University of Crete, Department of Biology

Zebrafish Behavioral Indicators of stress & anxiety

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Behavioral studies in fish

There are numerous fish species used as model organisms to study fish behavior. Some of them include:

- *Danio rerio* (zebrafish)
- *Notobranchius furzeri*
- *Oryzias latipes* (medaka)
- *Poecilia reticulata* (Guppy)
- *Gasterosteus aculeatus* (three-spined stickleback)
- *Oreochromis niloticus* (Nile tilapia)

Zebrafish as a model organism for behavioral studies

- Exhibit a wide repertoire of behaviors.
- Its small size along with the low cost housing permits high-throughput screening.
- Readily available video tracking technologies that can be coupled with zebrafish behavioral assays, providing data-rich endpoints (e.g. velocity, distance travelled, three-dimensional spatial and spatiotemporal swim path reconstructions) which are impossible to generate manually.
- Possess all major neurotransmitter systems, transporters, receptors and hormones.
- Fully sequenced genome with 70-75% of human genes having at least one zebrafish orthologue.

Zebrafish is one of the most frequently used fish species for behavioral purposes, leading to a rapid development of numerous protocols to study several behavioral phenotypes.

- Stress
- Anxiety
- Aggression
- Memory & Learning
- Reward
- Social behavior

Zebrafish as a model organism for anxiety and stress research

- Robust and easily quantifiable **cortisol** stress response.
- Clear-cut **drug-evoked phenotypes** with high predictive validity.
- Sensitivity to a wide range of **experimental stressors**, such as:

Novelty exposure	Predator exposure
Social isolation	Alarm substance
Confinement	

Disorder	Zebrafish Phenotypes
Anxiety/Fear-related behavior	• Reduction of **exploration** (especially in the top part of novel environments) • Increased **avoidance** • Erratic behavior and freezing • Elevated **cortisol** and brain *c-fos* • Highly sensitive to anxiolytic and anxiogenic agents

Kalueff *et al.*, 2014

Novelty based tests for studying anxiety

A novel environment constitutes a potentially dangerous situation for zebrafish. As a result, fish exhibit **avoidance behavior** which serves evolutionary conserved anti-predatory functions.

An animal's **exploratory behavior** in a novel environment is believed to reflect the emotional state of animals.

Tests in this category include:

1. Novel tank test (vertical exploration)
2. Open field test (horizontal exploration)
3. Light/dark preference test

Novel Tank test

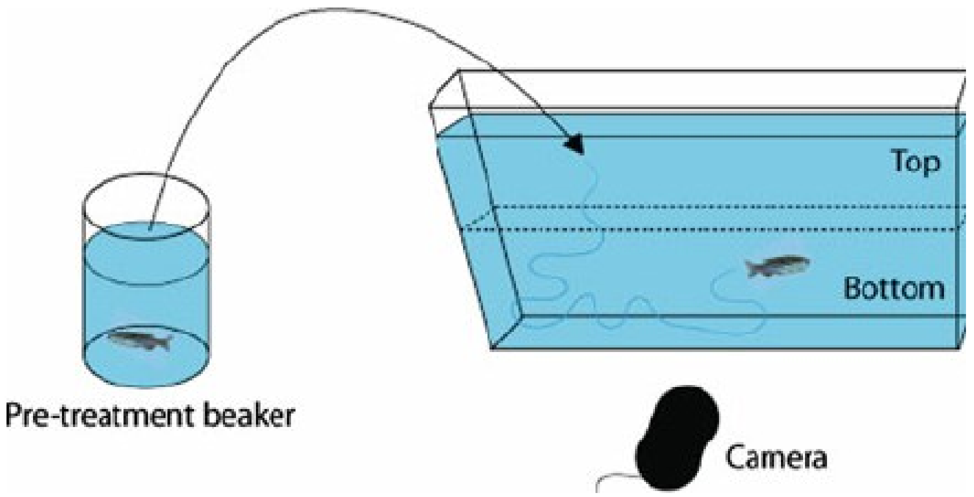
A novelty based paradigm, which measures **vertical exploration**.

Zebrafish express a robust **anxiety-like response**, once introduced to the novel tank apparatus, consisting of:

- Diving to the bottom of the tank (geotaxis)
- Reduced exploration
- Increased freezing
- Erratic movements

These behavioral responses are accompanied by **physiological** responses:

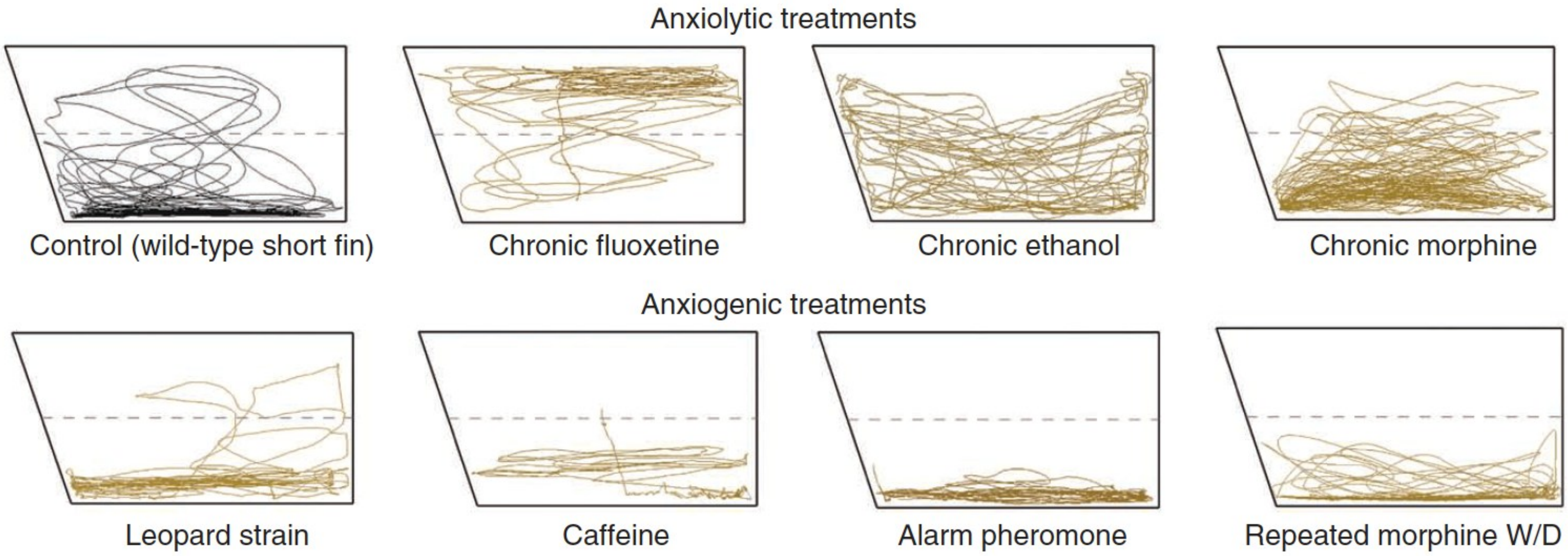
- Elevated cortisol levels
- Increased breathing
- Increased heart beat frequency.



Measured parameters:

- ✓ Time spent in the top part of the tank
- ✓ Transitions to the top
- ✓ Latency to the top
- ✓ Erratic movement
- ✓ Freezing incidents
- ✓ Time spent freezing

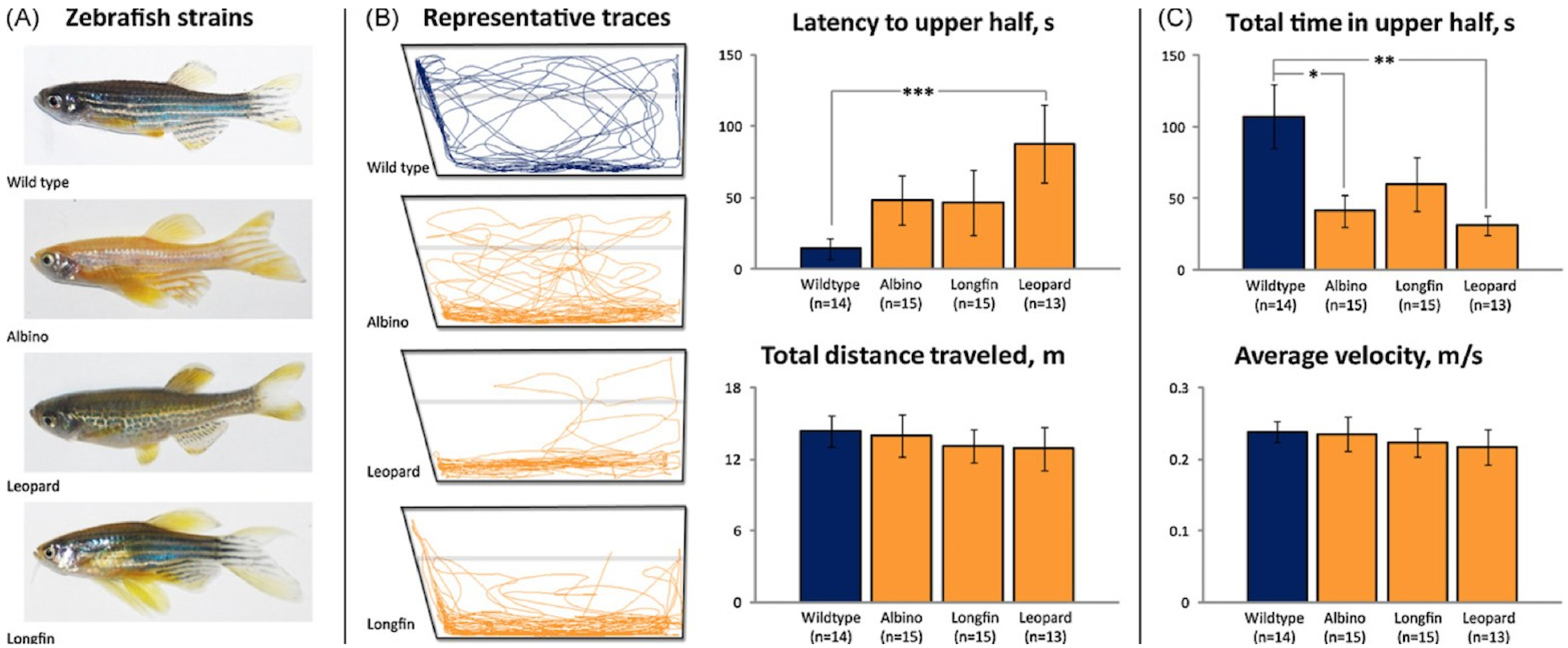
Novel Tank test



Representative trace of the zebrafish movement in the novel tank test (6 minute trial). Different experimental manipulations show a decrease or increase in the anxious phenotype, compared to the control tank.

