Incorporating ventilatory activity into a novel tank test for evaluating drug effects on zebrafish

Masayuki Yoshida *

Graduate School of Integrated Sciences for Life, Hiroshima University, 1-4-4 Kagamiyama, Higashihiroshima 739-8528, Japan

ARTICLE INFO
Keywords: Anxiety  
Caffeine  
Ethanol  
Novel tank test  
Ventilation  
Zebrafish

ABSTRACT
The effects of ethanol and caffeine exposure on zebrafish, Danio rerio, were investigated using a combination of measurements of behavioral and physiologic responses in a novel tank situation. Ventilation activity as a physiologic measure was measured remotely by monitoring ventilation-related bioelectric signals from freely moving zebrafish in the test tank. The directions of the behavioral responses, except for outer area preference, were substantially the same in both ethanol- and caffeine-treated fish and qualitatively indistinguishable, suggesting that relying solely on behavioral measures may lead to inappropriate interpretation of drug effects when depending on limited behavioral parameters. By incorporating ventilation activity-related physiologic measures into the quantification of drug effects in novel tank tests, more-accurate evaluations of differences in the effects of moderate doses of anxiolytic ethanol and anxiogenic caffeine were possible. Here, we propose that combining physiologic measures such as ventilation rate and its variability with behavioral measures makes it possible to characterize the effects of environmental challenges on zebrafish in a multi-dimensional and more-detailed manner.

1. Introduction

Zebrafish, Danio rerio, are used in a variety of tests to assess the pharmacologic and toxicologic effects of chemicals, particularly during the drug discovery process [1–7]. The novel tank test is one of the most commonly used behavioral tests, as it enables the evaluation of a drug’s effects on anxiety-related emotional responses of zebrafish in a novel environment [4,8–15].

In the novel tank test paradigm, behavioral phenotypes are quantified by measuring parameters such as traveling distance, frequency of immobility episodes, and place/depth preferences. Although quantifying the manifestation of behavioral responses to various challenges is a useful tool, behavioral responses represent but one of the multiple levels and dimensions of biological responses. Thus, in many studies, analyses of biochemical parameters such as systemic cortisol level in fish are incorporated into assessments of environmental and/or pharmacologic challenges [8,9,14,16–22]. Although measurement of systemic cortisol levels is considered a sensitive indicator of stress in zebrafish, a major inconvenience of the cortisol assay is that the fish must be killed, thus enabling only retrospective interpretations.

Physiologic responses, including changes in heart rate in embryonic zebrafish and medaka, Oryzias latipes, are also used as sensitive indices in evaluations of environmental and chemical stresses [23]. Video-aided heart rate monitoring techniques combined with modern software processing is possible because of the transparent body and limited motor activity of the embryonic stages of the fish. Although it is possible to monitor cardiac activity in relatively large-size freely behaving adult fish using telemetry or data logging techniques [24], it is difficult to apply these techniques to small fish such as adult zebrafish or medaka, which are used in a variety of assays, thus making minimally invasive surgical manipulations inevitable.

Ventilatory activity in fish is another useful parameter for examining physiologic responses to environmental challenges [25–27]. The electrical signals derived from gill ventilation due to opercular movement can be monitored using electrodes placed away from the fish [28,29]. However, partial restraint of fish movement is usually required for stable and continuous monitoring of electrical ventilatory activity.

A technique that enables researchers to monitor ventilatory activity in freely swimming zebrafish was recently developed [30,31]. This technique, which employs a recording chamber equipped with more than 100 electrodes and relies upon an advanced algorithm to estimate both ventilation and fish motion, has been used to evaluate emotional...
states of zebrafish during specific behaviors.

In addition to this sophisticated technique, a simpler and more convenient method to monitor ventilation activity in combination with video tracking for behavioral analysis was introduced recently for zebrafish placed in novel tank situations [32]. This method has been used to examine habituation of zebrafish in the novel environment of a shallow, open field of water. Combined measurement of physiologic (ventilation) and behavioral measures enabled the researchers to determine that the habituation process consists of two phases that lead to a relatively stable habituated state [32].

