

Environmental Toxicology

The Amphibian Short-Term Assay: Evaluation of a New Ecotoxicological Method for Amphibians Using Two Organophosphate Pesticides Commonly Found in Nature—Assessment of Behavioral Traits

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Abstract: Neurotoxic pesticides are used worldwide to protect crops from insects; they are recognized to impact nontarget organisms that live in areas surrounded by treated crops. Many biochemical and cell-based solutions have been developed for testing insecticide neurotoxicity. Nevertheless, such solutions provide a partial assessment of the impact of neurotoxicity, neglecting important phenotypic components such as behavior. Behavior is the apical endpoint altered by neurotoxicity, and scientists are increasingly recommending including behavioral endpoints in available tests or developing new methods for assessing contaminant-induced behavioral changes. In the present study, we extended an existing protocol (the amphibian short-term assay) with a behavioral test. To this purpose, we developed a homemade device along with an open-source computing solution for tracking trajectories of *Xenopus laevis* tadpoles exposed to two organophosphates insecticides (OPIs), diazinon (DZN) and chlorpyrifos (CPF). The data resulting from the tracking were then analyzed, and the impact of exposure to DZN and CPF was tested on speed- and direction-related components. Our results demonstrate weak impacts of DZN on the behavioral components, while CPF demonstrated strong effects, notably on speed-related components. Our results also suggest a time-dependent alteration of behavior by CPF, with the highest impacts at day 6 and an absence of impact at day 8. Although only two OPIs were tested, we argue that our solution coupled with biochemical biomarkers is promising for testing the neurotoxicity of this pesticide group on amphibians. *Environ Toxicol Chem* 2023;42:1595–1606. © 2023 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

Keywords: Amphibian; Behavioral toxicology; Neurotoxicity

INTRODUCTION

Neurotoxicity was substantially studied in the 1930s in the context of nerve gas development in anticipation of World War 2 (Soltaninejad & Shadnia, 2014). Along with warfare chemicals, the development of neurotoxic pesticides has increased and represents an effective solution for controlling insect pests in crops. Unfortunately, the fate and toxicity of neurotoxic pesticides such as dichlorodiphenyltrichloroethane were rapidly discovered (Carson, 2015). Currently, numerous chemicals are

detected in both terrestrial and aquatic environments, sometimes in drinking water resources (Vasseghian et al., 2021). Numerous methods have been developed for testing the neurotoxicity of chemicals, and the most common is the measurement of acetylcholinesterase (AChE) activity, an enzyme responsible for breaking down acetylcholine in synapses after nerve impulses are transmitted to the nearer nerve. Other solutions imply in vitro and cell-based methods (Poteser, 2017). Unfortunately, such a solution only partially pictures the impact at the organism level (Henry et al., 2022; Henry & Wlodkovic, 2019). Although behavior is the organism-level endpoint altered by neurotoxicity, behavioral ecotoxicology has gained interest only since the early 2000s (Chapman, 2007; Gerhard, 2007; Peeters et al., 2009; Thoré et al., 2019), and a substantial effort is currently invested toward a better integration of this discipline (Bownik & Wlodkovic, 2021; Ford et al., 2021; Henry et al., 2022; Peterson et al., 2017). In

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polluted environments, behaviors such as movement (Denoël et al., 2010), mate choice (Candolin & Wong, 2019), foraging (Kasumyan, 2001), parental care (Saaristo et al., 2010), and predator avoidance (Sievers et al., 2018) may be disrupted by chemicals through their effects on physiological, neurological, and morphological processes. Because these behaviors often have a strong influence on survival, reproductive performances, and ultimately individual fitness, individual-level effects of chemicals can further impact population viability and alter the composition and dynamics of communities (Saaristo et al., 2018). Despite the risks for animal conservation, the integration of chemical impact on behavior in current regulatory guidance is still low (Ågerstrand et al., 2020), and there is a call for the development of new standardized methods including behavioral endpoints (Ford et al., 2021).

Amphibians represent good candidates for developing behavioral standardized tests for testing neurotoxic pesticides. First, amphibians are the most threatened group of vertebrates, with >40% of species being endangered (International Union for Conservation of Nature, 2022). In combination with other human-related stressors, pollution is thought to crucially impact amphibian populations (Agostini et al., 2020; EFSA Panel on Plant Protection Products and Their Residues et al., 2018; Sparling et al., 2015). Second, amphibians live in wetlands receiving stormwater runoff in urban landscapes (Sievers et al., 2019), in rivers or river-supplied ponds contaminated by wastewater-treatment plant emissions (Khasawneh & Palaniandy, 2021), or in ponds surrounded by crops intentionally treated with plant protection products (Chen et al., 2019). In addition, amphibian behaviors such as dispersal (Cayuela et al., 2020), mate choice (Sullivan et al., 1995), and antipredator responses (Hossie et al., 2017) have been intensively studied over the last decades. By altering these behavioral components that may have a strong influence on individual fitness, neurotoxic pesticides are expected to have strong detrimental effects on long-term persistence and conservation of amphibian populations (Sparling et al., 2015).

A crucial component of amphibian behavior is locomotion, which is directly involved in predator avoidance, foraging, migration, and dispersal (Cayuela et al., 2020; Joly, 2019; Sinsch, 2006; Walton, 1988; Wassersug & Sperry, 1977). Locomotion is quantifiable at the different developmental stages such as larvae and metamorphosed individuals (Boes & Bernard, 2013; Brodin et al., 2013; Campbell Grant et al., 2010). Different approaches have been developed for assessing behavioral traits, from simple operator observation (Lowry et al., 1990) to more complex approaches involving Global Positioning System tracking (Strus & Strus, 2020), capture–recapture methods (Cayuela et al., 2018), or video processing (Brodin et al., 2013; Denoël et al., 2010). The latter are employed at both larval and juvenile stages and seem well adapted for developing standardized ecotoxicological tests. With the development of computing, video processing represents an effective means for tracking animals, and both commercial and open-source solutions are available (Bruijning et al., 2018; Noldus et al., 2001; Pennekamp et al., 2015). Although commercial software is often convenient and user-

friendly, the possibilities of automation are poor and, most crucial, the cost of such solutions is not negligible for organisms with low economical means.