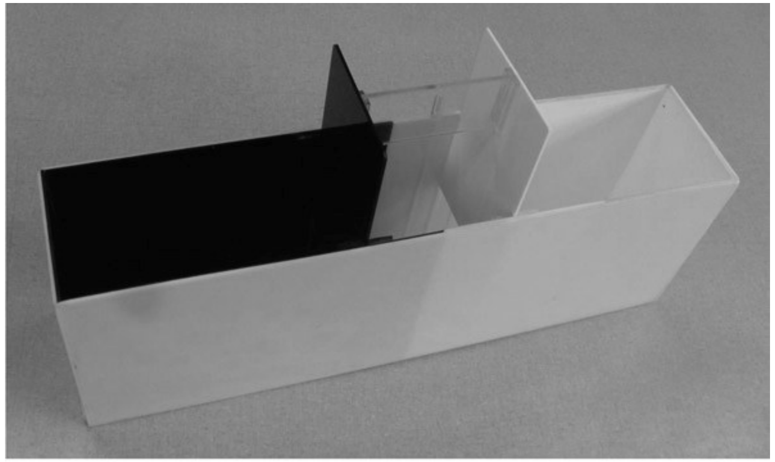
Cachat *et al.*, 2010

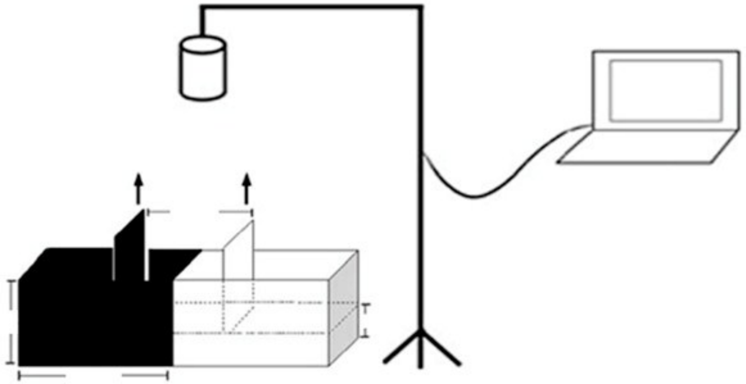
Novel Tank test



Egan *et al.*, 2009.

Light/dark preference test





Measured parameters:
- ✓ Time spent in the white zone
- ✓ Time spent in the black zone

Adult zebrafish, as well as other fish species (e.g. goldfish, guppies, minnows and tilapia), are generally believed to display an innate **aversion to bright** and white environments, and a **preference for darker** environments.

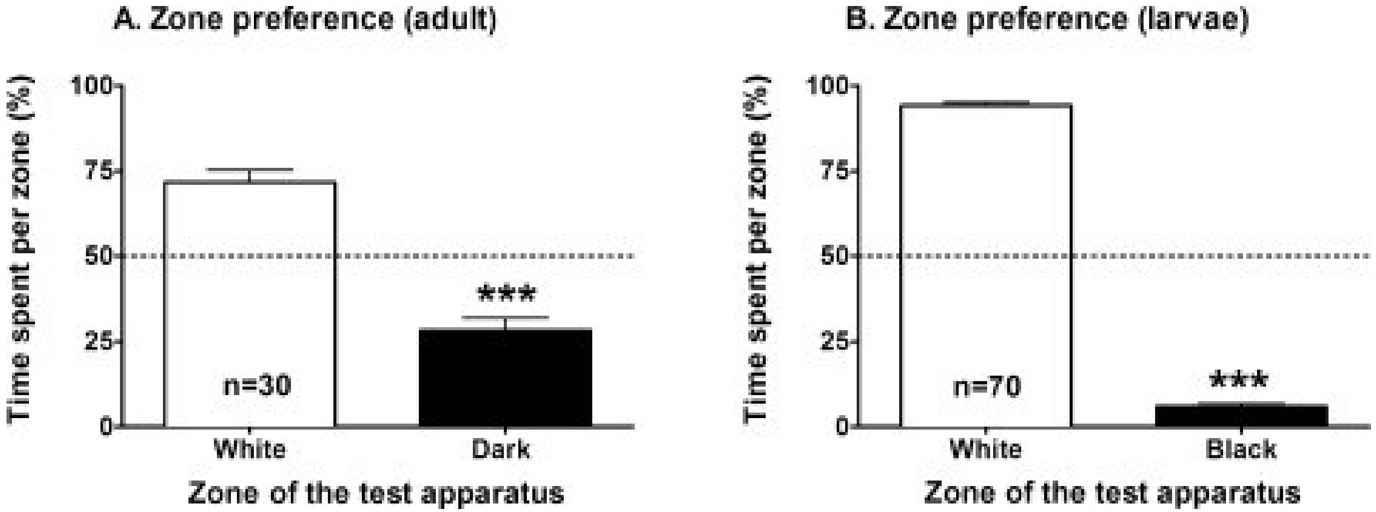
However, there are some reports which indicate a preference for the white area of the tank in zebrafish. The inconsistencies in the literature are largely attributable to differences in housing conditions (such as lighting), sex, age, social status and genetic strains.

Fish are left to acclimatize for 3-5 minutes in the centrally isolated compartment.

The separating doors are removed and fish are left to freely move in the tank for 15 minutes.

Light/dark preference test

Avoidance of the dark area & preference for the light/white area
in both adult zebrafish and larvae.



Champagne *et al.*, 2010

Exploratory tank test

Quantification of the **exploratory** behavior of adult zebrafish.

A shoal of conspecifics is placed in the first compartment and left there undisturbed for 24 hours. → The next day the experimenter opens the "door" of the second compartment and throws some food pellets to the first and second compartment. → Fish are left free to explore the tank for 8 days. They are fed daily according to their feeding regimen.

Chronic stress protocols

Protocols that expose zebrafish to **unpredictable chronic stress** (UCS) conditions.

- The majority of them last for 14 days.
- Fish are exposed to two stressors daily. The stressors are chosen randomly every day. Some of the stressors applied in protocols of chronic stress include:

- Restraint stress
- Social isolation
- Over-crowding
- Dorsal body exposure
- Tank change
- Cold stress
- Heat stress
- Chasing
- Predator exposure
- Alarm pheromone stress

Chronic stress protocols

Behavioral, physiological and cellular responses, similar to those observed in rodents and chronically stressed humans.

More specifically, the stress protocol induced:

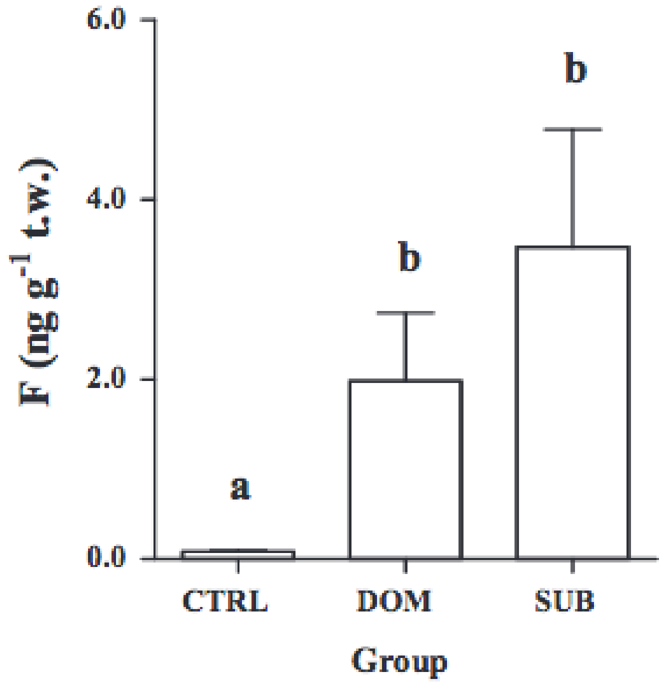
- Anxiety
- Cognitive impairment
- Neuroendocrine dysfunction (increased cortisol and *CRF* levels).

Impaired memory	Piato *et al.*, 2011
↑ Cortisol	Piato *et al.*, 2011; Pavlidis *et al.*, 2015
↑ CRF expression	Piato *et al.*, 2011
↓ Neurogenesis	Chakravarty *et al.*, 2013
↑ POMC, GR, MR, prolactin, BDNF, hypocretin/orexin, and c-fos expression	Pavlidis *et al.*, 2015

