

1 Ring a bell? Variable recognition of a multicomponent auditory stimulus associated with  
2 predation risk by zebrafish responding to full and partial matches

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15

16 **Abstract**

17 Many fishes learn to recognize correlates of predation risk by pairing novel stimuli with injury-  
18 released chemical cues released from damaged epidermal tissues. Here, zebrafish were  
19 conditioned to associate predation risk with a three-note auditory stimulus C<sub>5</sub> (523.25 Hz) +  
20 E<sub>5</sub> (659.25 Hz) + G<sub>5</sub> (783.99 Hz), then tested for responses to either only one tone (C), two of the  
21 components (C + G) or the full three-note chord (C+E+G). Zebrafish conditioned with alarm  
22 cues and C+E+G responded significantly more intensely to the full C+E+G stimulus or to partial  
23 representation of the original mix (C+G) than they did to the single element (C) of the original  
24 C+E+G conditioning stimulus. The lack of a response to the single component alone may be a  
25 failure to recognize the stimulus or an interpretation that the partial component is an indicator of  
26 low risk.

27

28 **Keywords:** Associative learning, predator-prey, chemical alarm cues, auditory stimuli, zebrafish

29 **1. Introduction**

30 Animals detect and attend to information about the environment to inform behavioral decision-  
31 making for navigation, habitat selection, foraging, predator avoidance, and reproduction (Dill,  
32 1987; Dukas, 1998; Shettleworth, 2001; Bshary & Triki, 2022; Dall et al., 2005; Webster and  
33 Laland 2018). Many fitness functions form trade-offs against one another (e.g., risk-sensitive  
34 foraging) and weighing relative costs and benefits of any decision is complicated by information  
35 that is often incomplete, unreliable, variable or of limited temporal relevance (Lima &  
36 Bednekoff, 1999; Sih et al., 2002; Ferrari et al., 2016).

37 Information about predation risk obtained directly through encounters with predators is  
38 inherently risky. Therefore, there is a large fitness benefit to detecting risk indirectly through  
39 publicly available cues, such as the signature odor of a predator, which can be detected from afar  
40 allowing prey to evade detection (Lima & Dill, 1990; Smith, 1992; Wisenden & Chivers, 2006;  
41 Wisenden et al., 2010). Similarly, alarm cues released when prey tissues are damaged during a  
42 predator attack are reliable indicators of an active predator nearby (Ferrari et al., 2010a).  
43 Moreover, alarm cues act as a releaser of associative learning to transfer perception of risk from  
44 alarm cues to the novel stimulus (Magurran, 1989; Suboski, 1990; Suboski et al., 1990; Mathis &  
45 Smith, 1993; Chivers & Smith, 1994; Chivers & Smith, 1995; see Kelley & Magurran, 2003,  
46 Ferrari et al. 2010a for reviews).

47 Predator risk cues, however, are expected to elicit prey responses in direct proportion to  
48 the level of risk. A predator risk threshold exists above which a response is appropriate in  
49 response to imminent danger. When the predator risk level is relatively low, ignoring the risk cue  
50 may be appropriate, allowing prey to invest energy and/or time on other life activities (Ferrari et  
51 al., 2008).

52           How fish decide whether or not an indicator of risk surpasses the threshold of mounting a  
53 behavioral response is ideally done using quantifiable test stimuli. Auditory stimuli, for example,  
54 provide a convenient modality that can be quantified precisely, recorded and presented in  
55 standardized ways that allow for experimental tests of learning and risk assessment.

56           Many fishes have well-developed sensory receptors for detecting auditory stimuli  
57 (Ladich, 2019). For example, the Otophysi is a speciose group of hearing specialists comprising  
58 approximately 67% of all freshwater fish species (Nakatani et al., 2011). The Otophysi possess  
59 modified ribs called Weberian ossicles that link the gas bladder to the inner ear allowing these  
60 fishes to detect a wide range of sound stimuli (Kasumyan, 2008; Higgs et al. 2011; Ladich, 2019;  
61 Fine & Parmentier 2022; Sprague et al. 2022). Fathead minnows *Pimephales promelas*,  
62 glowlight tetras *Hemigrammus erythrozonus* and zebrafish *Danio rerio* (all otophysans) can  
63 associate novel auditory stimuli with risk of predation via releaser-induced recognition learning  
64 (Wisenden et al., 2008; Seigel et al., 2021).

65           Seigel et al. (2021) showed that zebrafish conditioned to fear one tone responded when  
66 that tone was replayed but did not respond when a tone of a different frequency was played,  
67 suggesting that recognition of an auditory stimulus was specific to that frequency and not  
68 generalized to all tones. This raised the question of whether or not Otophysan fish can associate  
69 risk with multicomponent auditory stimuli, and if risk is associated with each individual  
70 component or if risk is associated only with the sum of all components. Here, we report how we  
71 conditioned zebrafish to fear a multicomponent auditory stimulus (a three-note chord) followed  
72 by tests of their ability to recognize and respond to playback stimuli when all three notes were  
73 replayed, when two of the original notes were replayed, or when only one of the original notes

74 was replayed. In essence, we tested the response threshold of zebrafish to recognize and respond  
75 behaviorally to full or partial information about predation risk.

76

## 77 **2. Methods**

78

### 79 *2.1 Animals and housing*

80 Zebrafish were purchased from a commercial supplier (EkkWill Water Life Resources, Ruskin,  
81 FL, USA) and held in 75-L holding tanks at 24 °C on a 12:12 L:D cycle, with dechlorinated tap  
82 water filtered by hang-on-back power filters and fed commercial flake food.