In a previous work, Boualit et al. (2022) developed an 8-day protocol that the authors tested using two organophosphate insecticides (OPIs), diazinon (DZN) and chlorpyrifos (CPF), for AChE inhibition. Organophosphate insecticides are a major group of chemicals used as neurotoxic insecticides and target AChE. In the present study, using the same protocol, we evaluated the effects of these compounds on tadpole swimming behavior of *Xenopus laevis*, a common model organism for amphibians. For this purpose, we developed a tracking application using OpenCV, an open-source Python library aimed at video vision (Bradski, 2000). From those videos, we measured the mean instantaneous speed and speed change, the total period of activity, the number of activity periods, and two direction-related components, the direction change and the standard deviation of the direction change (SDDC). Organophosphate insecticides target AChE activity, leading to an accumulation of acetylcholine in synapses overstimulating muscarinic and nicotinic receptors. The results of this accumulation are numerous, but hyperreflexation, muscle rigidity, and tremors have been documented (Russom et al., 2014). Because of these symptoms, we expect DZN and CPF to alter larval locomotor traits; and more particularly, we expect erratic speed and direction changes.

MATERIALS AND METHODS

Test organisms and exposure scenario

In our experiment, African clawed frog (*X. laevis*, Daudin, 1802) was used as a model species. The exposure scenario was identical to that of Boualit et al. (2022). Briefly, *X. laevis* eggs were obtained from in vitro fertilization. At Nieuwkoop and Faber stage 8, the egg mass was split into two equal batches, and half was dejellied using a bath of 2% L-cysteine solution buffered at pH 8.1, while the other batch was kept entire. This process aimed at testing the behavioral alteration of this commonly used process. Then, eggs were exposed for 8 days to six DZN nominal concentrations (0, 0.0001, 0.001, 0.01, 0.1, and 1 mg/L diluted in frog embryo teratogenesis assay—*Xenopus* [FETAX] solution) and five CPF nominal concentrations (0, 0.0001, 0.001, 0.01, and 0.1 mg/L diluted in a 0.002% v/v dimethylsulfoxide [DMSO]/FETAX solution) plus a solvent control (0.002% v/v DMSO/FETAX). For 4 days postfertilization, individuals were reared in 12-well plates. While in the DZN test, we used one replicate of 12 individuals per concentration/dejelling condition, in the CPF test we used three replicates of 12 individuals per concentration/dejelling condition. In both tests, we then randomly selected and transferred 10 individuals to containers for an exposure of 4 extra days. Thus, for each concentration, 20 individuals were tested for behavioral changes (10 per dejelling condition). The medium was renewed daily, and physicochemical parameters were recorded. The average values for these parameters were 8.58 ± 0.11 mg/L, 7.8 ± 0.07 , and 1647.12 ± 17.90 μ S/cm for dissolved oxygen, pH, and conductivity, respectively. Individuals were fed daily

with 60 μL of a 1:1 (m/m) mixture of spirulin:tetrafin (24:24 g/L; JBL Spirulina Premium and JBL Novo Bel). During the tests, individuals were reared in a climatic chamber at $21 \pm 1^\circ\text{C}$ with a 12:12-h light: dark cycle and an illumination of 680 lux. Pesticide concentrations were quantified in stock solution and in individual pots exposed for 24 h to the same as conditions as the organisms (t0 and t24). The arithmetic means between t0h and t24h were used for the interpretation of the results and figures. Breeders used for the in vitro fertilization were different individuals between DZN and CPF tests. Both tests were performed by the same operator. The entire experimental procedure was approved by the veterinary and ethics committee of canton Vaud under license VD3521a.

Behavioral traits

Behavior recording. On days 6, 7, and 8, tadpole swimming behavior was recorded. The protocol was based on a “home-made” video box. This box was conceived for standardizing the video recording sessions. The three-dimensional (3D) plan of this item is available in the data repository, and a picture is included in the Supporting Information. Briefly, at the bottom of the box were six enlightened wells, each containing a 12-cm glass Petri dish filled with 50 mL of FETAX solution, a standard medium for raising *X. laevis* larvae. At the top of the box was a camera (Nikon® D750). Larvae were individually transferred to a Petri dish using a plastic Pasteur pipette whose end had been cut to enlarge the diameter, and their swimming behavior was recorded for 10 min (six individuals per video). No indications of injury during transport were observed. The order of

recording was chosen for homogeneously distributed concentrations within the Petri dish positions in the setting.

Video processing. Trajectories were extracted from videos using OpenCV, an open source and cross-platform library aimed at real-time computer vision. In the present study, we used Python as the programming language for OpenCV. This new approach has the advantage of allowing the development of a tailored solution for tracking swimming tadpoles while using an easy programming language. Conceptually, our computing solution is simple (Figure 1) and relies on blob detection and blob tracking (a *blob* is a group of connected pixels in an image that share some common property, e.g., the same gray-scale value): (i) The larvae are sometimes reflected by the Petri dish wall, which can depress the tracking efficiency. That is why a mask was created using GIMP (2014) and applied to the original video using FFmpeg (Tomar, 2006) with the aim of obtaining a video of the Petri dishes without their walls. (ii) Multiple tracking on a video is obviously complex because of the need to correctly reassign trajectories to the larvae. For this reason, masked videos were split into six subvideos, each containing one and only one larva. (iii) Because noisy pixels are often registered as foreground, subvideos were denoised using an opening filter (erosion followed by dilation of frames). (iv) Because the trajectory extraction involves contour tracking, blurring was applied on subvideos to improve edge detection. (v) A foreground mask was computed using the KNN algorithm (Zivkovic & Van Der Heijden, 2006). The foreground mask is basically a binary set of frames containing the background in black and the foreground (here, the larva) in white. (vi) The last