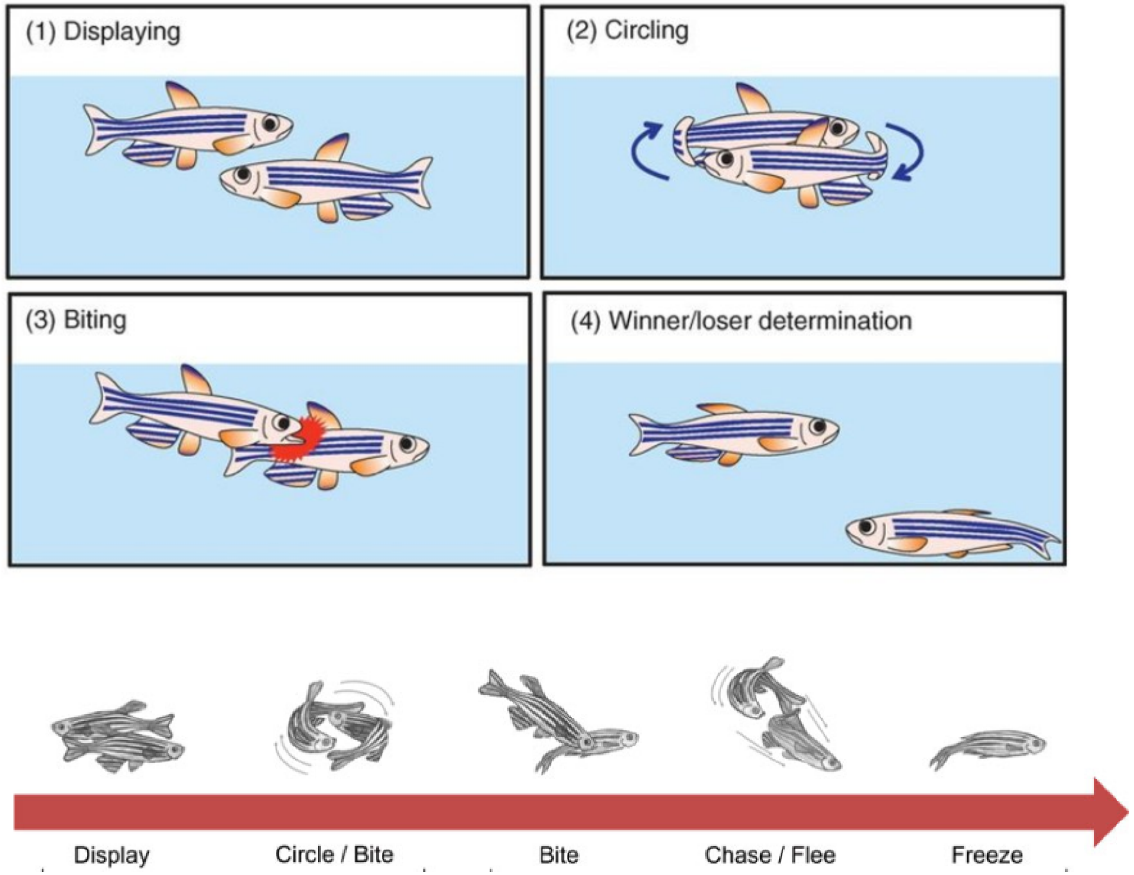
Aggression in zebrafish

Zebrafish in nature form shoals, yet when put in pairs they often exhibit an aggressive behavior that leads to the establishment of a social hierarchy. The “winner” of this dyadic interaction is considered the **dominant** individual, while the “loser” is the **subordinate**.

The established hierarchy consists a form of **social stress** for both the dominant and the subordinate, therefore, zebrafish exhibit anxiety-like behavior. Both dominants and subordinates show higher cortisol levels compared to controls (Pavlidis *et al.*, 2011).



Aggressive behavior in zebrafish



Oliveira *et al.*, 2011

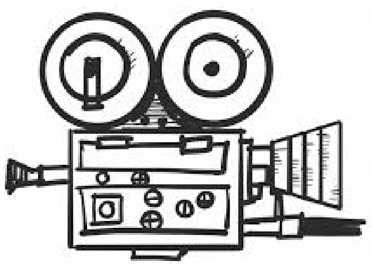
Displaying consists of an approach to the conspecific followed by a turn to the left or right with fins erect.
Circling is considered another form of lateral display that may last an extended period and during which the fish rise in the water column.
Chasing is a pursuit or a quick approach to an individual.
Bites consist of closing the mouth against another individual.
Freezing is the term that describes the immobility state with retracted fins.
A fleeing individual moves away from a pursuing individual for an extended period of time.

Dominant behavior consists mainly of **chasing** and **biting**, while subordinate behavior is expressed as **fleeing** and **freezing**.

Unconditioned social interaction test / Paired aggression test

Pairs of same sex adult zebrafish are introduced in 2L tanks

2 h →



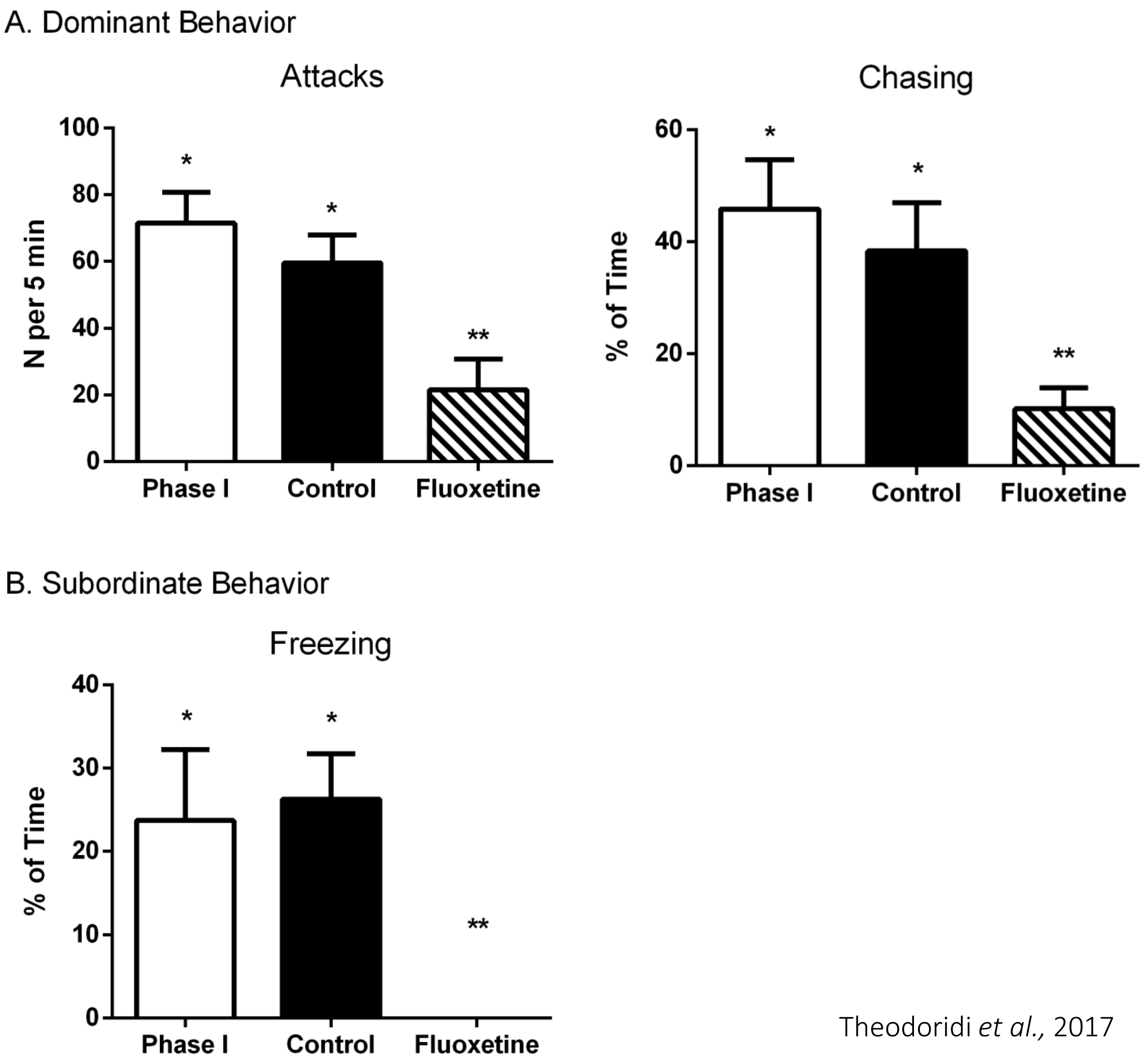
Recording of the pair's behavior for 5 minutes.

→

Behavioral analysis and quantification of the **dominant** and **subordinate** behavior

↓

- Number of attacks from the dominant
- Total duration of chasing
- Total duration of subordinate's freezing behavior



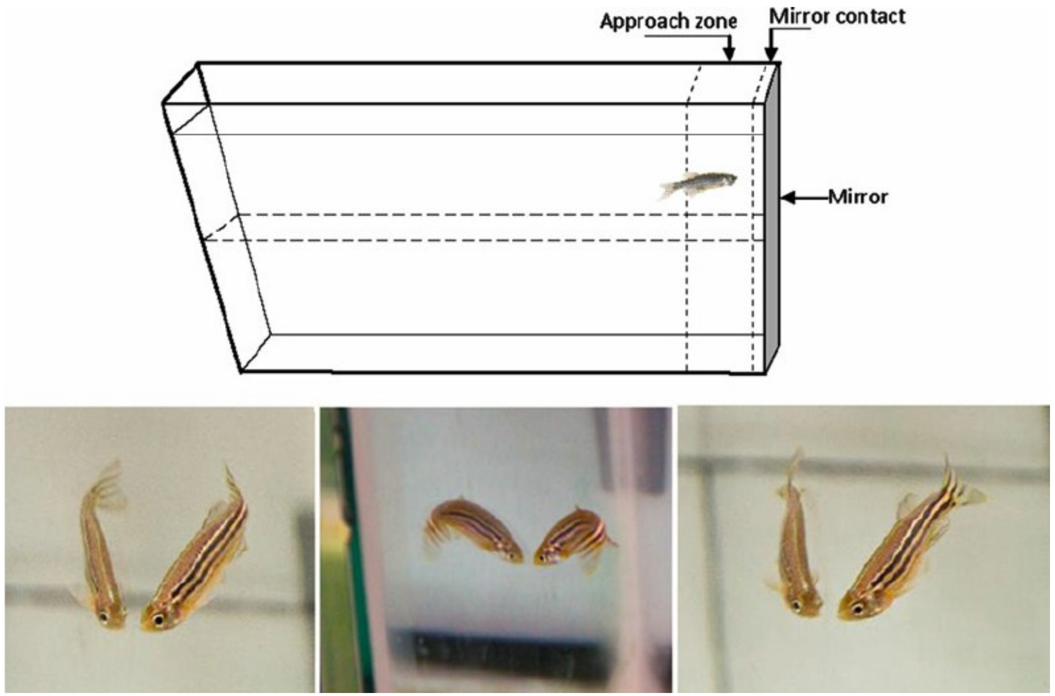
Acute exposure to fluoxetine can alter the aggressive behavior of adult male zebrafish.

Theodoridi *et al.,* 2017

Mirror test

An established protocol to study **social** and **aggressive** behavior in adult zebrafish.