83

### 84 *2.2 Preparation of chemical and auditory test cues*

85 Chemical alarm cue was prepared in two batches by euthanizing adult zebrafish with an overdose  
86 of methanetricaine sulfonate (MS222). The first batch used 49 zebrafish (mean  $\pm$  SE total length  
87 =  $38.3 \pm 0.4$  mm). The second batch used eight zebrafish (TL =  $37.9 \pm 0.5$  mm). Fish carcasses  
88 were placed together in a single beaker with deionized water and homogenized using a hand  
89 blender. The homogenate was filtered through a loose wad of polyester fiber to remove  
90 connective tissue, and diluted with deionized water to produce a concentration of alarm cue  
91 equivalent to one fish per 10 mL. Alarm cue was aliquoted into 10-mL doses and frozen at -20  
92 °C until needed. Blank water control cue was prepared by freezing 10-mL aliquots of deionized  
93 water at -20 °C until needed.

94 We used the Audacity® application to generate tones C<sub>5</sub> (523.25 Hz), E<sub>5</sub> (659.25 Hz),  
95 and G<sub>5</sub> (783.99 Hz) to create mpg files of (1) C, (2) C+G and (3) C+E+G, each of 5 min duration  
96 (the subscript '5' refers to the 5<sup>th</sup> octave on a standard piano keyboard, i.e., one octave above

107 middle C). Zebrafish can detect sounds from 100 Hz to 8000 Hz (Higgs et al. 2011) and in a  
108 previous study associated risk with a tone of 285 Hz (Seigel et al. 2021). The three sound files  
109 were downloaded to an iPhoneXR. Playback was conducted with volume set to maximum  
110 through waterproof earbud headphones (model PWPE10B, PyleUSA.com) suspended inside the  
111 test aquaria 12 cm from the tank bottom i.e., equidistant between the surface and bottom, in zone  
112 3 of the grid drawn on the front pane. This method produced clearly discernable auditory stimuli,  
113 with each tone of equal amplitude (Fig. 1).

114

### 115 *2.3 Power spectra analysis of auditory test stimuli*

116 The auditory stimuli C, CG, and CEG were played through waterproof earbuds fixed at one end  
117 of a 37-L aquarium (same size as used for fish studies), and recorded with a hydrophone  
118 (kithub.cc) fixed at the opposite end. No aerator was in the aquarium during these tests in order  
119 to reduce the background noise level. The microphone output was amplified and filtered  
120 (gain=1000, band pass filtering 1 Hz to 5 kHz) using an amplifier built in-house (Land et al.,  
121 2001). Open-source software (Audacity®, version 2.4.1) was used to produce a power spectrum  
122 of each tone combination (sampling time 10 s, sampling rate 44 kHz, spectrum size 131,072).  
123 The power spectra are shown in Figure 1. The sound level was taken relative to the acoustic  
124 background noise level. The relative amplitudes of the individual tones C, E, and G were,  
125 respectively:  $45 \pm 5$  dB,  $39.0 \pm 0.7$  dB,  $39.7 \pm 0.7$  dB.

126

### 127 *2.4 Experimental Protocol*

128 Individual test fish were placed in 37-L aquaria and left for at least 24 h to acclimate to the tank  
129 before testing. The front pane of the tank facing the aisle had a 5 x 5 cm grid drawn on it for

120 scoring fish behavior. Each tank was equipped with an air stone and a second length of airline  
121 tubing attached to the air stone to allow for injection and dispersal of test stimuli. Each tank had  
122 a small shelter object made from a ceramic tile 11 x 11 cm supported by legs 4 cm tall on each  
123 corner.

124         The experiment occurred in two steps; a conditioning trial followed by a test trial (Table  
125 1). All fish were conditioned with the multicomponent auditory stimulus C+E+G played  
126 simultaneously for 30 s during the stimulus-injection period between pre- and post-stimulus  
127 behavioral observations. The negative control treatment group received water while the three  
128 tones were being played. This treatment controlled for the potential of a pre-existing aversion to  
129 the CEG stimulus. The other three treatment groups received 10 mL of alarm cue solution while  
130 the three-note auditory stimulus was played. In all trials, fish activity (total number of grid lines  
131 crossed), vertical distribution (horizontal row of the grid, 1 = tank bottom, 5 = surface, occupied  
132 by the fish every 10 s), and use of the shelter object (total accumulated time under the shelter)  
133 was scored for 5 min before (pre-stimulus observations) and for 5 min after (post-stimulus  
134 observations) presentation of test stimuli. Each fish was used only once in a condition-test  
135 sequence. Injection hoses were replaced after each trial.

136         After each conditioning trial was completed, tanks were drained and refilled with room-  
137 temperature dechlorinated tap water. After at least 24h (24 – 168h, mean  $\pm$  SE = 67.2  $\pm$  3.9h), a  
138 test trial was conducted. The group conditioned with water + CEG stimuli was tested with the  
139 CEG stimulus for 30 s. Of the three treatment groups conditioned with alarm cue + CEG, one  
140 was tested with the CEG stimulus for 30 s, one group was tested with two of the three notes play  
141 (CG) for 30 s and one group was tested with one of the three notes (C) for 30 s. No chemical  
142 stimuli were presented during test trials.