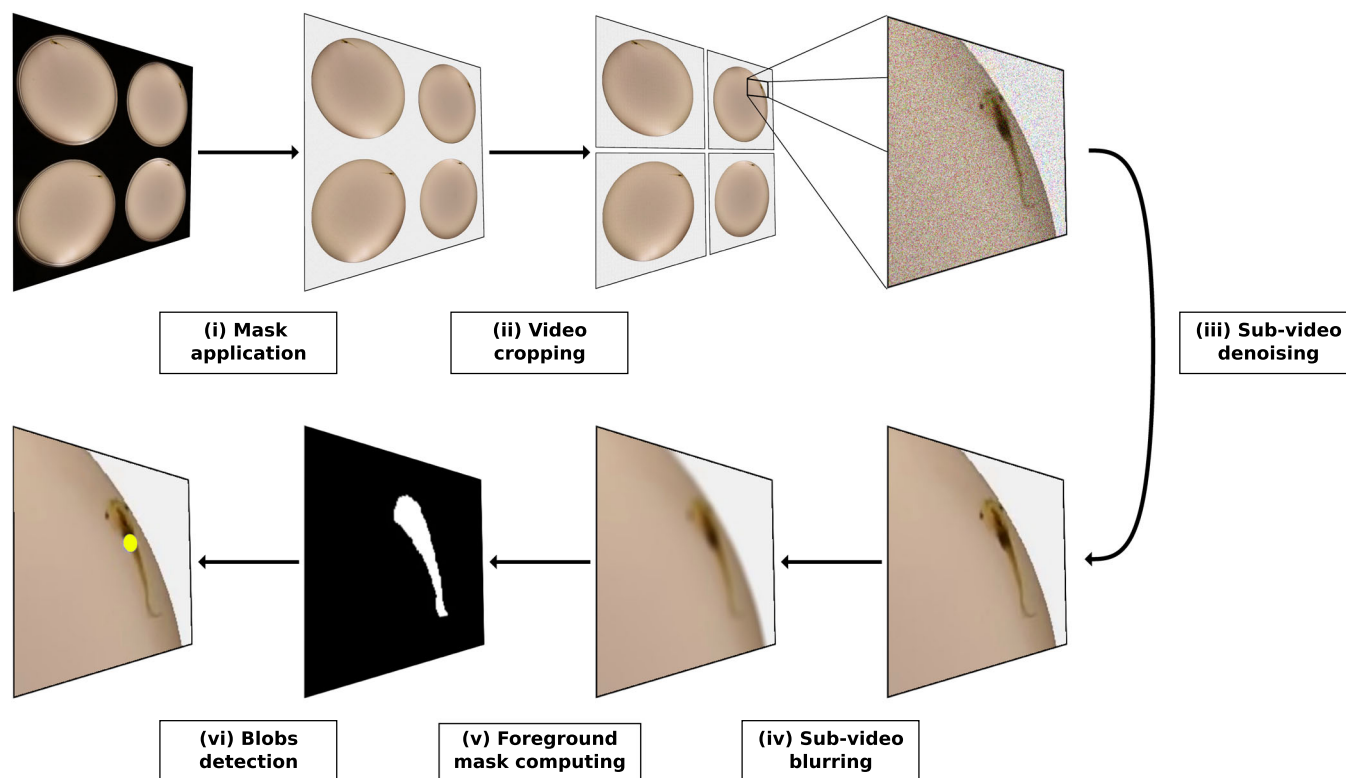


FIGURE 1: Diagram of the video-tracking processing.

TABLE 1: Outputs of pairwise comparison testing the effects of developmental stage on mean speed change

Comparison	Estimate	SE	df	t	p
Days 6–7	-2.122	1.936	71.478	-1.096	0.5197
Days 6–8	-8.828	1.904	56.162	-4.636	0.0001
Days 7–8	-6.706	1.956	71.855	-3.429	0.0029

p-value adjustment: Tukey's honestly significant difference. Bold indicates significant p values.

step is the detection of contours and tracking. Because the edges of video masks are sometimes registered as foregrounds, the x,y coordinates of the blob with the largest area were assigned to the larvae. The Python code regarding sub-video processing is available in the Supporting Information.

Coordinate cleaning. Because foreground extraction relies on motion, in the case where the subvideo starts with a motionless larva, the largest blobs correspond to the subvideo mask edge. To correct these coordinates, we plotted histograms of the blob area to select a threshold value under which we suppose the blob corresponds to the subvideo mask edge.

TABLE 3: Outputs of pairwise comparison testing the main effects of exposure to chlorpyrifos and dejellying on behavioral components

Variables	Predictors	Day 6		Day 7		Day 8	
		Estimate	p	Estimate	p	Estimate	p
Speed	Control	3.5	<0.001	3.65	<0.001	–	–
	DMSO	-0.05	0.816	-0.31	0.332	–	–
	4.1e-05 mg/L	0.12	0.602	-0.3	0.351	–	–
	5e-04 mg/L	-0.18	0.436	-0.26	0.417	–	–
	3.5e-03 mg/L	-0.32	0.171	-0.46	0.151	–	–
	3.65e-02 mg/L	-2.16	<0.001	-1.82	<0.001	–	–
	Nondejellied	0.31	0.058	0.27	0.238	–	–
Speed change	Control	67.01	<0.001	68.93	<0.001	–	–
	DMSO	1.7	0.54	-1.68	0.624	–	–
	4.1e-05 mg/L	2.58	0.347	0.06	0.986	–	–
	5e-04 mg/L	-1.47	0.592	-0.1	0.977	–	–
	3.5e-03 mg/L	-0.16	0.954	-1.65	0.635	–	–
	3.65e-02 mg/L	-27.4	<0.001	-20.3	<0.001	–	–
	Nondejellied	3.25	0.157	3.16	0.321	–	–
Activity	Control	516.5	<0.001	527.8	<0.001	506.9	<0.001
	DMSO	1.98	0.929	-20.3	0.334	-64.3	0.053
	4.1e-05 mg/L	5.84	0.793	-22.4	0.287	-3.47	0.914
	5e-04 mg/L	-30.2	0.166	-18.5	0.379	-83.5	0.026
	3.5e-03 mg/L	-29.3	0.184	-22.5	0.299	-20.8	0.514
	3.65e-02 mg/L	-295	<0.001	-208	<0.001	-84.2	0.06
	Nondejellied	9.65	0.606	9.31	0.588	18.58	0.303
DC	Control	752.9	<0.001	–	–	–	–
	DMSO	65.29	0.37	–	–	–	–
	4.1e-05 mg/L	14.23	0.845	–	–	–	–
	5e-04 mg/L	76.77	0.293	–	–	–	–
	3.5e-03 mg/L	112.3	0.13	–	–	–	–
	3.65e-02 mg/L	-177	0.016	–	–	–	–
	Nondejellied	-14.4	0.782	–	–	–	–
SDDC	Control	–	–	1012	<0.001	–	–
	DMSO	–	–	126	0.128	–	–
	4.1e-05 mg/L	–	–	148.2	0.074	–	–
	5e-04 mg/L	–	–	99.47	0.229	–	–
	3.5e-03 mg/L	–	–	38.93	0.637	–	–
	3.65e-02 mg/L	–	–	290.3	0.001	–	–
	Nondejellied	–	–	-63.4	0.184	–	–