The present study expanded upon this technique to examine its usefulness for evaluating the effects of drugs on behavioral and physiologic responses during the habituation of zebrafish to a novel tank test situation. In combination with conventional behavioral analysis, ventilation rate and ventilation rate variability (VRV) were measured to better quantify and discriminate the effects of drugs on the zebrafish habituation process in a novel tank situation. Several studies have shown that VRV is a useful measure for evaluating emotional states in zebrafish [30–32], similar to heart rate variability (HRV) in humans [23, 34]. These studies suggest that VRV is a useful alternative to measurement of HRV in fish [32], even though the precision of VRV measurement could be inferior to that of HRV.

Ethanol and caffeine were chosen for the purpose of this study as representative anxiolytic and anxiogenic agents, respectively, as they would be expected to facilitate or impair habituation processes in novel environments. Acute exposure to moderate concentrations of ethanol, typically 0.25–0.5%, reportedly has anxiolytic effects on zebrafish [8, 10, 35–38]. Although some studies have reported that higher doses of ethanol are required for inducing anxiolytic effects [12,39], higher concentrations of ethanol (≥1%) are also associated with the possibility of motor performance impairment. Therefore, in this study, an ethanol concentration of 0.3% was chosen to examine the expected anxiolytic effects of acute ethanol exposure [35,36,40].

Acute caffeine exposure reportedly exerts anxiogenic behavioral effects on zebrafish [8,14,40]. In pilot experiments, we found that acute exposure to higher doses of caffeine (≥200 mg/L) can induce convulsion- or seizure-like movements in zebrafish, as reported by Rosa et al. [14]. Thus, we chose a caffeine concentration of 100 mg/L to monitor expected moderate yet clearly discernable anxiogenic effects [8,14,21], although some studies have used higher doses of caffeine to elicit anxiogenic effects [9,14,18].

2. Materials and methods

2.1. Animals

A total of 67 laboratory-reared AB zebrafish aged 5–6 months were used in this study. The fish were housed in groups of 5–10 individuals per 2.5 l tank at 27 ± 2°C under a 14 h light/10 h dark cycle. The zebrafish were assigned to three groups: control group (n = 22, 11 males and 11 females), ethanol group (n = 22, 12 males and 10 females), and caffeine group (n = 23, 14 males and 9 females). All animal experiments were conducted in accordance with the Guidelines for Animal Experimentation at Hiroshima University (approval no. F19-1).

2.2. Novel tank test apparatus

The novel tank test apparatus was the same as that reported by Yoshida [32]. Briefly, the apparatus consisted of a white round test tank (height 125 mm, diameter 240 mm) equipped with four pairs of radially placed stainless-steel electrodes on the wall for recording ventilation-related electrical signals and a USB video camera (DMK23UM021, The Imaging Source, Bremen, Germany) mounted above the tank. Electrical signals were differentially amplified and filtered between 0.5 and 30 Hz (AB-610J, Nihon Kohden, Tokyo, Japan) and stored on a PC (MacBook Pro, Apple, Cupertino, CA, USA) via an A/D converter (sampling rate 1 kHz; PowerLab 16/35, AD Instruments, Bella Vista, Australia).

A plastic cylinder (height 30 mm, diameter 90 mm) was placed at the center of the test tank, and a square stainless-steel plate (55 × 55 mm) was placed inside the cylinder to serve as the ground electrode. The test tank was illuminated from above by white LED lights, providing an illuminance at the water surface of 710-770 lux. The test tank was also illuminated from below by an infrared LED light so that the silhouette images of the fish were clearly recorded at 30 frames/s (resolution 640 × 480 pixels).