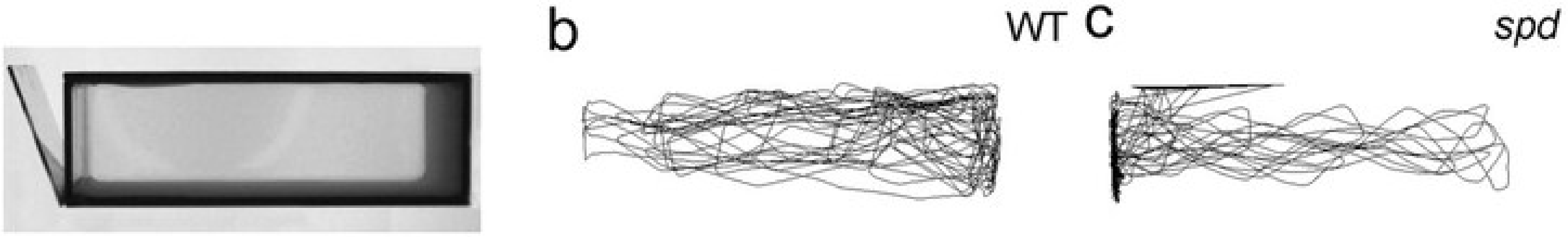
Not possible to observe the full repertoire of aggressive behavior and it has been shown that zebrafish have different endocrinological and genetic responses to a mirror "fight" (Teles *et al.*, 2013), but the risk of injury is avoided.



Measured parameters:

- ✓ Frequency of attacking the mirror
- ✓ Total duration of biting the mirror
- ✓ Number of contacts with the mirror
- ✓ Latency of approaching the mirror

Mirror test

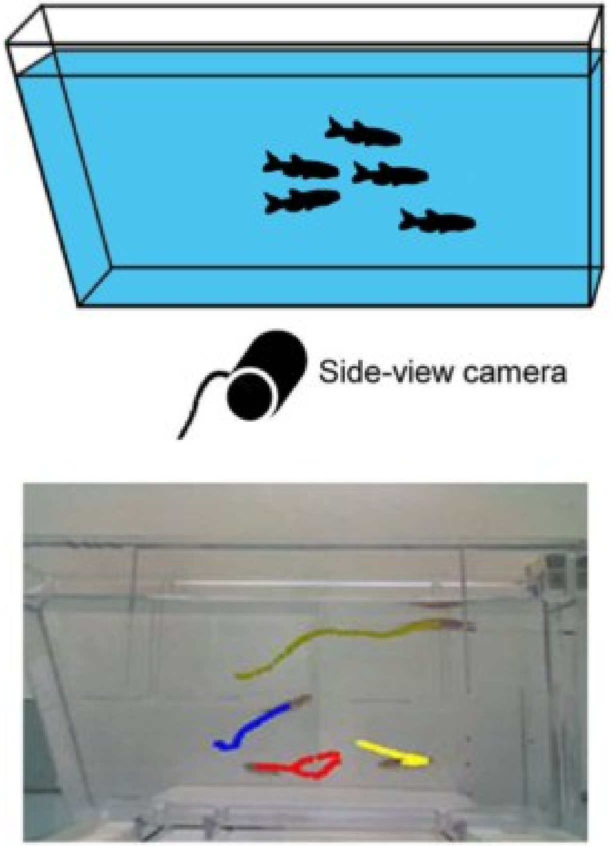


Norton *et al*., 2011

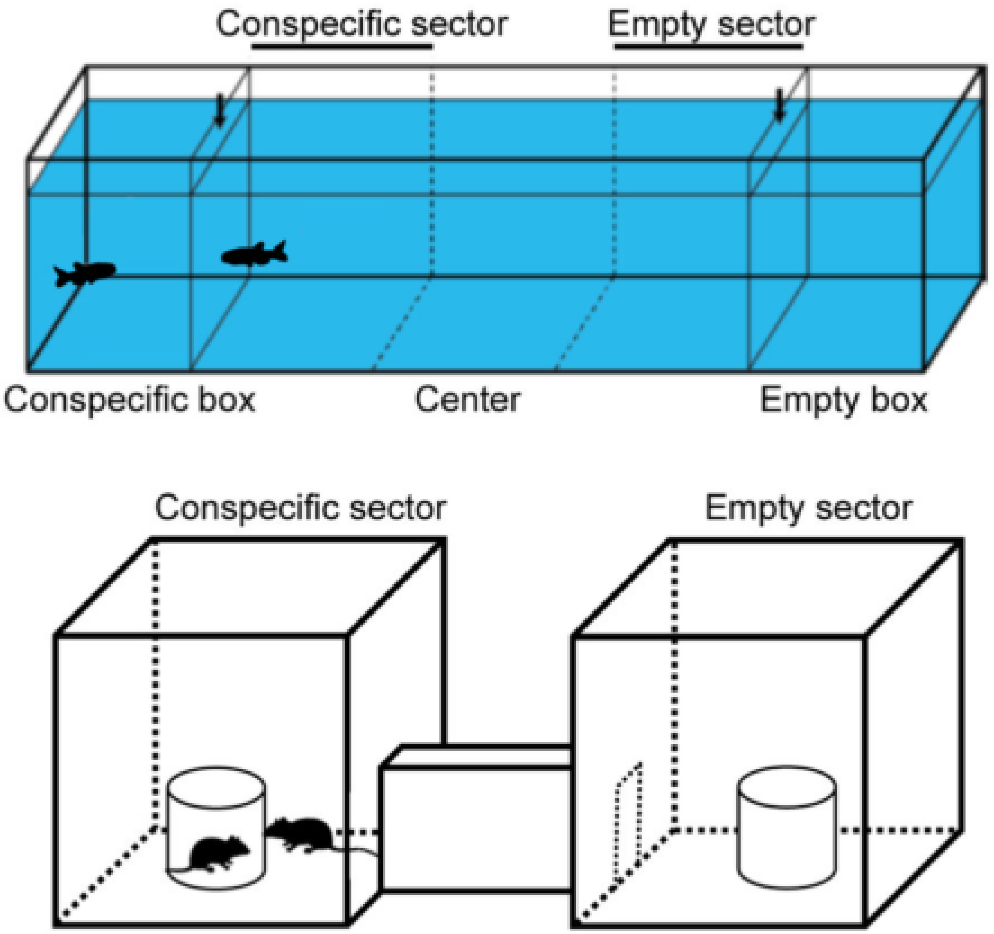
Representative trace of the swimming behavior of WT (b) and mutants with a robust aggressive phenotype (knock-out of **fibroblast growth factor receptor 1a** (Fgfr1a)) (c).

Tests assessing social behavior

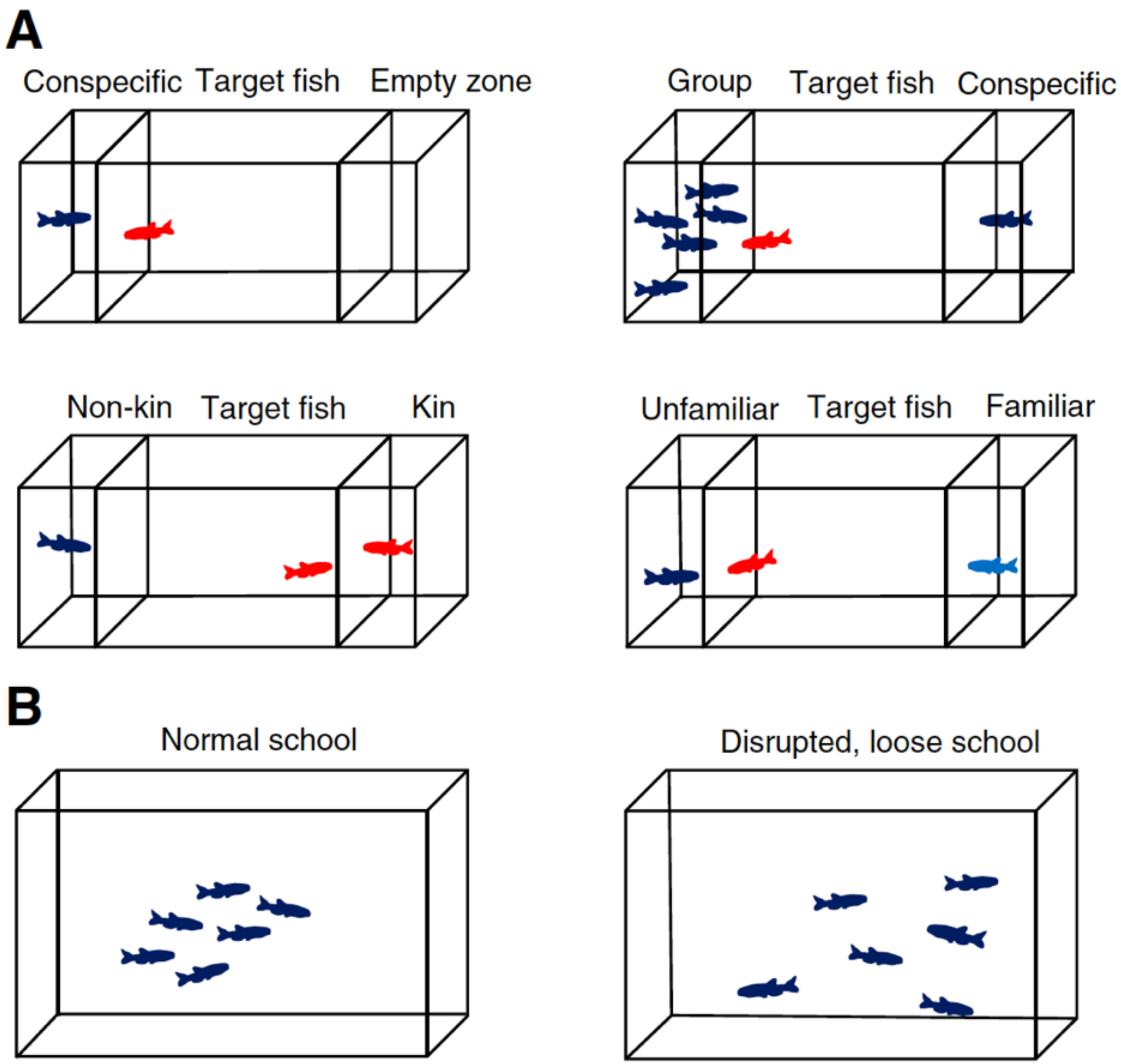
Shoal cohesion test



Social preference test



Tests assessing social behavior



Stewart *et al.*, 2014

Social buffering

SCIENTIFIC REPORTS

OPEN

Mechanisms of social buffering of fear in zebrafish

Ana I. Faustino[1,2,3,†], André Tacão-Monteiro[1,3] & Rui F. Oliveira[1,2,3]

Social buffering: the phenomenon by which group living and presence of conspecifics reduces stress responses.