p-value adjustment: Tukey's honestly significant difference. Bold indicates significant p values.
DMSO = dimethyl sulfoxide; DC = direction change; SDDC = standard deviation of the direction change.

TABLE 2: Outputs of pairwise comparison testing the main effects of exposure to diazinon and dejellying on activity at day 6 and direction change at day 8

Predictors	Activity at day 6		DC at day 8	
	Estimate	p	Estimate	p
Intercept	506.52	<0.001	700.73	<0.001
7.38E-04 mg/L	-12.76	0.649	-23.62	0.787
2.07E-03 mg/L	0.34	0.991	18.77	0.84
6.19E-03 mg/L	12.42	0.662	106.32	0.238
7.28E-02 mg/L	-14.11	0.615	114.55	0.198
8.01E-01 mg/L	-87.36	0.002	97.35	0.281
Nondejellied	-8.59	0.621	120.09	0.023
Observations	113		110	
Marginal R ² /conditional R ²	0.136/0.144		0.083/NA	
Deviance	1317.942		1538.643	

p-value adjustment: Tukey's honestly significant difference. Bold indicates significant p values.
DC = direction change; NA = not available.

In that case, coordinates were replaced with coordinates of the first point with an area higher than the threshold (when the larvae start to swim). Then, to cope with larvae stopping swimming, the coordinates for which the area was lower than

the threshold value were replaced with the coordinates of the last point with an area higher than the threshold (the last known coordinate corresponding to the larva). Thus, with this solution, we obtain x,y coordinates for each frame of the video and obviously the timestamps that are necessary for the following analyses.

Behavioral component analyses. In the present study, after removing the first minute of each video as a period of acclimation, the R package TrajR (McLean & Skowron Volponi, 2018) was used for extracting behavioral components. TrajR is a toolkit for animal trajectory description and analysis. The trajectory study was divided into two parts: (i) Analysis of speed-related components: mean speed, mean speed change, total period of activity (the total period faster than 0 cm/s), and number of periods of activity; (ii) Analysis of direction-related components: mean direction change and SDDC as defined in Kitamura and Imafuku (2015). Directional change is an index of nonlinearity, while SDDC describes irregularity.

Statistical analyses

All statistical analyses were performed with R: A language and environment for statistical computing (2022). Extreme outliers were removed from the data set using the boxplot method. An extreme outlier was defined as data point lying outside three times the interquartile range. Normality and homogeneity of variance of residuals were graphically checked. When the residuals were not normally distributed, square root or log transformation was applied. After transformation, all residuals were distributed normally. The α significance level of all tests was set at 0.05.

Impact between control conditions. The impacts of developmental stage and of clutch identity (DZN or CPF test) were tested by computing mixed-effects models for testing the impact of stage on the behavioral components. We computed models with stage and clutch identity as fixed factors and well position in the setting as a random factor. In the case of a significant effect of stage or clutch identity, a pairwise comparison using the estimated marginal means was run with Tukey's honestly significant difference method for p value adjustments (summarized in Table 1).

Impact of pesticides on behavioral components. For each test, we computed mixed-effects models for testing the impact of DZN and CPF exposure and dejellying on the behavioral components. We computed models with pesticide concentration and dejellying as fixed factors and well position in the setting as a random factor. In the case of a significant effect of pesticide or dejellying concentration, a pairwise comparison with the control or the dejellied eggs as the reference group was run with Tukey's honestly significant difference method for p value adjustments (summarized in Tables 2 and 3).

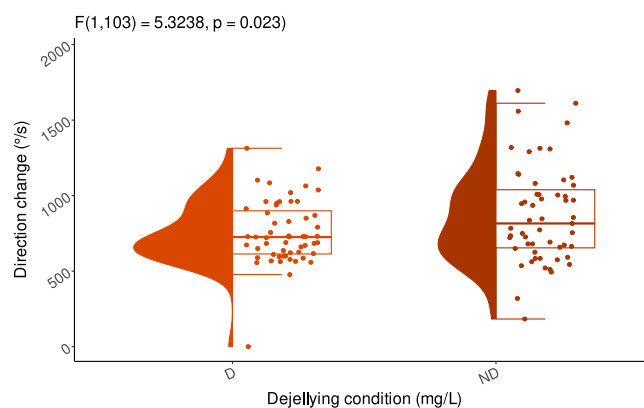


FIGURE 2: Impact of dejellying on mean direction change ($*p=0.01-0.05$, $**p=0.001-0.01$, $***p<0.001$). The figure contains data from all diazinon concentrations. p values are adjusted with Tukey's honestly significant difference. The position of the Petri dish in the setting is set as a random effect in statistical models. The central bar of the boxplot is the group median. Upper and lower hinges correspond to the 25th and 75th quantiles, respectively. Upper and lower whiskers extend from the closest hinge, to the largest and the smallest values at most 1.5 times the interquartile range. Violins represent the smoothed histogram of the data distribution. Extreme outliers are not displayed in the graphs. D = dejellied eggs; ND = nondejellied eggs.