2.3. Novel tank test procedure and data analysis

Novel tank tests were conducted as reported by Yoshida [32]. The fish was initially housed for 20 min in a pretreatment tank containing 400 ml of home water, 0.3% ethanol in home water, or home water containing 100 mg/L of caffeine (Wako Pure Chemical Industries, Osaka, Japan). The fish was then gently introduced into the test tank containing home water (for the control group), 0.3% ethanol in home water (ethanol group), or 100 mg/L of caffeine in home water (caffeine group). The depth and temperature of the water in the test tank were 30 ± 1°C, and the light in the test tank was provided by white LED lights, illuminating the tank from above. Video tracking for behavioral analysis was introduced recently for video visualization of heart beat and respiratory patterns [45,46]. The minor and major axes of the fitted ellipse of a Poincaré plot are used as standard descriptor 1 (SD1) and standard descriptor 2 (SD2), respectively [46,47]. SD1 and SD2 describe the short- and long-term
variability in intervals [46,47]. We also calculated the area of the fitted ellipses to describe the overall VRV in each 10 min period.

All statistical analyses were performed using R statistical platform (www.r-project.org). Differences were considered significant at \( P < 0.05 \).

3. Results

3.1. Motor activity

Ethanol and caffeine treatment induced a significant increase in swimming velocity compared with the control group during the earlier periods in the novel tank test trial (Dunnett’s test, ethanol vs. control in period 1: \( t = 4.185, P < 0.001 \); ethanol vs. control in period 2: \( t = 2.865, P = 0.011 \); caffeine vs. control in period 1: \( t = 2.879, P = 0.010 \); caffeine vs. control in period 2: \( t = 2.748, P = 0.015 \)) (Fig. 1A). In the ethanol and caffeine groups, the swimming velocity decreased toward the end of the test trial (repeated measures analysis of variance [ANOVA], ethanol: \( F_{2,42} = 3.472, P = 0.040 \); caffeine: \( F_{2,44} = 5.258, P = 0.009 \)), and the differences compared with the control group were insignificant in period 3 (Dunnett’s test, ethanol vs. control: \( t = 1.8, P = 0.135 \); caffeine vs. control: \( t = 0.988, P = 0.513 \)) (Fig. 1A). In the control group, no significant change in the swimming velocity was observed over the course of the test (repeated measures ANOVA, \( F_{2,42} = 0.076, P = 0.927 \)) (Fig. 1A).

The turning angle decreased significantly over the test period in all three groups (repeated measures ANOVA, control: \( F_{2,44} = 11.39, P < 0.001 \); ethanol: \( F_{2,42} = 14, P < 0.001 \); caffeine: \( F_{2,44} = 19.48, P < 0.001 \)), but drug treatment had no significant effect on turning angle in any period (Dunnett’s test, \( P > 0.05 \)) (Fig. 1B).

The number of darting behaviors changed significantly only in the caffeine group, decreasing over the test period (repeated measures ANOVA, control: \( F_{2,44} = 3.337, P = 0.045 \)), although the differences between the caffeine group and control group and between the ethanol group and control group were not significant in any of the three periods (Dunnett’s test, ethanol vs. control in period 1: \( t = 0.422, P = 0.879 \); ethanol vs. control in period 2: \( t = 1.042, P = 0.478 \); ethanol vs. control in period 3: \( t = 1.692, P = 0.167 \); caffeine vs. control in period 1: \( t = 1.857, P = 0.121 \); caffeine vs. control in period 2: \( t = 1.888, P = 0.113 \); caffeine vs. control in period 3: \( t = 1.288, P = 0.336 \)) (Fig. 1C).