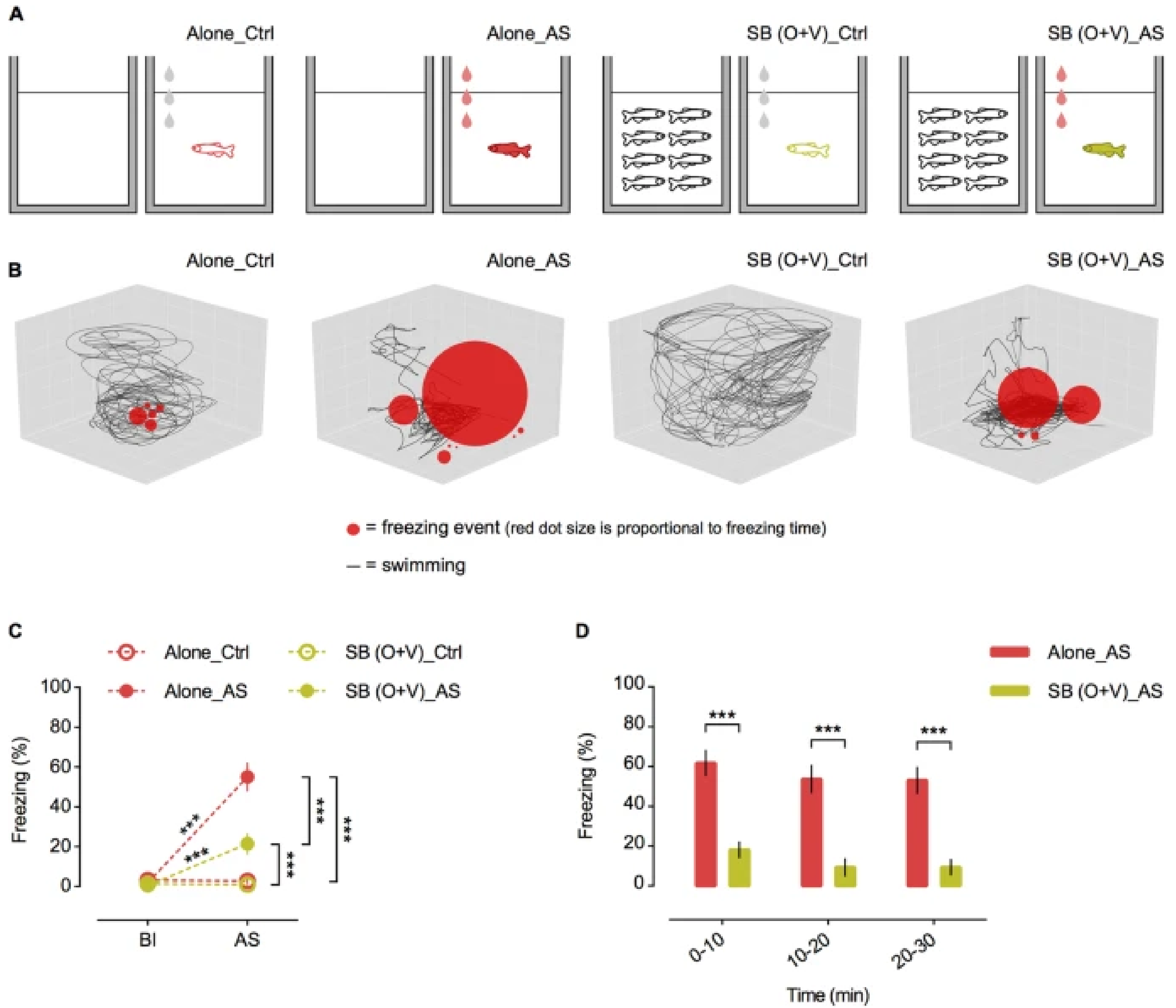
Important potential for the enhancement of welfare in captive fish.

An analysis of social buffering of stress response in zebrafish verified that the effect is independent of shoal size and related to brain regions known to be involved in mammalian social buffering.

Adult zebrafish were exposed to an aversive stimulus (alarm substance) either in the absence or presence of conspecific cues.

The behavioral analysis focused on these five stress indicators:

- location in tank and
- freezing.

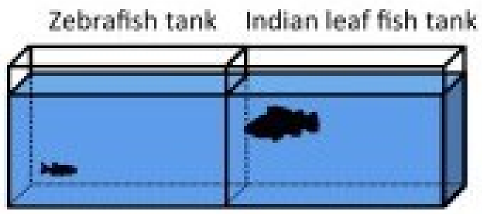


"When exposed to AS in the presence of both olfactory (shoal water) and visual (sight of shoal) conspecific cues, focal fish exhibited a ***lower fear response*** *than when tested alone, demonstrating social buffering in zebrafish. When separately testing each cue's effectiveness, we verified that the* ***visual cue*** *was more effective than the olfactory in reducing freezing in a persistent threat scenario."*

Faustino *et al*., 2017

Predator avoidance test

Zebrafish natural predators include among others Indian leaf fish (*Nandus nandus*), freshwater garfish (Xennentodon spp.), catfish (*Mystus bleekeri*), as well as avian predators.



Kalueff *et al*., 2014

In laboratory conditions, the presence of the Indian leaf fish induces fear-like responses in zebrafish, which consist of:

- Increased frequency of escape efforts and
- erratic movements post exposure to a predator (Bass & Gerlai, 2008).

Indian leaf fish, *Nandus nandus*

Depending on the species of predator that they are exposed to, zebrafish adjust their defensive and fear-like behaviors (Ahmed *et al*., 2012).

Predator avoidance test

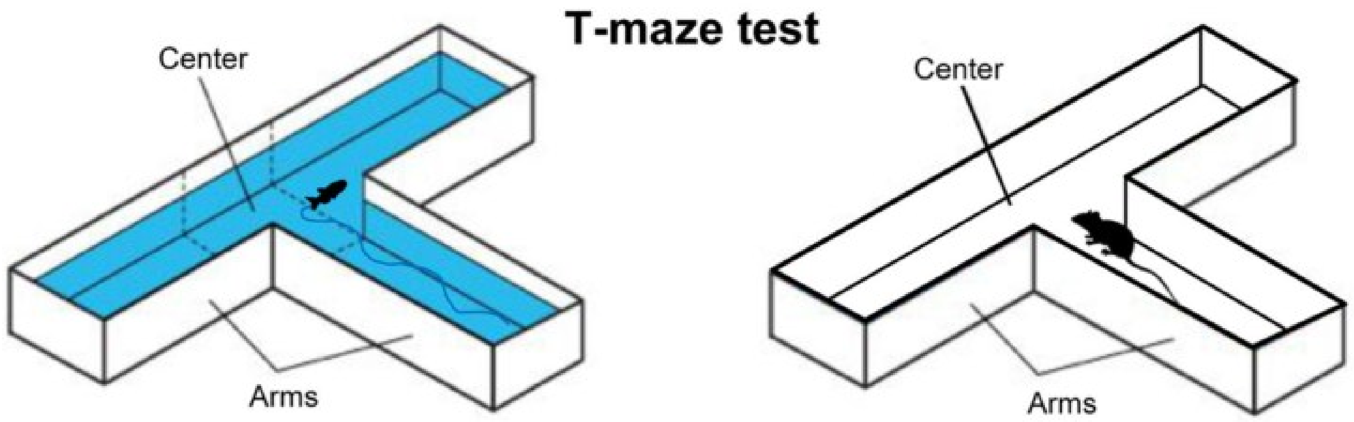
Exposure to both live predator and the robotic model induced a robust avoidance response in zebrafish. Contrary to this, computer-animated images failed to elicit a fear-like behavioral response.

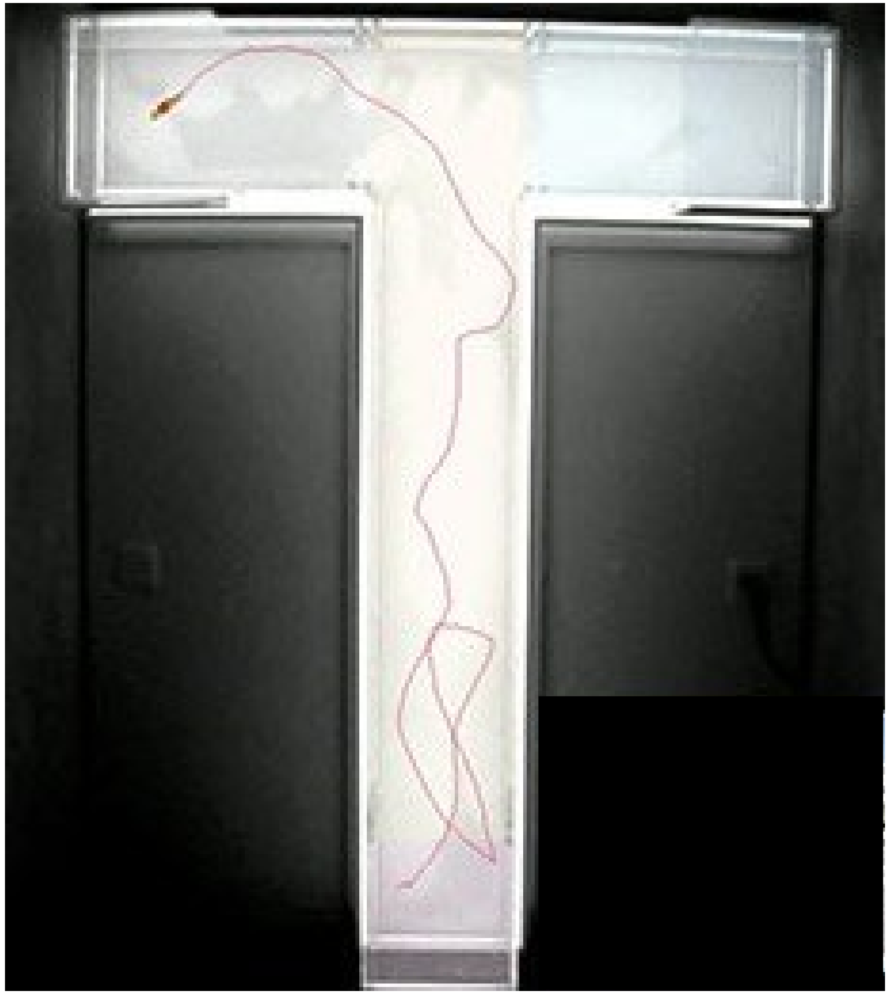
(a) Live red tiger oscar; (b) robotic predator designed after the live oscar; and (c) snapshot of the computer-animated of live Oscar