143

## 144 *2.5 Statistical treatment*

145 Variables for change in behavior (post-stimulus – pre-stimulus) were not normally distributed  
146 (Kolmogorov-Smirnov test,  $P < 0.05$ ). Mann-Whitney U tests were used to compare the  
147 behavioral responses of water-conditioned fish to alarm cue-conditioned fish in conditioning  
148 trials. Comparisons of behavioral responses among the four treatment groups in test trials were  
149 done by Kruskal-Wallis one-way ANOVAs followed by Bonferroni-corrected pairwise  
150 comparisons between treatment pairs. The software used for these analyses was SPSS v26.

151

## 152 **3. Results**

153

### 154 *3.1 Conditioning trials*

155 Relative to control trials that received blank water, zebrafish that received conspecific alarm cues  
156 reduced vertical distribution ( $U = 167.5$ ,  $n = 59$ ,  $P = 0.005$ ), trended strongly to reduce activity  
157 ( $U = 223.5$ ,  $n = 59$ ,  $P = 0.064$ ), and increased time under the shelter ( $U = 502.5$ ,  $n = 59$ ,  $P =$   
158  $0.003$ ; Fig. 2).

159

### 160 *3.2 Test trials*

161 Response to acoustic stimuli in test trials differed by the completeness of the acoustic stimulus  
162 used in the test trial (CEG, CG or C). Treatment group had a significant effect on change in  
163 vertical distribution ( $KW = 10.374$ ,  $df = 3$ ,  $P = 0.016$ ; Fig. 3) but not activity ( $KW = 0.915$ ,  $P =$   
164  $0.822$ ; Fig. 4), or Shelter Use ( $KW = 4.669$ ,  $df = 4$ ,  $P = 0.198$ ; Fig. 5).



165 Pairwise comparisons of change in vertical distribution revealed that high variability within  
166 the water control treatment group caused it to overlap with the other three treatment groups  
167 (Table 2). For fish tested with the three-note chord, those conditioned with alarm cues + CEG  
168 trended toward a reduction in vertical distribution compared to those conditioned with water +  
169 CEG ( $U = 157$ ,  $n = 59$ ,  $P = 0.065$ ). For fish conditioned with alarm cues + CEG, response  
170 intensity in test trials to CEG did not differ from CG but response intensity to CEG and CG were  
171 each significantly more intense than the behavioral response to C alone (Table 2). These results  
172 indicate that zebrafish significantly reduced vertical distribution in response to the full three-note  
173 CEG and to the partial two-note CG compared to the one-note C stimulus.

174

#### 175 **4. Discussion**

176 Zebrafish conditioned with alarm cues and a three-note auditory stimulus responded with  
177 antipredator behavior when re-exposed to either the same three-note stimulus, or two of the three  
178 notes, relative to a playback stimulus containing only one of the three notes. These data  
179 corroborate earlier studies that demonstrated that fishes can associate risk with auditory stimuli  
180 (Wisenden et al., 2008; Seigel et al., 2021, 2022). Response intensity of learned responses were  
181 of lower intensity than behavioral responses during conditioning trials, manifest only as a  
182 significant change in vertical distribution but not change in activity or shelter use. In contrast,  
183 Seigel et al. (2021) showed significant changes in all three behavioral measures during test trials  
184 of zebrafish conditioned to a single tone. Nevertheless, these data are the first study to show that  
185 recognition of novel auditory indicators of predation risk can occur in response to  
186 multicomponent auditory stimuli and that response intensity is sensitive to the completeness of  
187 the match between conditioned and test stimuli. As such, these data provide an example of how

188 information uncertainty mediates the tradeoff between risk assessment and other life tasks (Lima  
189 & Bednekoff, 1999; Sih et al., 2000; Ferrari et al., 2016).

190 Information about the temporal and spatial distribution of risk confers fitness benefits to  
191 prey, but monitoring and updating this information incurs a cost in that the best information is  
192 available at times and places of greatest risk. Small fishes employ surreptitious olfactory  
193 sampling behaviors (“stealthy sniffing”) to monitor olfactory information while minimizing  
194 whole-body movements that are easily detected by predators. For example, darters and cichlids  
195 engage in opercular pumping (Hawkins et al., 2004; Gibson & Mathis, 2006; Bareto & Volpato,  
196 2006; Bareto et al., 2012; Sanches et al., 2015) and minnows perform fin-flicking (Sutrisno et al.,  
197 2014) in response to alarm cues. Both of these behaviors increase the rate of water flow over  
198 chemoreceptors in the external nares. In lab and field studies, fish avoid areas labeled with  
199 chemical alarm cues released from damaged epidermal tissues of conspecifics but continue to  
200 sample risk from afar aided by semiochemicals carried on water currents (Wisenden et al., 1994;  
201 1995; 2010). Alternatively, prey fish may approach the source of danger to acquire information  
202 about risk (Brown & Godin, 1999; Wisenden et al. 2010).

203 The “predator recognition continuum hypothesis” predicts that responses to indicators of  
204 risk lie on a continuum from innate recognition mechanisms (no learning), to acquired  
205 recognition (no generalization) to generalization from known predators to general classes of  
206 predictors of risk (Ferrari et al., 2007). Generalization of antipredator responses occurs when a  
207 novel stimulus is either missing some of the components of the conditioned stimulus, and/or has  
208 novel components added, reducing the certainty of recognizing the novel stimulus as an indicator  
209 of risk, resulting in a response of lower intensity or no response at all (Griffin et al., 2001; Ferrari  
210 & Chivers, 2009; Ferrari et al., 2007, 2009; Brown et al., 2011; Chivers et al., 2012; Chivers &

211 Ferrari, 2013; Mitchell et al., 2013). In the current study, we did not add novel notes, but simply  
212 sequentially deleted them until test subjects were too uncertain that the test stimulus matched the  
213 conditioned stimulus, and therefore did not show a behavioral response. There are parallels  
214 between our current study and studies where visual matching of novel predator identity to a  
215 conditioned model is impaired by the presence of turbidity (Ferrari et al., 2010b; Chivers et al.,  
216 2012) or when chemical cues are diluted below response threshold (Lawrence & Smith, 1989;  
217 Mirza & Chivers, 2003, Mirza et al., 2006). However, in those studies response strength was  
218 reduced when stimulus amplitude was reduced, whereas in our study stimulus intensity was held  
219 constant but the presence of selected components were deleted. Future experiments with various  
220 combinations of deleted and added components would inform the cognitive decision-making  
221 thresholds for response versus ignore.