RESULTS

The following figures present only the behavioral components crucially affected by exposure to DZN and CPF. The complete set of figures presenting all behavioral components are available in the Supporting Information.

Impact of dejellying

Models demonstrated no impact of the dejellying process on the behavioral component regarding exposure to CPF. Conversely, analyses of the DZN test demonstrated a significant impact on direction change (Figure 2). This figure shows the impact of dejellying on direction change regardless of the condition of exposure. At day 8, direction change was significantly higher at 120.09°/s in nondejellied eggs than in dejellied eggs.

Negative and solvent control

Comparison between negative controls in DZN and CPF tests denotes several differences regarding the selected behavioral components. First, the data analysis demonstrated a significant impact of clutch identity (DZN vs. CPF test), with an increase in the mean speed of 0.62 cm/s in the CPF test (Figure 3A). Clutch identity also significantly impacted the total period of activity (Figure 3B) and the number of periods of activity (Figure 3C), respectively, with an increase of 1.15 s and a decrease of 466 periods in the CPF test compared with the DZN test. To evaluate the impact of time, Figure 3D shows the mean speed change in the negative control condition of combined DZN and CPF data. Significant differences between days 6 and 8 (increase of 8.83 cm/s²) as well as between days 7

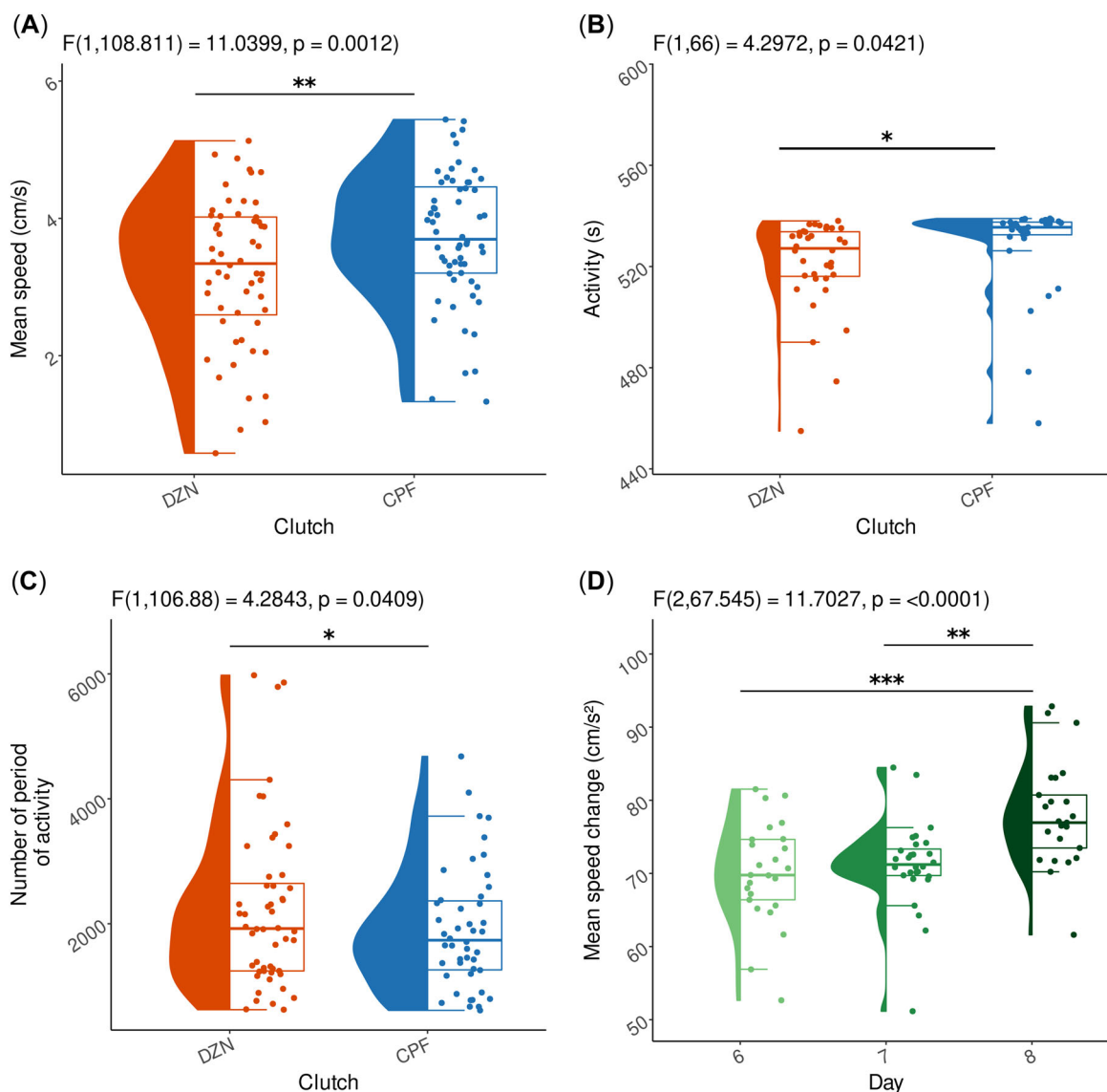


FIGURE 3: Impact of clutch affiliation on mean speed (A), total period of activity (B), number of periods of activity (C), and impact of developmental stage on mean speed change (D). The data represent individuals raised in negative control conditions during both diazinon and chlorpyrifos tests (* $p = 0.01$ – 0.05 , ** $p = 0.001$ – 0.01 , *** $p < 0.001$). p values are adjusted with Tukey's honestly significant difference. The position of the Petri dish in the setting is set as a random effect in statistical models. The central bar of the boxplot is the group median. Upper and lower hinges correspond to the 25th and 75th quantiles, respectively. Upper and lower whiskers extend from the closest hinge, respectively, to the largest and the smallest values at most 1.5 times the interquartile range. Violins represent the smoothed histogram of the data distribution. Extreme outliers are not displayed in the graphs. DZN = diazinon; CPF = chlorpyrifos.

and 8 (increase of 6.71 cm/s^2) were observed. Models and their associated pairwise comparisons did not demonstrate any impact of the solvent control on swimming traits (Supporting Information, Figures 4 and 5).