Ladu *et al.*, 2015

T-maze

- Learning capacity
- Long-term memory
- Short-term memory
- Memory plasticity

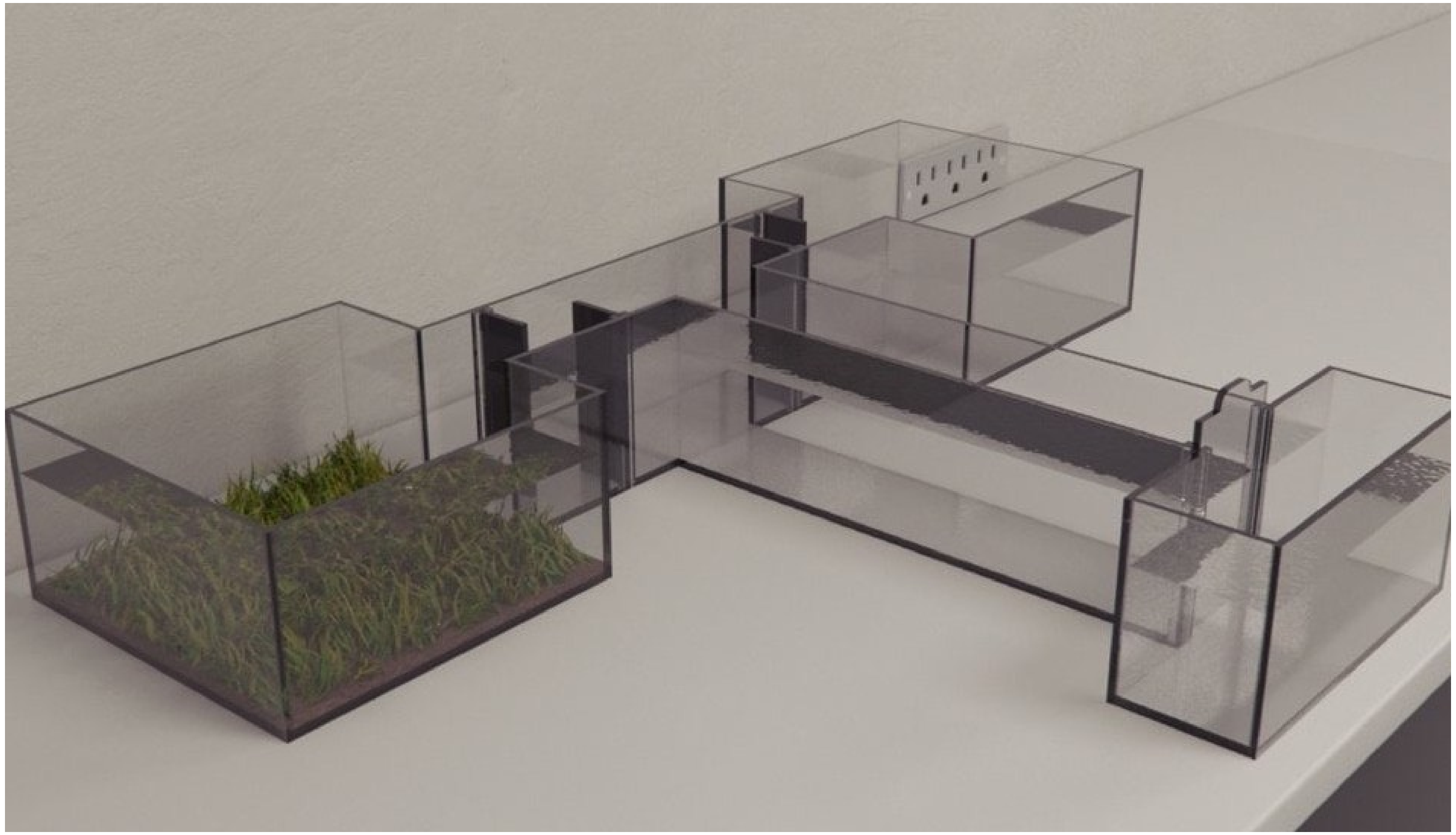




T-maze

Alternative structure

One arm has an enriched environment which is favorable to zebrafish (plants and marbles).



Assessing stress response in zebrafish larvae

Fewer behavioral protocols have been established for zebrafish larvae. The most commonly used are:

- Acute stress response to a swirling stressor
- Tapping assay
- Light/Dark preference
- Open field test

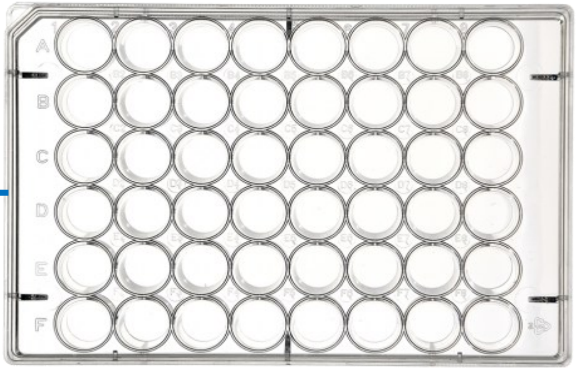
Behavioral studies in zebrafish larvae

Adult zebrafish used for reproduction

Fertilised eggs

Larvae 5 days post fertilization (dpf)

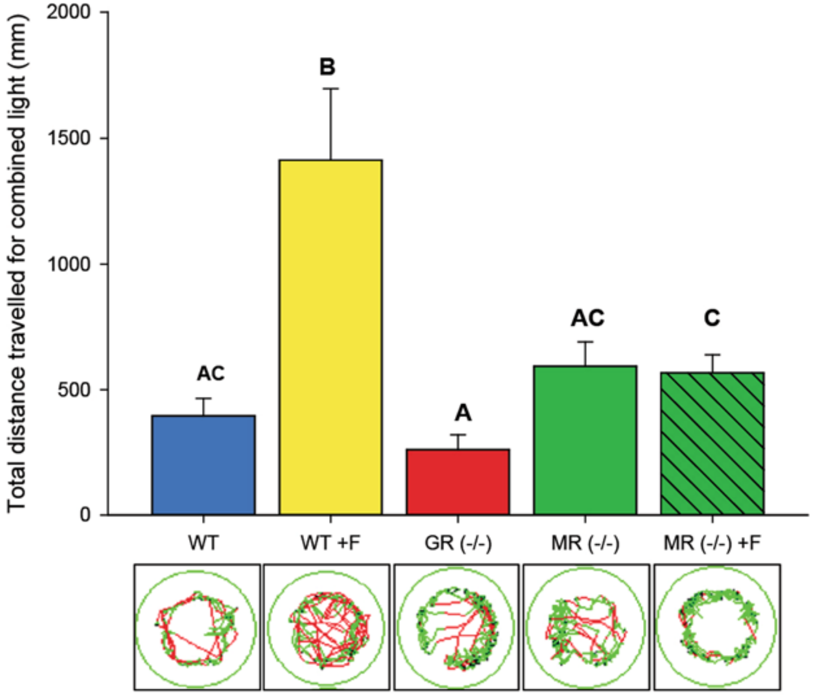
Transfer of larvae in cell culture plates



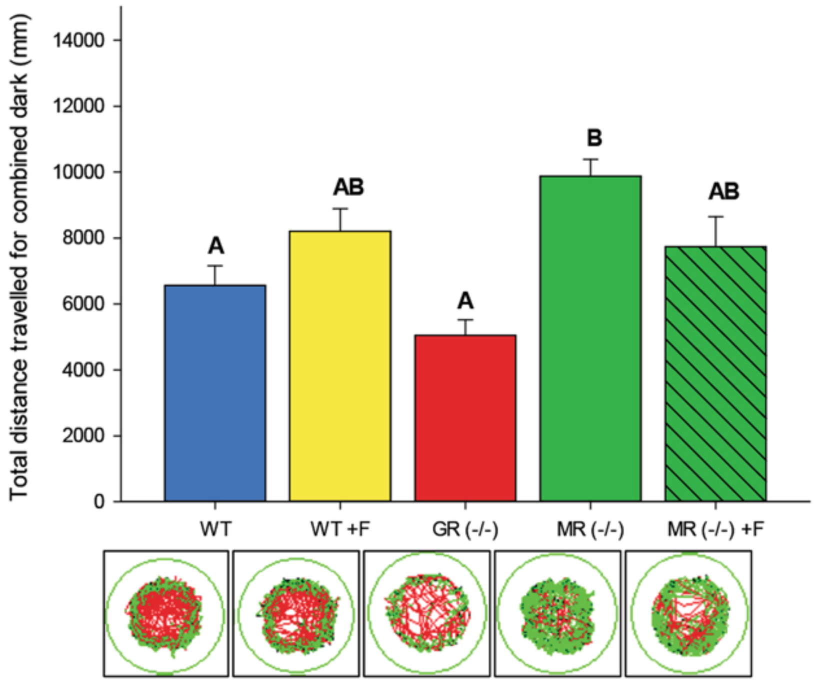
Observation Chamber

Assessing stress response in zebrafish larvae

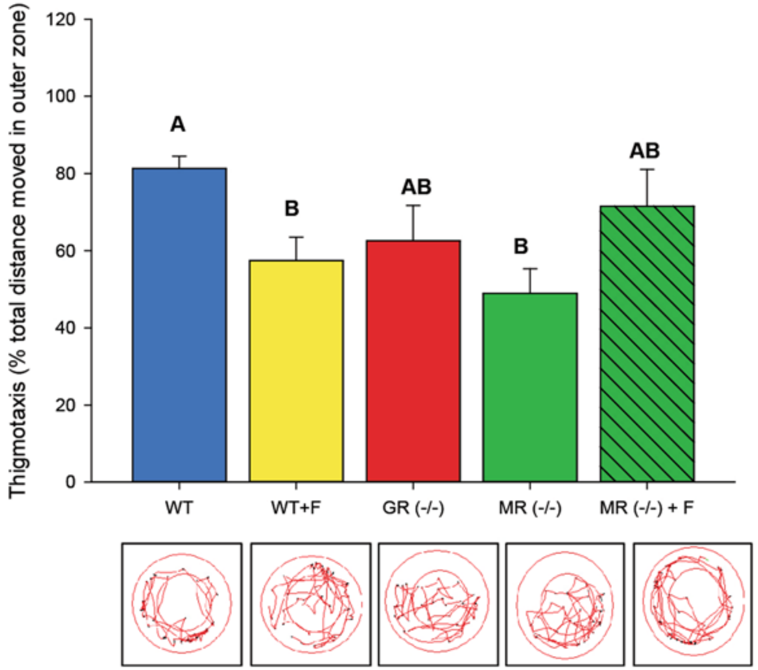
Total distance moved in the light period