Caffeine treatment induced a marked increase in the number of quick turns, and the fish exhibited jerking movements, especially during the early period of the trial; this behavior declined significantly toward the end of the trial (repeated measures ANOVA, \( F_{2,44} = 10.03, P < 0.001 \)) (Fig. 1D). The control and ethanol groups also exhibited a decreasing trend in the number of quick turns (repeated measures ANOVA, control: \( F_{2,42} = 7.24, P = 0.002 \); ethanol: \( F_{2,42} = 11.85, P < 0.001 \)) (Fig. 1D). The differences between the control and caffeine groups were significant in all three periods of the test trial (Dunnett’s test, period 1: \( t = 4.885, P < 0.001 \); period 2: \( t = 5.605, P < 0.001 \); period 3: \( t = 3.953, P < 0.001 \)) (Fig. 1D). No significant difference was observed between the control group and ethanol group in any of the three periods (Dunnett’s test, period 1: \( t = 0.912, P = 0.563 \); period 2: \( t = 0.896, P = 0.574 \); period 3: \( t = 0.843, P = 0.609 \)) (Fig. 1D).

3.2. Location preference

Zebrafish in the control and ethanol groups preferred to remain in the outer area of the arena during period 1 but gradually moved toward the middle area as the test progressed (Fig. 2). No difference in area preference was observed between the control group and ethanol group in any of the three periods, except that during period 1, ethanol-treated fish tended to remain in the outer area to a slightly greater degree than the control fish (Dunnett’s test, \( t = 2.315, P = 0.044 \)) (Fig. 2A).

In period 1, the caffeine-treated fish stayed in the middle and inner areas of the arena longer than did control fish (Dunnett’s test, middle area: \( t = 5.119, P < 0.001 \); inner area: \( t = 9.652, P < 0.001 \)) (Fig. 2A). In contrast, the amount of time the fish spent in the outer area during period 1 was significantly shorter in the caffeine group than the control group (Dunnett’s test, \( t = 10.966, P < 0.001 \)) (Fig. 2A). This pattern of area preference in the caffeine group was due at least in part to the jerking movements of the caffeine-treated fish, which exhibited a rapid swimming velocity accompanied by frequent quick turnings (Fig. 1A, D). This pattern did not change markedly in periods 2 and 3 (Fig. 2B, C). Thus, even though the preference for remaining in the middle and inner areas of the tank increased and the preference for the outer area declined in the later periods in the control group, the length of time spent in the inner area among fish in the caffeine group was still longer than that in the control group in the later periods (Dunnett’s test, period 2: \( t = 4.855, P < 0.01 \); period 3: \( t = 3.176, P = 0.004 \)) (Fig. 2B, C), and the length of

Fig. 1. Behaviors of zebrafish in the three periods in the 60 min novel tank test trial: period 1, 0–10 min; period 2, 20–30 min; period 3, 50–60 min. (A) Swimming velocity. (B) Turning angle. (C) Number of darting movements. (D) Number of quick turns. See text for definitions of darting movements and quick turns. Circles, control group; rectangles, ethanol group; triangles, caffeine group. Means ± SEMs are shown. Significant differences from control group are indicated by ++ (for ethanol group, \( P < 0.01 \)), * (for ethanol group, \( P < 0.05 \)), ++ (for caffeine group, \( P < 0.01 \)), and + (for caffeine group, \( P < 0.05 \)).
time spent in the outer area was shorter in period 2 (Dunnett’s test, $t = 3.615, P = 0.001$) (Fig. 2B).

3.3. Ventilation activity

The ventilation interval and nRMSSD increased as time elapsed after subject fish were introduced into the test tank in both the control group (repeated measures ANOVA, interval: $F[2,42] = 39.23, P < 0.001$; nRMSSD: $F[2,42] = 15.17, P < 0.001$) and ethanol group (repeated measures ANOVA, interval: $F[2,42] = 17.43, P < 0.001$; nRMSSD: $F[2,42] = 10.49, P < 0.001$) (Fig. 3A, B). The trend toward an increase in ventilation interval and nRMSSD were similar in the control and ethanol groups, except for the first time block, in which the ethanol-treated fish exhibited a greater nRMSSD (Dunnett’s test, $t = 2.486, P = 0.029$) (Fig. 3B). By contrast, no significant increase or decrease in either ventilation interval or nRMSSD was observed in caffeine-treated fish during the course of the test period (repeated measures ANOVA, interval: $F[2,44] = 1.102, P = 0.341$; nRMSSD: $F[2,44] = 2.81, P = 0.071$) (Fig. 3A, B). Although no significant differences were observed between the control and caffeine groups in terms of ventilation interval and nRMSSD during period 1, both of these parameters were markedly lower in the caffeine group during periods 2 and 3 compared with the control group (Dunnett’s test, interval, period 1: $t = 0.540, P = 0.811$; period 2: $t = 2.671, P = 0.018$; period 3: $t = 4.345, P < 0.001$, nRMSSD, period 1: $t = 1.181, P = 0.394$; period 2: $t = 4.779, P < 0.001$; period 3: $t = 4.207, P < 0.001$) (Fig. 3A, B).