222         Zebrafish presented with a multicomponent stimulus of three notes in this study failed to  
223 respond when only a single note was replayed. This finding contrasts with how fathead minnows  
224 can learn to recognize the alarm cues of a heterospecific member of their prey guild (brook  
225 stickleback *Culaea inconstans*) from a multicomponent olfactory cue that contains the odor of  
226 the predator (yellow perch *Perca flavescens*) fed a mixed diet of fathead minnows and  
227 stickleback (Mirza & Chivers, 2001). In that study, test subjects were able to isolate and  
228 remember components of a mixed olfactory stimulus, associate each of them with risk, and later  
229 respond with antipredator behavior when a subset of those chemicals were later presented on  
230 their own. There may well be an interaction between the sensory modality and the trade-off  
231 between the detect-respond / detect-ignore threshold. This topic is ripe for future work.

232         Releaser-induced recognition learning, and generalization, enable prey to label novel  
233 stimuli as dangerous. However, because animals must simultaneously evade risk while engaging

234 in other potentially conflicting, fitness-positive activities such as foraging, social and  
235 reproductive behaviors. Thus, animals cannot afford to minimize risk; instead, they must manage  
236 risk. There is a growing literature dedicated to revealing how animals avoid over-responding to  
237 stimuli that may or may not indicate risk (Wisenden & Harter, 2001; Brown et al., 2011; Ferrari  
238 et al., 2011; Chivers & Ferrari, 2014; Mitchell et al., 2015). Generalization windows may be  
239 broad if the diversity of predators is relatively small (Ferrari et al., 2007) or narrow if predator  
240 diversity is large (Mitchell et al., 2013). If the window for predator-recognition generalization is  
241 set too broadly, then prey risk responding to non-threatening predators. For example, velvet  
242 geckos *Oedura lesueurii* avoid all elapid snakes even though only some are dangerous (Webb et  
243 al., 2010). Conversely, a predator-recognition window that is too conservative may lead prey to  
244 fail to respond to a predator that poses real and imminent danger. For example, invasive  
245 predators can benefit from being outside of the generalized predator window of native prey  
246 (Goodchild & Stockwell, 2016; Anton et al., 2016; 2020; Carthey & Blumstein, 2018; Melotto et  
247 al., 2020; Stockwell et al. 2022). Similarly, prey can adjust response intensity to the strength of  
248 alarm cue or perception of risk represented by test stimuli (Mirza & Chivers, 2003, Mirza et al.,  
249 2006).

250           Understanding of the role of sound in mediating fish behavior is in its infancy (Rountree  
251 et al., 2019) but it is well established that there is a rich aquatic soundscape of acoustic signals  
252 used in the context of intrasexual male-male competition, and intersexual courtship displays  
253 (Putland, et al., 2018; Ladich, 2019). In the context of predator-prey interactions, acoustic stimuli  
254 are used by prey to detect the presence of predators, by predators to detect prey (in response,  
255 prey silence their acoustic signals in the presence of risk) and production of sounds by prey when

256 they are captured or held (Ladich, 2022). The present study reveals that acoustic stimuli may be a  
257 relatively unexplored dimension of risk assessment by fishes.

258 In this study, we showed for the first time that fish can associate multicomponent  
259 auditory stimuli with predation risk, and respond when a novel stimulus contains all or most of  
260 the original components but not when the play back stimulus was missing most of the salient  
261 information in the original conditioning stimulus. This work underscores the potential for  
262 auditory stimuli as an experimental tool for testing cognitive mechanisms in risk assessment by  
263 fishes.

264

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275

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447 **Figure legends**

448

449 **Figure 1.** Power spectra for tones used for conditioning and playback trials using tones C<sub>5</sub>  
450 (523.25 Hz), E<sub>5</sub> (659.25 Hz), and G<sub>5</sub> (783.99 Hz). Upper panel, C alone; middle panel, C+G;  
451 lower panel C+E+G.

452

453 **Figure 2.** Median  $\pm$  quartiles change in behavior by zebrafish in response to either blank water  
454 (open fill) or chemical alarm cues for conditioning trials (shaded fill).

455

456 **Figure 3.** Median  $\pm$  quartiles of change (post-pre) in vertical distribution (horizontal row of the  
457 grid) in response to tones in test trials. All fish were conditioned with a three-note chord (CEG) +  
458 either water (W) or conspecific alarm cue (A) and later tested with either all three notes (CEG),  
459 two of the three notes (CG) or only one note (C). Shared letters above bars are not statically  
460 different in Kruskal-Wallis post-hoc pairwise comparison tests among all four treatment groups  
461 ( $P > 0.05$ ).

462

463 **Figure 4.** Median  $\pm$  quartiles of change (post-pre) in activity (number of lines crossed) in  
464 response to tones in test trials. All fish were conditioned with a three-note chord CEG + either  
465 water (W) or conspecific alarm cue (A) and later tested with either all three notes (CEG), two of  
466 the three notes (CG) or only one note (C).

