unexposed controls, the novel tank-exposed fish euthanized with tricaine showed unaltered levels of whole-body cortisol (data not shown). This observation, consistent with other fish studies[78], suggests that current protocol (involving a brief novel tank testing followed by euthanasia with tricaine) does not confound the zebrafish endocrine data obtained.

● **TIMING**
Steps 1 and 2, acclimation: 1 h for each cohort of experimental animals. If pretreating animals with drugs, allow enough time for drug pretreatment before behavioral testing.
Steps 3–6, novel tank testing: 6 min per fish. If performing testing of multiple animals, allow an additional 3–4 min in between each trial for setting up the next test. Longer (e.g., 30 min) testing trials may be used, if necessary, and will require additional time.
Steps 7–14, cortisol extraction: 150 min per group of 15 samples, with an additional 12 h for ether evaporation.

Steps 15–27: incubation ~12 h (usually performed overnight); ELISA protocol: 6–7 h; ELISA data analysis: ~1–2 h, depending on the number of samples.
Steps 28–47, video tracking fish behaviors: ~6 min for the program and/or observer to analyze each video; program setup and design will vary.
Steps 48–50, statistical analyses of behavioral data: uploading and analysis of data will vary depending on amount collected. Data can be analyzed both off- and online, depending on experimental design and software configuration.

ANTICIPATED RESULTS

During a standard 6-min novel tank test, increases in normal (naïve) zebrafish exploratory behavior and decreases in their freezing behavior are expected to occur (**Figs. 1–3**, **Supplementary Video 1**). Both manual and various video-tracking tools (e.g., CleverSys TopScan, Noldus EthoVision, **Fig. 2a–d**) can be used for behavioral analysis in zebrafish. Fish behavior can be either attenuated or exaggerated depending on stress or drug exposure. By comparing the treated fish with controls, the experimenter should expect to find statistically significant behavioral differences across a variety of endpoints (refer to **Table 1** for the interpretation of each behavioral endpoint). For example, the administration of anxiogenic drugs has been demonstrated to lower overall transitions to, and time spent in, the top of the tank, as well as to increase the occurrence and duration of freezing bouts, relative to controls (**Fig. 2c**,**d**). In contrast, anxiolytic treatments induce a greater number of transitions to and longer time spent in the top, consistent with reduced anxiety levels (**Fig. 2a**,**b**). Overall, this trend is generally consistent among strains, but may vary in terms of the latency, occurrence and duration of behaviors (**Figs. 1** and **2**). Moreover, an examination of the computer-generated traces will reveal the patterning of their activity, with anxiogenic substances leading to overt bottom dwelling and anxiolytic agents producing traces that demonstrate robust exploratory behavior (**Figs. 3** and **5**). Finally, cortisol levels are generally expected to parallel amounts of anxiety behaviors, and will vary accordingly (**Fig. 4**). For example, treatment with an anxiogenic agent will lead to greater anxiety-like behavior, and it will also raise zebrafish cortisol levels.

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Note: Supplementary information is available via the HTML version of this article.

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