Fig. 4 shows Poincaré plots of the ventilation interval in representative individual fish in the control, ethanol, and caffeine groups. Averaged fitted ellipses (SD ellipses) are also shown in Fig. 5. Qualitative comparison of the Poincaré plots and SD ellipses suggested that the dispersion in the plots during period 1 was the greatest in the ethanol group, and the ellipses in the control and ethanol groups shifted toward the upper-right quadrant in the graphs for the later periods (Figs. 4 and 5). In the caffeine group, on the other hand, the SD ellipses were relatively small, and the locations did not change appreciably throughout the test trial (Figs. 4 and 5).

For quantitative between-group comparisons, the average SDs of the Poincaré plots were calculated (Fig. 6). SD1 and SD2 represent the dispersion of the plots along the minor and major axes of the fitted ellipses. Repeated measures ANOVA revealed increases in SD1, SD2, and the area of the SD ellipses during the test trial in the control and ethanol groups, indicating that both the long-term and short-term (as well as overall) variability in the ventilation interval increased as the fish became habituated to the test environment (control: $F[2,42] = 28.38, P < 0.001$; SD1, $F[2,42] = 22.64, P < 0.001$; SD ellipse area, $F[2,42] = 29.09, P < 0.001$, ethanol: $F[2,42] = 12.38, P < 0.001$; SD2, $F[2,42] = 11.29, P < 0.001$; SD ellipse area, $F[2,42] = 14.33, P < 0.001$) (Fig. 6). In addition, compared with the control group, the ethanol group exhibited significantly larger SD1 and SD2 values and greater SD ellipse area just after introduction to the test tank (period 1) (Dunnett’s test, SD1: $t = 2.785, P = 0.0133$; SD2: $t = 2.896, P = 0.001$; SD ellipse area: $t = 3.086, P = 0.006$) (Fig. 6).

In the caffeine group, by contrast, no significant changes were observed in SD1, SD2, or SD ellipse area over the three periods (repeated measures ANOVA, SD1, $F[2,44] = 2.229, P = 0.12$; SD2, $F[2,44] = 2.326, P = 0.11$; SD ellipse area, $F[2,44] = 2.111, P = 0.146$), although the initial response of the caffeine-treated fish to the novel environment in terms of the SD of VRV measurements was not different from that of control fish (Dunnett’s test, SD1: $t = 0.008, P = 1.0$; SD2: $t = 1.479, P = 0.245$; SD ellipse area: $t = 0.765, P = 0.663$) (Fig. 6). Furthermore, as no time-dependent increase in SD1, SD2, or SD ellipse area were observed in caffeine-treated fish, the measures were significantly smaller than those in control fish in both periods 2 and 3 (Dunnett’s test, SD1, period 2: $t = 4.17, P < 0.001$; period 3: $t = 4.823, P < 0.001$, SD2, period 2: $t = 5.42, P < 0.001$, period 3: $t = 6.482, P < 0.001$, SD ellipse area, period 2: $t = 4.915, P < 0.001$; period 3: $t = 5.717, P < 0.001$) (Fig. 6).