467

468 **Figure 5.** Median  $\pm$  quartiles of change (post-pre) in time under the shelter object in response to  
469 tones in test trials. All fish were conditioned with a three-note chord CEG + either water (W) or

470 conspecific alarm cue (A) and later tested with either all three notes (CEG), two of the three  
471 notes (CG) or only one note (C).

472 **Table 1.**

473 Experimental design using tones C<sub>5</sub> (523.25 Hz), E<sub>5</sub> (659.25 Hz), and G<sub>5</sub> (783.99 Hz).

Treatment group	Conditioning Trial	Test trial	n
Control	Water + (C+E+G)	(C+E+G)	15
Full three-note stimulus	Alarm cue + (C+E+G)	(C+E+G)	15
Partial two-thirds	Alarm cue + (C+E+G)	(C+G)	14
Partial one-third	Alarm cue + (C+E+G)	C	15

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479 **Table 2.**

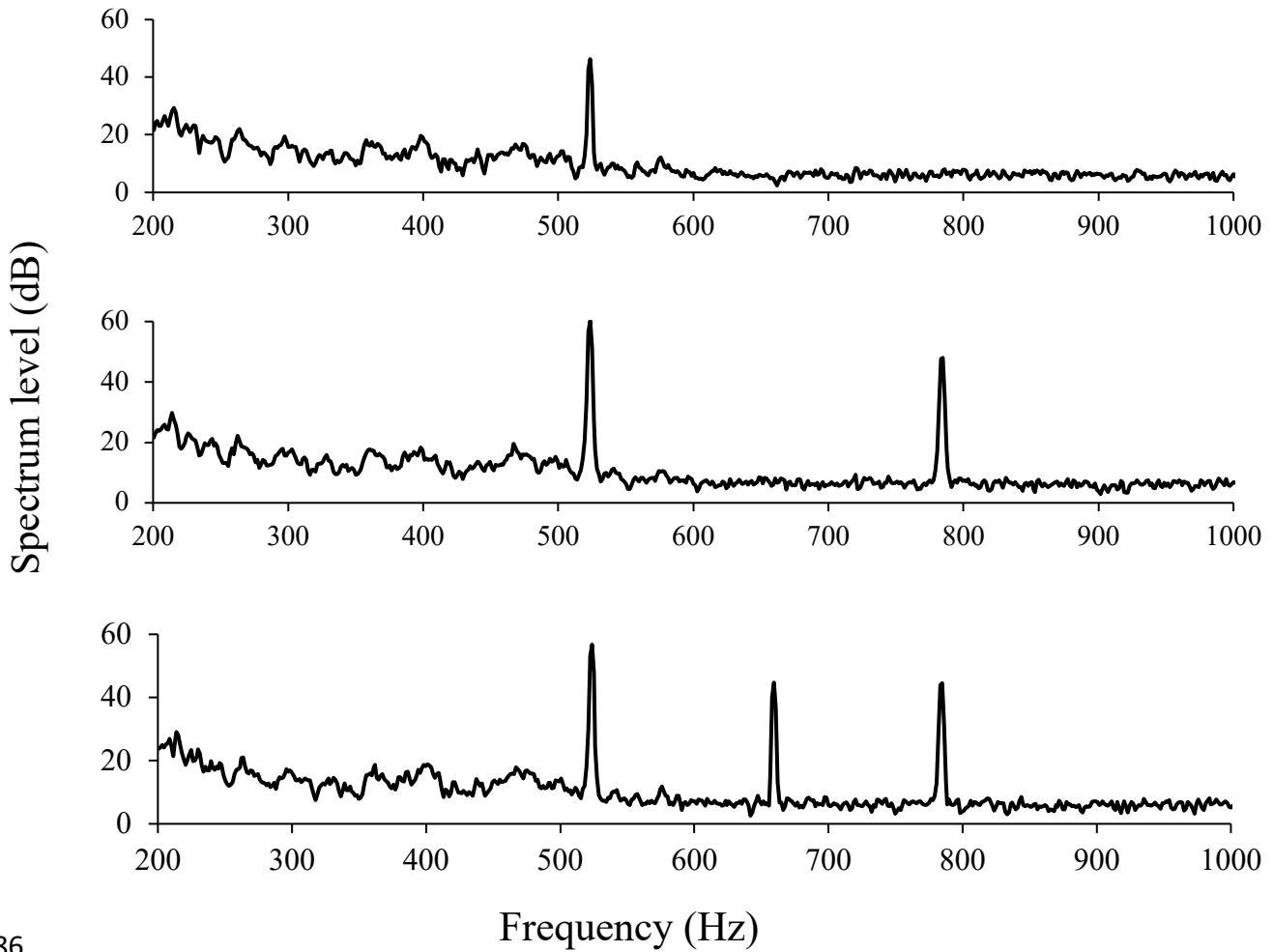
480 P-values for alpha-corrected post-hoc pairwise comparisons among treatment groups in test trials  
481 for change in vertical distribution. Abbreviations are Conditioning cues:Test cues, W = water; A  
482 = alarm cues; C, E, G = tones. See text for details.