Impact of DZN on swimming behavior

Behavioral changes were not observed for direction-related components (Supporting Information, Figures 2 and 3). Regarding speed-related components, only the total period of activity at day 6 (Figure 4) demonstrated a significant difference at the highest concentrations compared to the control with periods of 502 and 415 s, respectively, for the control condition

and the highest DZN concentration (the complete set of figures is available in the Supporting Information).

Impact of CPF on swimming behavior

Speed-related components. Exposure to CPF produces numerous changes in behavioral components related to speed (Figure 5). Regarding mean speed (Figure 5A), at days 6 and 7, significant decreases occurred at the highest concentration compared to the control condition. At day 6, the mean speed was decreased by 2.17 cm/s , while it was decreased by 1.82 cm/s at day 7. No significant changes occurred at day 8, although the mean speed decreased by

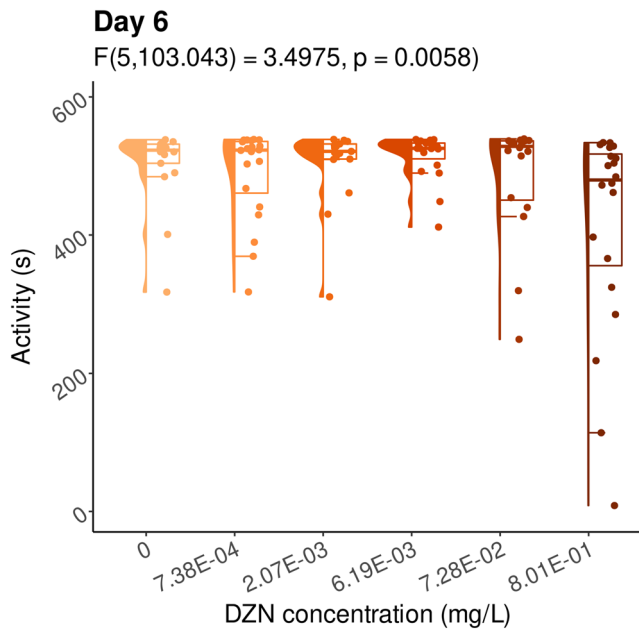


FIGURE 4: Impact of exposure to diazinon total period of activity ($*p = 0.01–0.05$, $**p = 0.001–0.01$, $***p < 0.001$). p values are adjusted with Tukey's honestly significant difference. Plates of origin are set as a random effect in statistical models. The position of the Petri dish in the setting is set as a random effect in statistical models. The central bar of the boxplot is the group median. Upper and lower hinges correspond to the 25th and 75th quantiles, respectively. Upper and lower whiskers extend from the closest hinge, respectively, to the largest and the smallest values at most 1.5 times the interquartile range. Violins represent the smoothed histogram of the data distribution. Extreme outliers are not displayed in the graphs. DZN = diazinon.

0.89 cm/s at the highest concentration. The same pattern was observed regarding mean speed change (Figure 5B), with significantly decreased speed change at days 6 (-27.4 cm/s^2) and 7 (-20.4 cm/s^2) at the highest concentration compared to the control conditions. The total period of activity was also impacted, with significantly decreased periods at days 6 and 7 at the highest concentration compared to the control condition, with decreases of 297 and 208 s, respectively, at days 6 and 7. At day 8, the total period of activity was significantly decreased (-84 s) at a CPF concentration of $5 \times 10^{-4} \text{ mg CPF/L}$ compared to the control condition. Last, no differences are observable regarding the number of periods of activity.

Direction-related components. The mean direction change and SDDC were impacted by CPF (Figure 6). At day 6, the direction change was significantly decreased at the highest concentration compared to the control condition, with respective values of 568 and 744°/s. Regarding the SDDC, at day 7, individuals demonstrated an SDDC of 1270°/s, which was significantly higher than that in the control condition (980°/s). Although not significant, the SDDC at days 6 and 8 at the highest concentration was higher than the SDDC at days 6 and 8 in the control condition, with differences of 131 and 210°/s, respectively (Supporting Information, Figure 5B).

DISCUSSION

In the present study, we tested a method for assessing the impact of DZN and CPF on swimming behavior in 6- to 8-day-old larvae of *X. laevis*. This method involved the use of a homemade setting along with open-source software for extracting trajectories and computing examples of speed- and direction-related components. Our research demonstrated a strong impact of CPF on mean speed, speed change, and activity.

Impact of exposure

Our approach allowed us to detect the detrimental impact of CPF on behavioral components. Our results are congruent with the findings of previous studies showing a similar impact of CPF on the movement speed in several anuran larvae (Da Silva et al., 2020; Silva et al., 2021) and urodele species (Robles-Mendoza et al., 2011). Poor changes were observed regarding the direction-related components. Nevertheless, we observed a decreasing nonlinearity of trajectories while we were expecting erratic trajectories. More research is needed for investigating these counterintuitive results. In addition, Boualit et al. (2022) demonstrated both inhibition of AChE activity and a decrease of snout-to-vent length under exposure to CPF. While the inhibition of AChE activity induces impairment of the skeletal neuromuscular junctions, the cardiac conduction system, and smooth muscle functioning (Russom et al., 2014), morphological traits in amphibian larvae were demonstrated to be related to locomotion (Fitzpatrick et al., 2003; Zamora-Camacho & Calsbeek, 2022). Thus, the alteration of AChE activity and morphological traits likely has a detrimental effect on locomotor capacities.

The results observed for speed, speed change, and activity suggest a potential time dependence because they occurred only on days 6 and 7. The result for direction change also demonstrates a decreased value at day 6 only. Thus, in general, 6-day-old individuals exposed to CPF may be the most sensitive stage in our method because the magnitude of the changes was the highest regarding all of the studied components. More OPIs should be tested to determine if this stage is the most relevant for comparing OPI toxicity. The results also demonstrate a higher toxicity of CPF on behavioral components than DZN because only a few impacts were observable with DZN at these concentrations. This is supported by previous work (Boualit et al., 2022) showing that CPF exhibits inhibition of AChE at lower concentrations compared with DZN. Speed and activity are commonly measured in methods testing the impact of chemicals on swimming behavior (Denoël et al., 2010; Moreira et al., 2019; Shuman-Goodier & Propper, 2016). However, few studies have analyzed the effect of chemicals on acceleration and direction-related components of movement (but see Brewer et al., 2001; Rehage et al., 2002). Loss of activity obviously has an impact on speed and should always be measured along with speed-related components as a mechanistic endpoint for predicting changes in mean speed.