Total distance moved in the dark period



Thigmotaxis

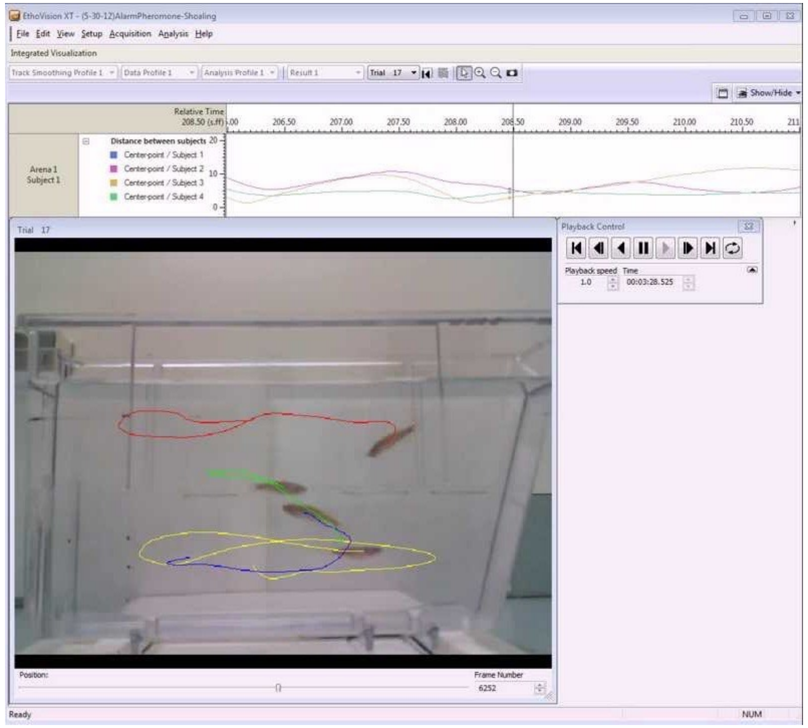


Larvae with different genetic manipulations related to genes regulating the HPI axis display differences in light/dark preference and anxiety levels (thigmotaxis).

Faught & Vijayan, 2018

Behavioral analysis software packages

Noldus



Ethovision

Daniovision

Behavioral analysis software packages

Viewpoint

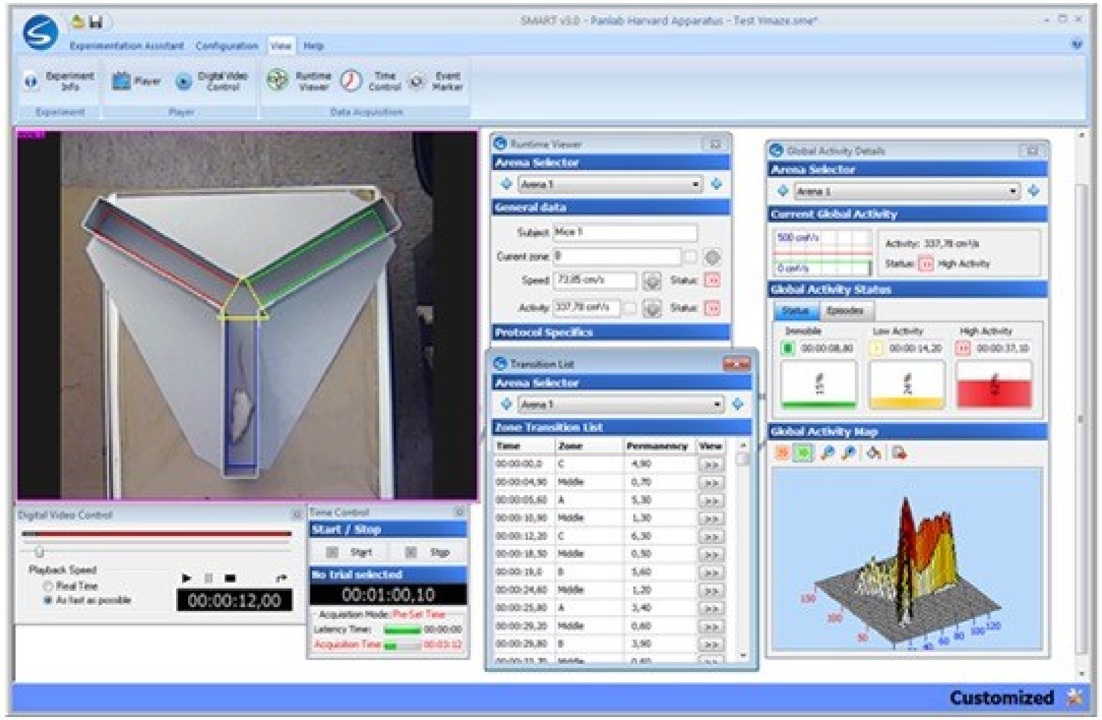
Zebrabox

Zebracube

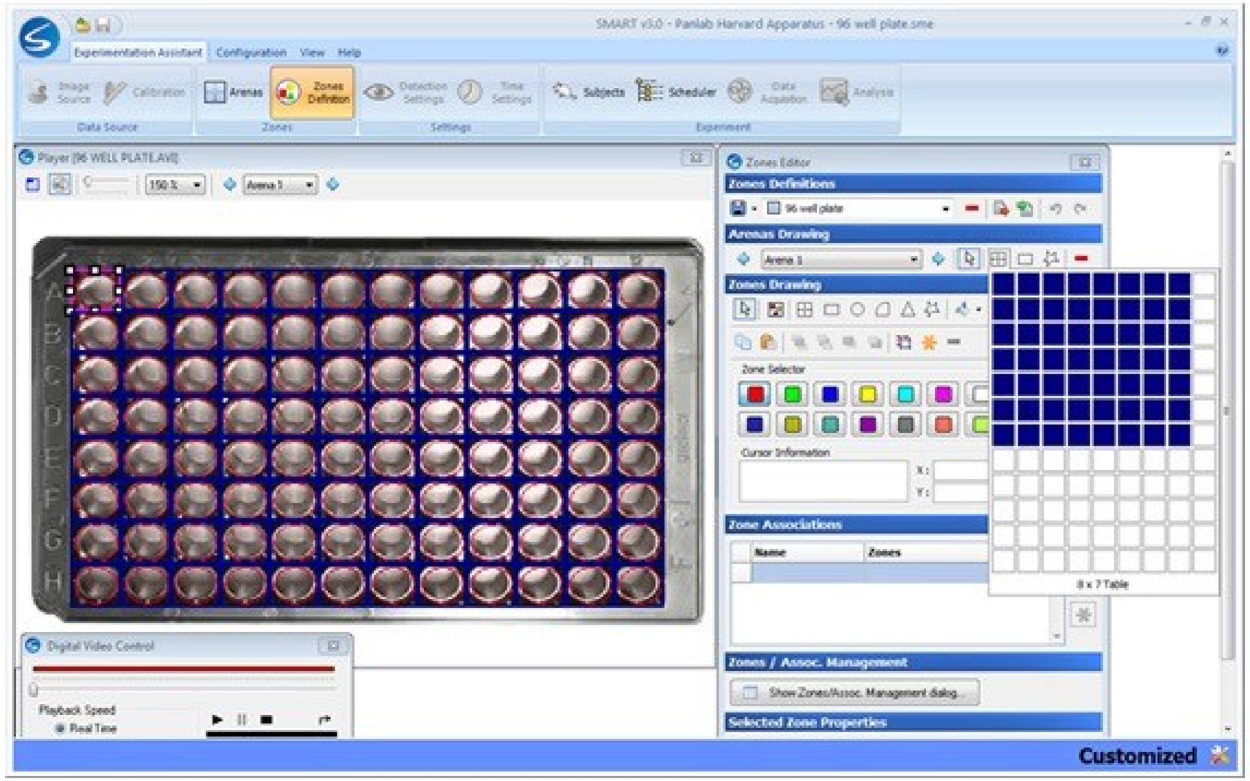
Behavioral analysis software packages

Smart 3.0

Y-maze Spontaneous Alternation



Zebrafish Larvaes Activity



Open-source behavioral analysis software packages

Software	*Operative system/program*	*Types of analyses*	*Types of input*	*Types of output*	*Webpage*
wrMTrck	Windows and Mac OS X/ JAVA-ImageJ	Total length, average speed, area, perimeter, and trajectories.	AVI files with jpg compression	txt, xls, tiff files and AVI videos	www.phage.dk/plugins/ wrmtrck.html
Mouse Behavior Tracker	Windows, Mac OS X and Linux/JAVA-ImageJ	Distance and average velocity.	AVI or MPEG-compressed AVI files, Mp4	Txt or xls files	www.BioTechniques.com/ article/114607.
AnimalTracker	Windows, Mac OS X and Linux/JAVA-ImageJ	Total length, average speed, and time spent in ROI.	AVI files with jpg compression	txt, xls, tiff files and AVI videos	animaltracker.elte.hu
idTracker	Windows/MATLAB	Trajectories, identification of one animal in different videos and ROI.	Compatible with MATLAB, uncompressed AVI or MPEG-compressed AVI files	*X* and *Y* coordinates and images files	www.idtracker.es
Mousetracker	Windows, Linux and Mac OS X/Pascal-Delphi-MS Excel	Velocity, acceleration, and time spent in ROI.	AVI format	*XY* coordinates can be copied directly	www.neuro.ufrn.br/ softwares/mouselabtracker
JAABA	Windows, Mac OS X and Linux/MATLAB	Bites, persecution, sexual behavior, angle of turn, grooming, jump, walk, immobilization, and touch. Locomotion and ROI.	Several formats and resolutions. *X* and *Y* coordinates	MATLAB files	http://jaaba.sourceforge.net https://www.janelia .org/lab/branson-lab
Ctrax	Windows and Mac OS X/ Phyton—MATLAB	Trajectories, velocities, speed, position, and turning speed histograms.	Common digital video formats, mainly AVI	csv and mat files. Converts the file to .ann extension	ctrax.sourceforge.net
VideoHacking	Windows, Mac OS X and Linux/Phyton—Open CV	Velocity, acceleration, total length, average speed, and time spent in ROI.	Common digital video formats	Graphical interface to view data summary	faculty.ithaca.edu/iwoods/ docs/
ToxTrack/ToxId	Windows/C++	Total distance, speed, acceleration, time near the walls (measure of anxiety), and ROI.	AVI or MPEG-compressed AVI files	txt, xls, tiff files and AVI videos	https://sourceforge .net/projects/toxtrac/
EthoWatcher	Windows/C++	Frequency, duration, and latency of each behavior.	AVI or MPEG-compressed AVI files	csv files	http://ethowatcher .paginas.ufsc.br
MouseMove	Windows/LabView—ImageJ	Distance, average velocity, acceleration, curvature, stationary fraction, laterality y ROI.	AVI or MPEG-compressed AVI files	csv files	https://www.nature.com/ articles/srep16171#s3, Supplementary File 2
Cowlog	Windows, Mac OS X and Linux/Java—html	Analysis of different behaviors can be set (tapping a button when the event occurs)	Common digital video formats	csv files	cowlog.org

AVI, audio video interleaved; MJPEG, motion joint photographic experts group; ROI, region of interest.