Treatment groups	A(CEG):CEG	A(CEG):CG	A(CEG):C
W(CEG):CEG	0.081	0.225	0.229
A(CEG):CEG	1	0.616	0.003
A(CEG):CG		1	0.017
A(CEG):C			1

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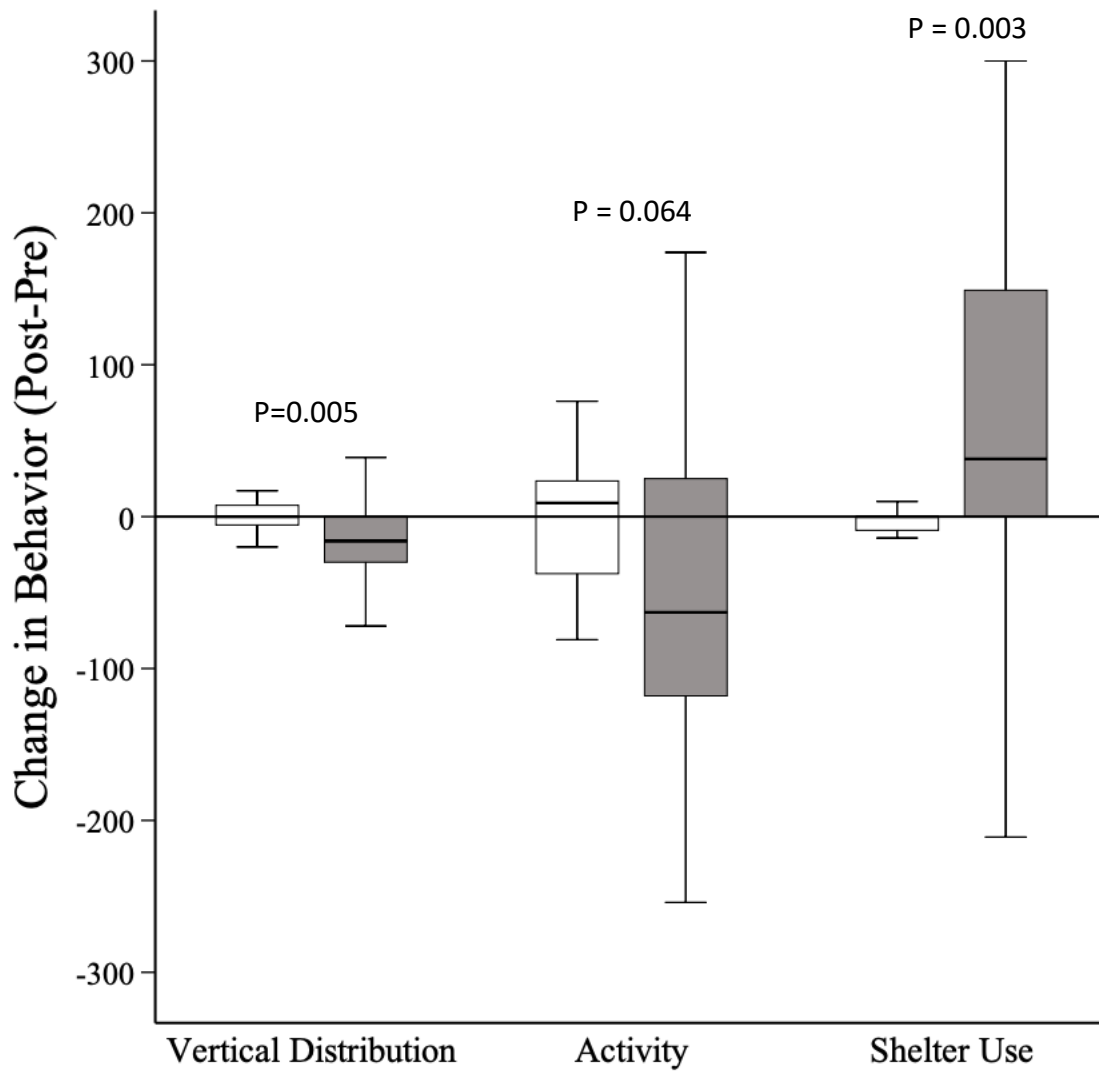
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487 Figure 1. Power spectra for tones used for conditioning and playback trials using tones C<sub>5</sub>  
488 (523.25 Hz), E<sub>5</sub> (659.25 Hz), and G<sub>5</sub> (783.99 Hz). Upper panel, C alone; middle panel, C+G;  
489 lower panel C+E+G.