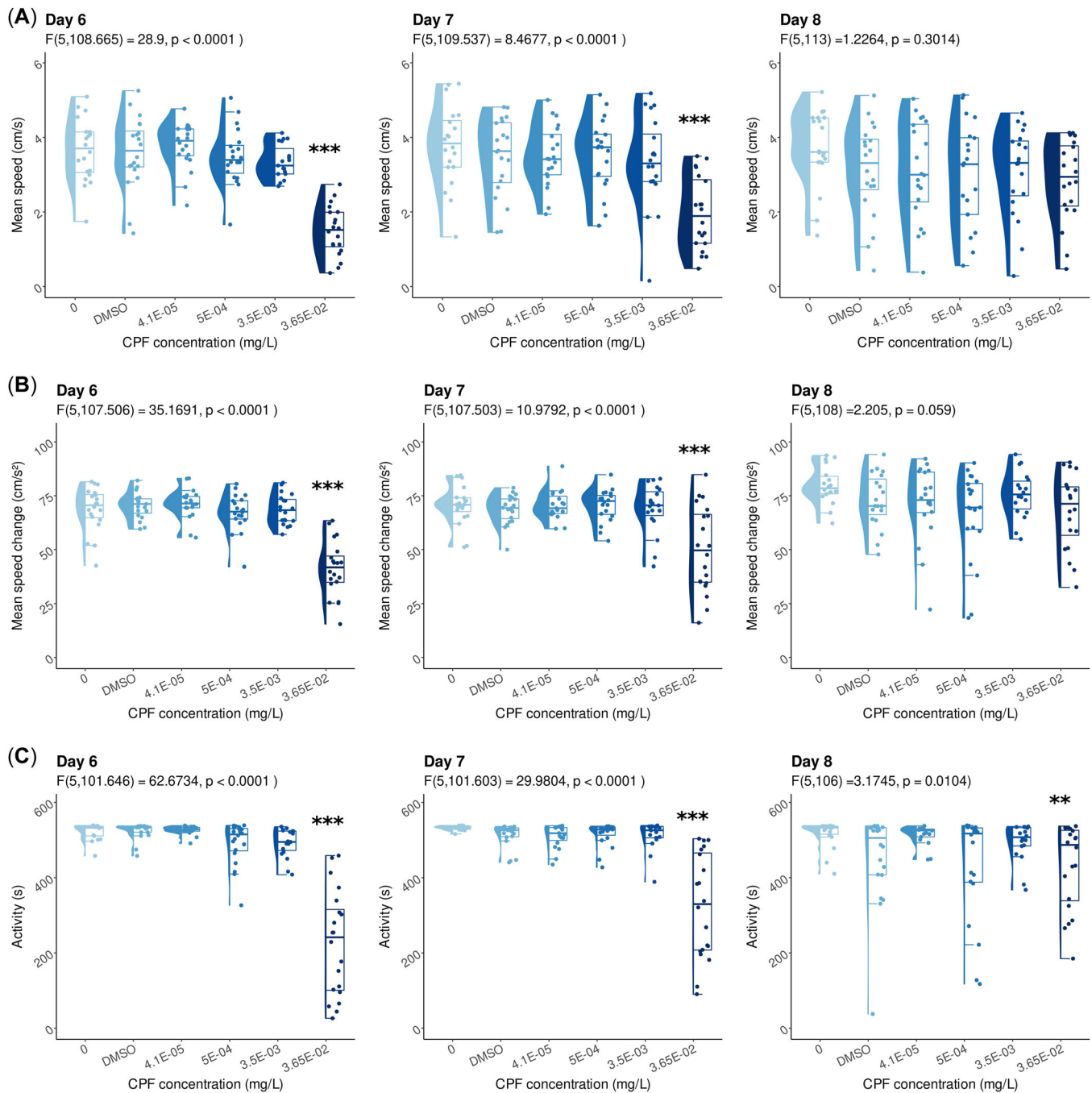


FIGURE 5: Impact of exposure to chlorpyrifos on mean speed (A), mean speed change (B), total period of activity (C), and number of periods of activity (D) ($*p = 0.01–0.05$, $**p = 0.001–0.01$, $***p < 0.001$). p values are adjusted with Tukey's honestly significant difference. The position of the Petri dish in the setting is set as a random effect in statistical models. The central bar of the boxplot is the group median. Upper and lower hinges correspond to the 25th and 75th quantiles, respectively. Upper and lower whiskers extend from the closest hinge, respectively, to the largest and the smallest values at most 1.5 times the interquartile range. Violins represent the smoothed histogram of the data distribution. Extreme outliers are not displayed in the graphs. DMSO = dimethyl sulfoxide; CPF = chlorpyrifos.

Chemically mediated alterations of locomotor capacities and activity rate likely have negative effects on individual performances. First, they may increase predation risk—which is high at the larval stage in nature—because of depressed predator avoidance behavior (Polo-Cavia et al., 2016; Raimondo et al., 1998; Sievers et al., 2018), leading to a decrease in metamorphosis rate and recruitment. Second,

decreased locomotor capacities and activity rate probably impact foraging efficiency, which may negatively influence growth rate and body size at metamorphosis (Kupferberg, 1997). In amphibians with a biphasic life cycle, size at metamorphosis is a critical parameter affecting juvenile survival (Cabrera-Guzmán et al., 2013; Székely et al., 2020) and mobility (Beck & Congdon, 2000; Chelgren et al., 2006), as well as the age at sexual