Franco-Restrepo *et al.*, 2019

Open-source behavioral analysis software packages

Review Article | Published: 29 July 2021

A review of 28 free animal-tracking software applications: current features and limitations

Veronica Panadeiro, Alvaro Rodriguez, Jason Henry, Donald Wlodkowic & Magnus Andersson

Lab Animal **50**, 246–254 (2021) | Cite this article

Deep-learning methods in behavioral analyses

www.nature.com/npp

Neuropsychopharmacology

Check for updates

ARTICLE OPEN

Deep learning-based behavioral analysis reaches human accuracy and is capable of outperforming commercial solutions

Oliver Sturman[1,2], Lukas von Ziegler[1,2], Christa Schläppi[1,2], Furkan Akyol[1,2], Mattia Privitera[1,2], Daria Slominski[1,2], Christina Grimm[2,3], Laetitia Thieren[2,4], Valerio Zerbi[2,3], Benjamin Grewe[2,5,6] and Johannes Bohacek[1,2]

Deep-learning methods in behavioral analyses

nature neuroscience
TECHNICAL REPORT
https://doi.org/10.1038/s41593-018-0209-y

DeepLabCut: markerless pose estimation of user-defined body parts with deep learning

Alexander Mathis[1,2], Pranav Mamidanna[1], Kevin M. Cury[3], Taiga Abe[3], Venkatesh N. Murthy[2], Mackenzie Weygandt Mathis[1,4,8*] and Matthias Bethge[1,5,6,7,8]

University of St Andrews

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PoseR - A deep learning toolbox for decoding animal behavior

Pierce Mullen, Maarten Frans Zwart

School of Psychology and Neuroscience, Centre for Biophotonics, Institute of Behavioural and Neural Sciences

DeepLabCut in zebrafish behavioral studies

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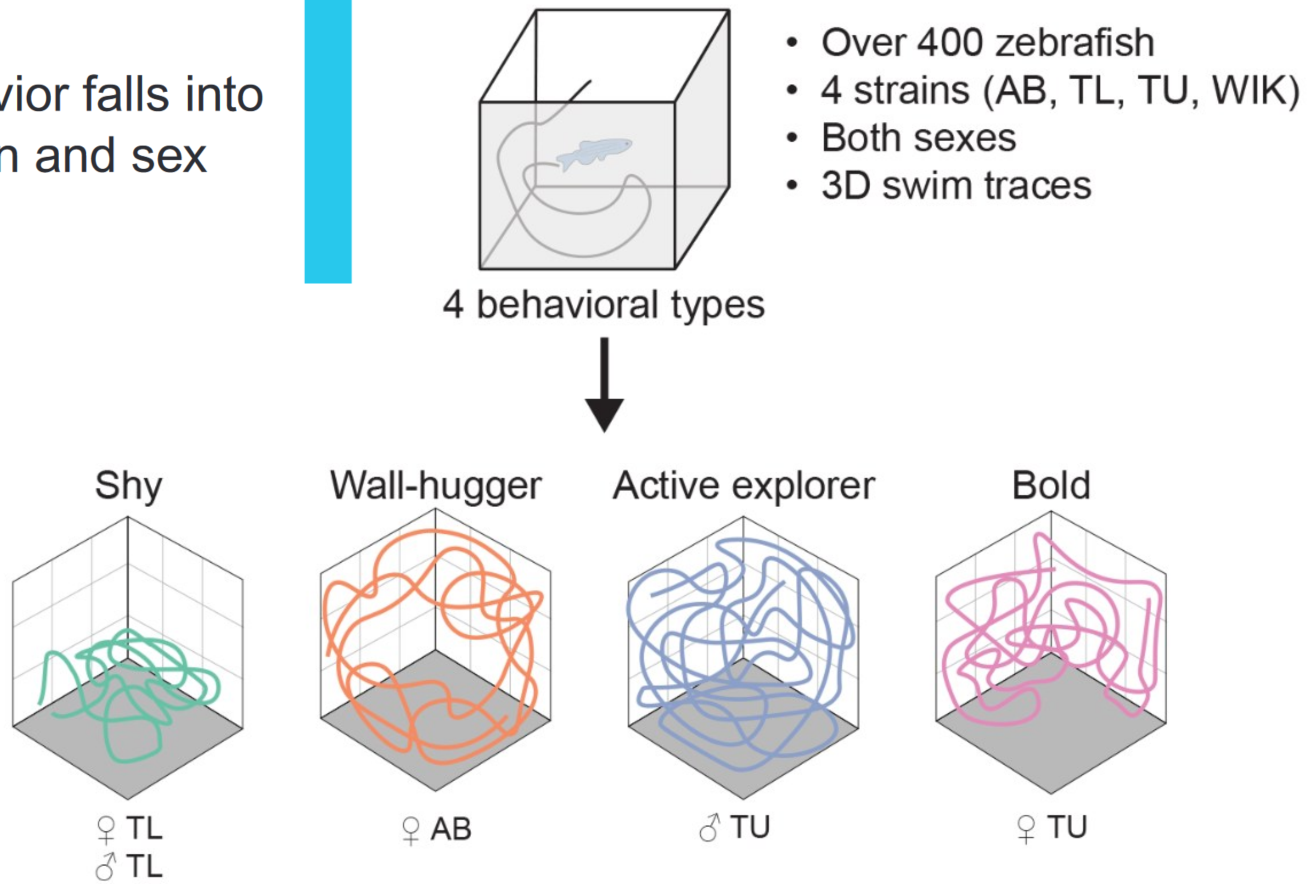
RESEARCH ARTICLE

Beyond bold versus shy: Zebrafish exploratory behavior falls into several behavioral clusters and is influenced by strain and sex

Neha Rajput, Kush Parikh and Justin W. Kenney*

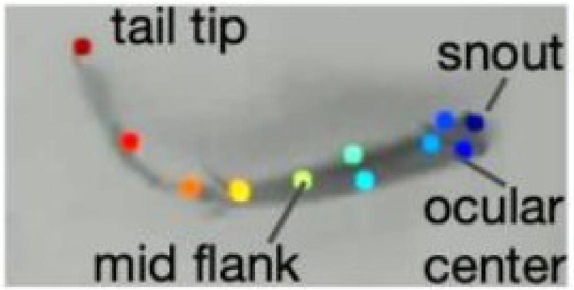


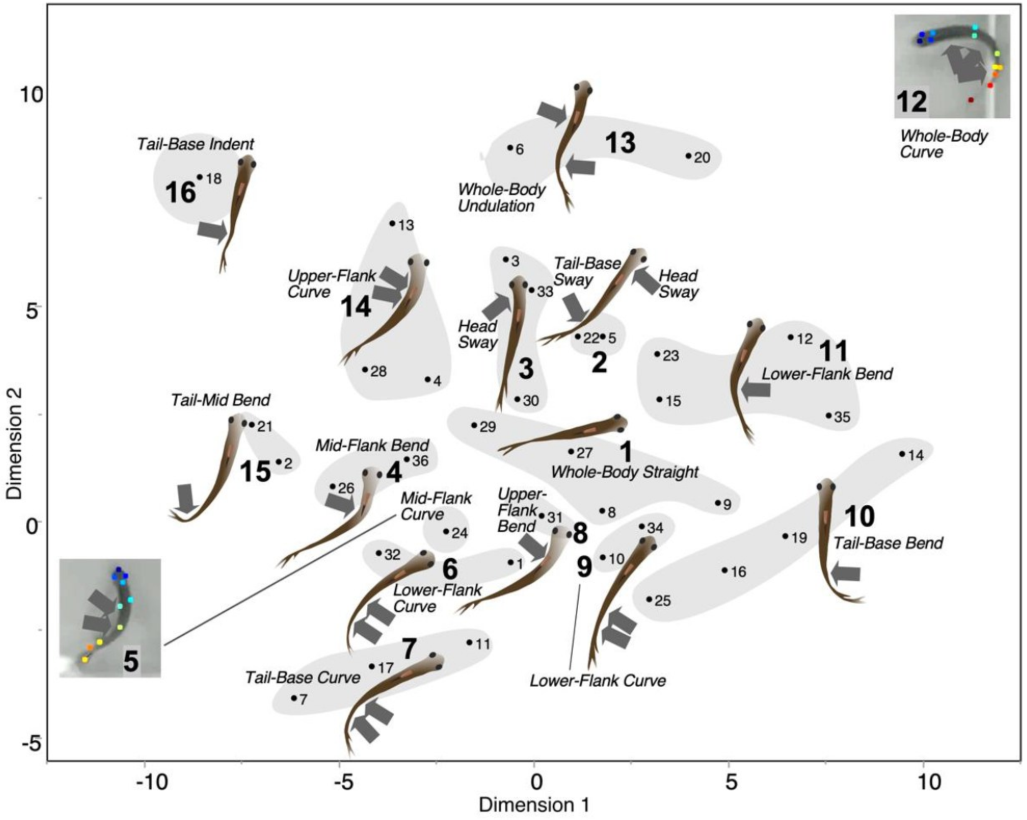
DeepLabCut in zebrafish behavioral studies

Pose analysis in free-swimming adult zebrafish, *Danio rerio*: "fishy" origins of movement design

Jagmeet S. Kanwal[1*], Bhavjeet Sanghera[†1,2], Riya Dabbi[1] and Eric Glasgow[3]

[1]Department of Neurology, Georgetown University Medical Center, Washington, DC, USA, [2]University of Miami, Coral Gables, FL, USA [3]Department of Oncology, Georgetown University Medical Center, USA





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Thank you!

Any questions?
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