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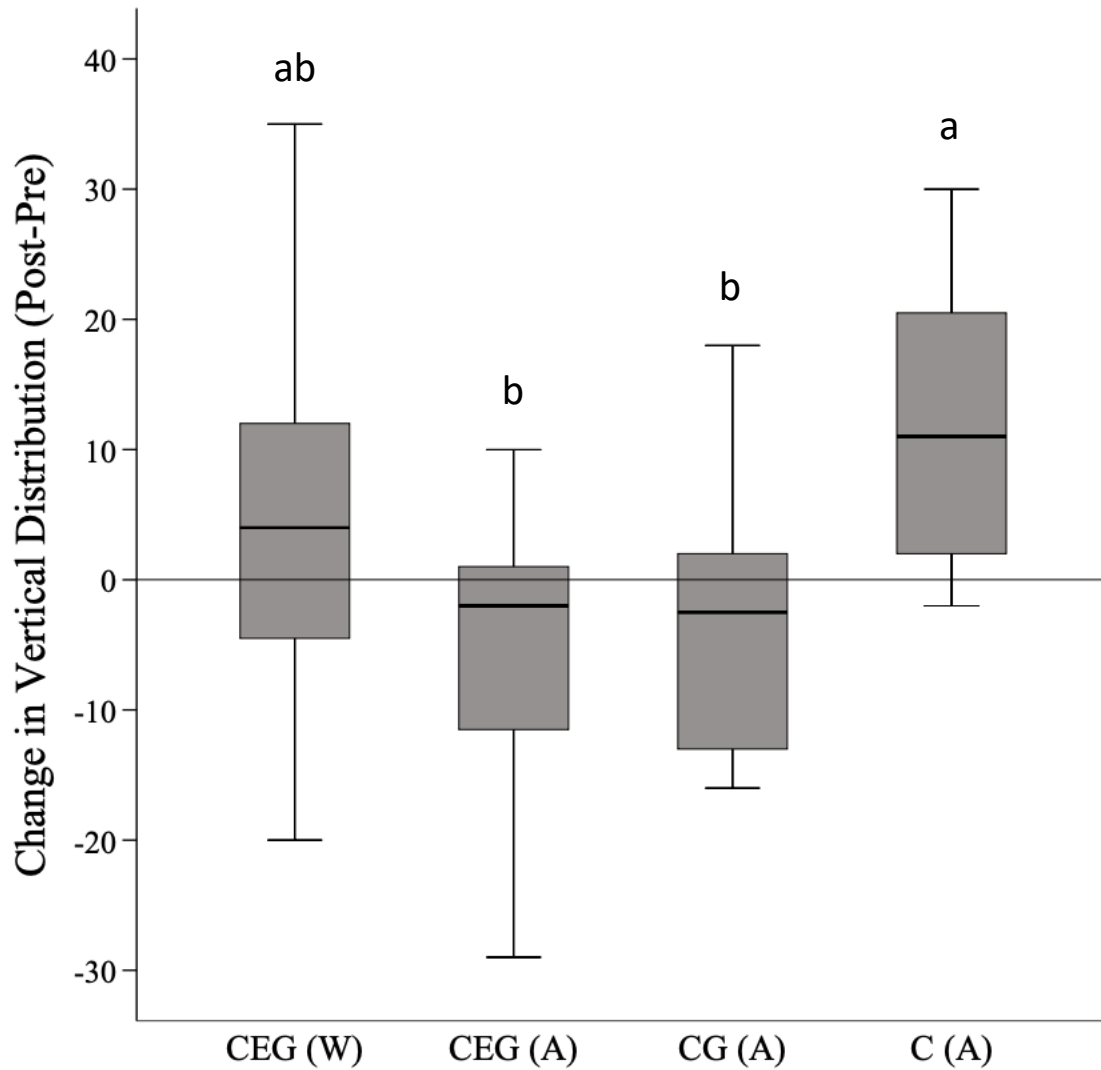
496 Figure 2. Median  $\pm$  quartiles change (post-pre) in behavior by zebrafish in response to either

497 blank water (open fill) or chemical alarm cues for conditioning trials (shaded fill).

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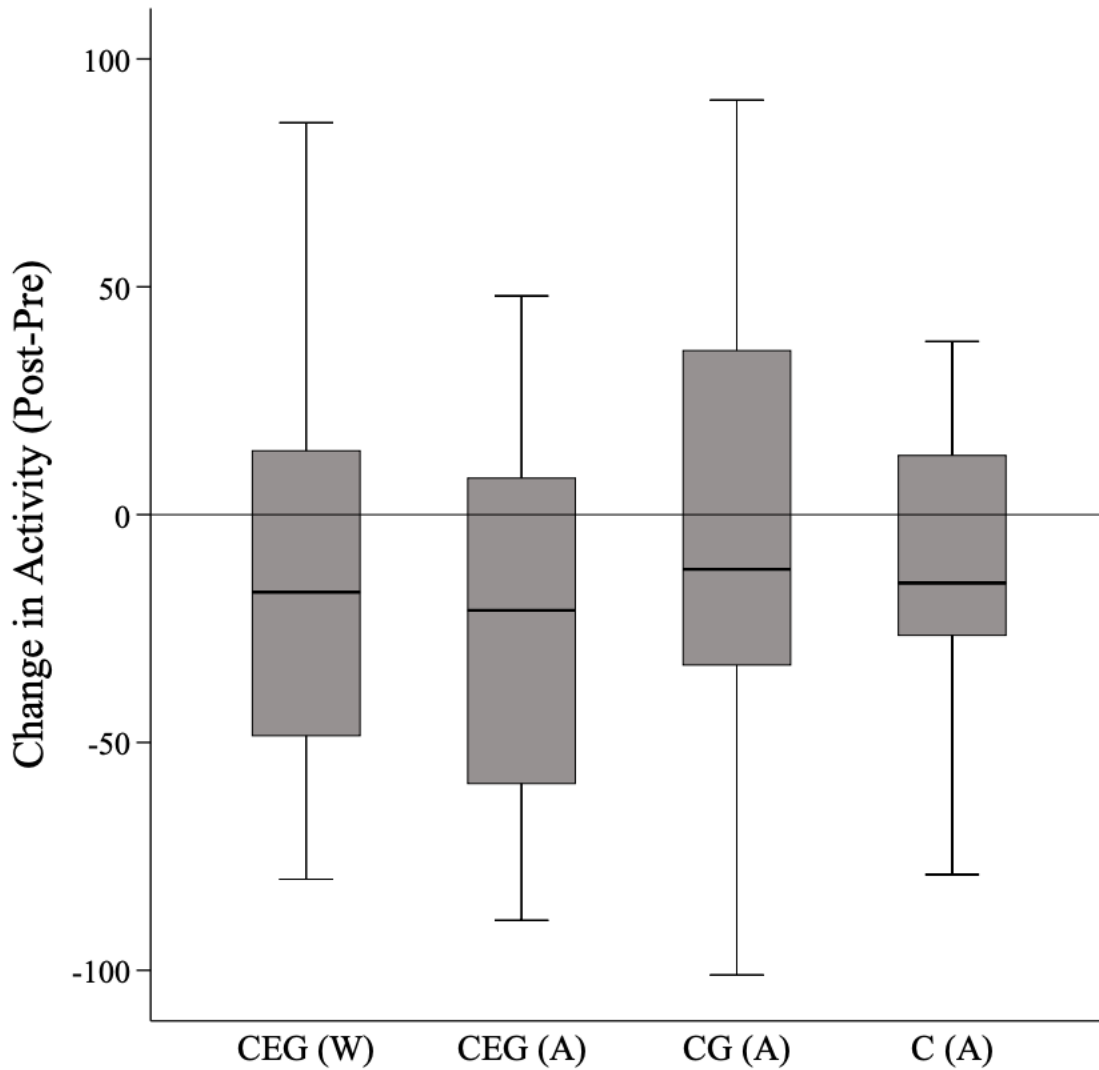
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501 Figure 3. Median  $\pm$  quartiles of change (post-pre) in vertical distribution (horizontal row of the  
502 grid) in response to tones in test trials. All fish were conditioned with a three-note chord (CEG) +  
503 either water (W) or conspecific alarm cue (A) and later tested with either all three notes (CEG),  
504 two of the three notes (CG) or only one note (C). Shared letters above bars are not statically  
505 different in Kruskal-Wallis post-hoc pairwise comparison tests among all four treatment groups  
506 ( $P > 0.05$ ).