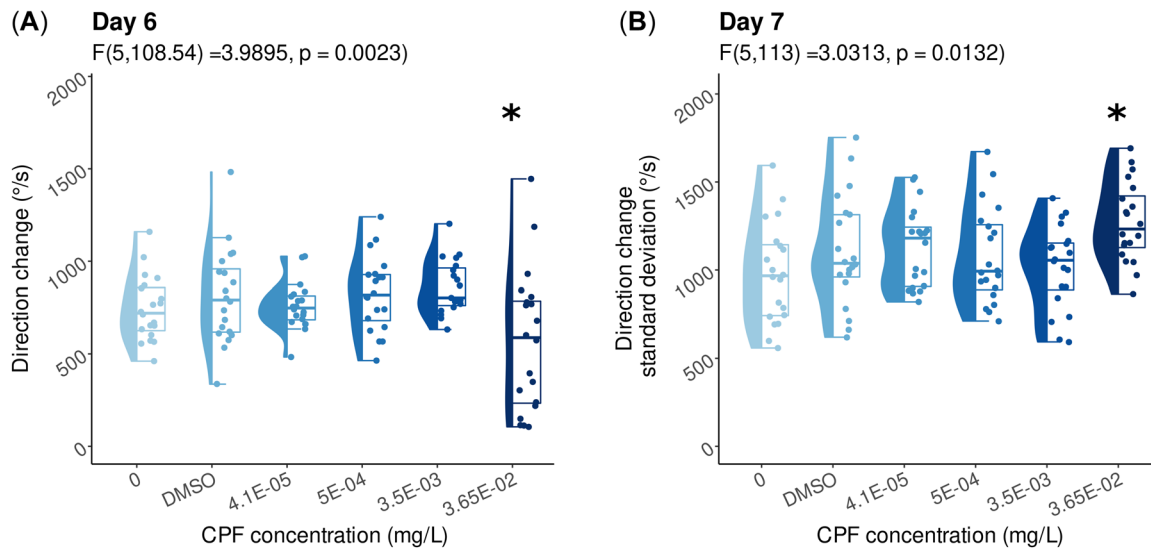


FIGURE 6: Impact of exposure to chlorpyrifos on mean direction change at day 6 (A) and the standard deviation of the direction change at day 7 (B) ($*p = 0.01$ – 0.05 , $**p = 0.001$ – 0.01 , $***p < 0.001$). p values are adjusted with Tukey's honestly significant difference. The position of the Petri dish in the setting is set as a random effect in statistical models. The central bar of the boxplot is the group median. Upper and lower hinges correspond to the 25th and 75th quantiles, respectively. Upper and lower whiskers extend from the closest hinge, respectively, to the largest and the smallest values at most 1.5 times the interquartile range. Violins represent the smoothed histogram of the data distribution. Extreme outliers are not displayed in the graphs. DMSO = dimethyl sulfoxide; CPF = chlorpyrifos.

maturity (Earl & Whiteman, 2015). Although the population-level impacts of neurotoxic pesticide exposure are still poorly understood, our study suggests that chemically mediated alterations of movement behavior could have far-reaching consequences for amphibian persistence in human-dominated polluted environments.

Link with AChE activity

In Boualit et al. (2022), approximately 50% and 80% of AChE activity was inhibited in 8-day-old individuals exposed to 8.96×10^{-1} mg DZN/L and 3.65×10^{-2} mg CPF/L, respectively. Nevertheless, in the present study, no evidence of behavioral changes was established during the DZN test or the CPF test at day 8. Without any quantification of AChE at day 6, it is difficult to discern if behavioral alterations result from AChE inhibition, but behavioral impairment associated with high levels of AChE inhibition has already been documented in mammals (Carr & Chambers, 1991), birds (Hart, 1993), fish (Sismeiro-Vivas et al., 2007), and amphibians (Peltzer et al., 2013). In any case, this suggests that AChE activity alone does not correctly represent the toxicity of OPIs.

Egg dejellinging

Regarding the impact of egg dejellinging, our analysis demonstrates a minor difference between dejellied and non-dejellied eggs; but unfortunately, our pseudorandomization during the video recording does not allow us to state if these changes were induced by dejellinging or if the well position in the setting had an impact on the recorded metrics. More research

focusing on the protective role played by the jelly coat should be conducted.

Limits of the method

Although the computing application developed in the present study represents a promising way to improve the reproducibility of behavioral tests in ecotoxicology, the cleaning of data was often a little arbitrary, and more efficient ways should be implemented and tested. The device used in the present study for measuring behavior was homemade. Although we provide the device's 3D plan, its building remains complex; and differences in the structure of the device could imply reproducibility issues—a recurrent problem in behavioral science in general (Laraway et al., 2019; Richter et al., 2009). A solution for coping with this problem would be to develop a 3D plan of a device that is printable. Currently, 3D printers are common in universities, and numerous companies offer 3D printing services. The present device is rectangular and has a door on one side. We do not know how the distance to corner and/or the distance to the door affect the larvae behavior. A circular device could avoid any effect of the position of the arenas in the device. In addition, the duration of the test can represent a problem regarding human resources; and if the stage at day 6 proves to be the most sensitive to OPIs, the method should be shortened.

CONCLUSION

Our study presents a new approach for assessing the impact of OPIs on amphibian larval swimming behavior. This method proved its efficiency at demonstrating chemically mediated changes in locomotor parameters related to speed and

direction. Because the efficiency of predicting the impacts at the organism level based on suborganismal tools is currently uncertain, this method involving the measurement of behavior (the apical target of neurotoxic pesticides) represents an interesting base toward fast and cost-efficient solutions for assessing neurotoxicity. Although breeders were different individuals between the DZN and CPF tests, impact of test identity demonstrated only a few differences. These results suggest good repeatability. Last, the amphibian short-term assay (Boualit et al., 2022) extended with our behavioral approach represents an efficient way to assess behavioral changes along with biochemical and morphological mechanistic traits, and we are confident in its potential to be improved and tested for standardization.

Supporting Information—The Supporting Information is available on the Wiley Online Library at <https://doi.org/10.1002/etc.5642>.

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Data Availability Statement—Data, the 3D plan, and the tracking application code are available at <https://doi.org/10.5281/zenodo.7596506>.

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