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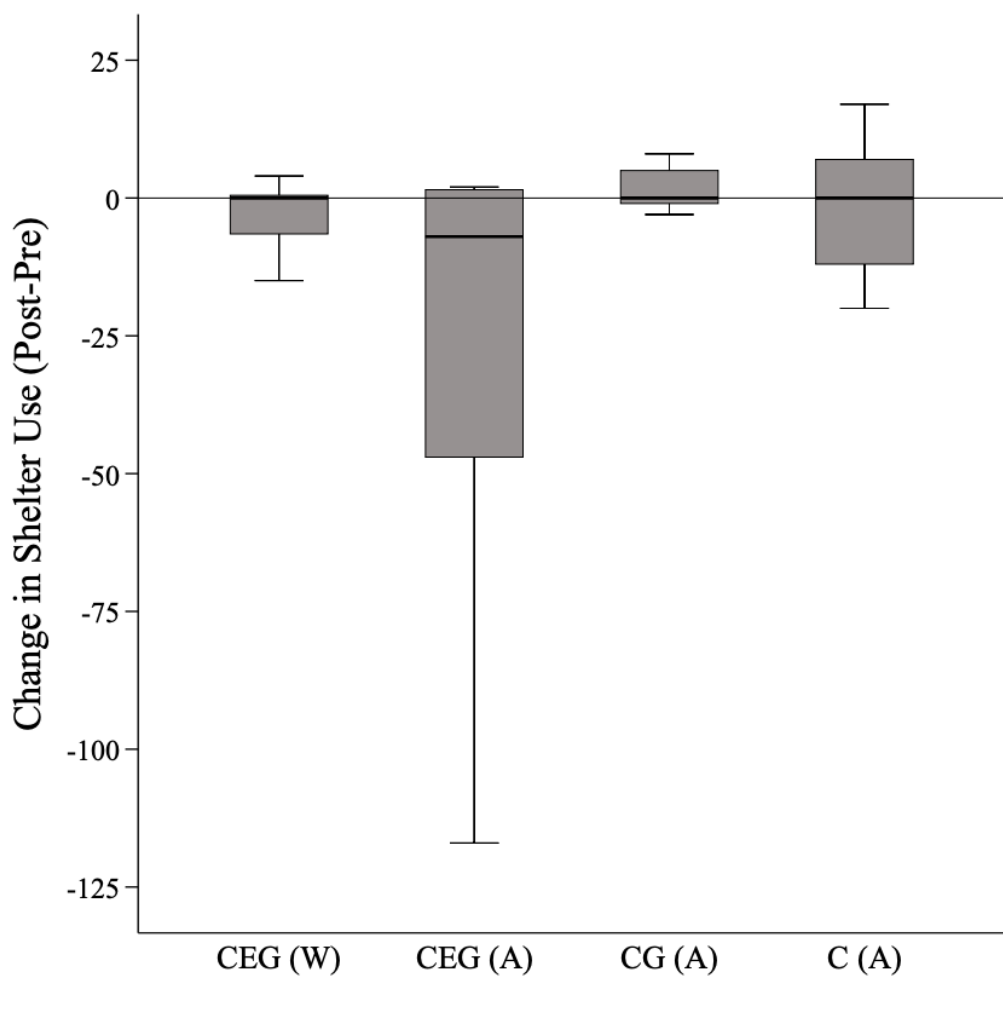
508

509 Figure 4. Median  $\pm$  quartiles of change (post-pre) in activity (number of lines crossed) in  
510 response to tones in test trials. All fish were conditioned with a three-note chord CEG + either  
511 water (W) or conspecific alarm cue (A) and later tested with either all three notes (CEG), two of  
512 the three notes (CG) or only one note (C).

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517 Figure 5. Median  $\pm$  quartiles of change (post-pre) in time under the shelter object in response to  
518 tones in test trials. All fish were conditioned with a three-note chord CEG + either water (W) or  
519 conspecific alarm cue (A) and later tested with either all three notes (CEG), two of the three  
520 notes (CG) or only one note (C).

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