Fig. 2. Mean proportions of fish at distinct tank locations in the three periods of the novel tank test trial. (A) Period 1. (B) Period 2. (C) Period 3. See text for definition of the divisions of the test arena. Dark gray, inner area; pale gray, middle area; white, outer area. Significant differences from control group are shown by * (for ethanol group, $P < 0.05$), ++ (for caffeine group, $P < 0.01$), and + (for caffeine group, $P < 0.05$).

Fig. 3. Ventilation activity of zebrafish in the three periods in the 60 min novel tank test trial. (A) Ventilation interval. (B) nRMSSD. Circles, control group; rectangles, ethanol group; triangles, caffeine group. Means ± SEMs are shown. Significant differences from control group are shown by * (for ethanol group, $P < 0.05$), ++ (for caffeine group, $P < 0.01$), and + (for caffeine group, $P < 0.05$).
4. Discussion

4.1. Behavioral effects of ethanol and caffeine on habituation to the novel environment

After introduction to the novel-tank test arena, the initial disorganized swimming patterns attenuated in all three groups (control, ethanol, and caffeine) as fish became habituated to the novel environment, as demonstrated by significant decreasing trends in turning angle, darting, and quick-turn behaviors. Two major differences between the control and test groups were observed. First, swimming velocity in periods 1 and 2 was greater in both the ethanol and caffeine groups than in the control group. Thus, ethanol and caffeine treatment exerted similar effects on this parameter. Second, quick-turn behaviors were more frequent in the caffeine group compared with the control group, whereas ethanol treatment had no effect on this parameter. Therefore, to differentiate the effects of moderate doses of ethanol and caffeine on the test environment, it is necessary to quantify "quick-turn" behavior, the definition of which is inevitably somewhat arbitrary, depending on the situation.

Preference for the outer area of the tank was apparent in the control and ethanol groups in period 1. Ethanol-treated fish tended to prefer the outer area of the tank in period 1, whereas caffeine-treated fish showed a slight preference for the outer area in period 2. However, in period 3, there was no significant difference in preference among the groups.

Fig. 4. Representative Poincaré plots of individual fish in the control (A–C), ethanol (D–F), and caffeine (G–I) groups in the three periods in the novel tank test trial. Data obtained from a single individual fish in each group are shown. Fitted ellipses are shown by solid elliptic circles. SD1 and SD2 are minor and major axes of the fitted ellipse, as indicated in E.

Fig. 5. Comparisons of average fitted ellipses of Poincaré plots of the control (blue), ethanol (green), and caffeine (orange) groups in period 1 (A), period 2 (B), and period 3 (C) in the novel tank test trial.
outer area in period 1. Fish in the control and ethanol groups invaded middle and inner areas as the trial proceed, suggesting that thigmotactic behavior decreased as the fish habituated to the test environment. Compared with control fish, caffeine-treated fish exhibited a significant difference in location preference. During period 1, caffeine-treated fish remained in the middle and inner areas of the tank longer and remained in the outer area for a shorter time. This could in part be due to the disorganized swimming pattern observed in the caffeine-treated fish, as indicated by a greater number of quick-turn behaviors compared with control fish, rather than a manifestation of location preference. During period 1, caffeine-treated fish remained in the middle and inner areas of the tank longer and remained in the outer area for a shorter time. This could in part be due to the disorganized swimming pattern observed in the caffeine-treated fish, as indicated by a greater number of quick-turn behaviors compared with control fish, rather than a manifestation of location preference. Therefore, in contrast to control fish, the stay time of caffeine-treated fish in the outer area tended to be longer in period 3 compared with earlier periods, but the stay time of caffeine-treated fish in the inner area was still longer than that of control fish.

The present results regarding the effect of 0.3% ethanol and 100 mg/L caffeine were largely consistent with those of previous reports regarding the anxiolytic and anxiogenic effects of ethanol [8,10,35–38] and caffeine [8,40]. The present study also observed reduced thigmotaxis [8,38] and increased locomotion [35] in zebrafish in response to the moderate dose of ethanol in the novel tank situation. The present observation of a marked increase in quick-turn movements throughout the test trial in caffeine-treated fish compared with control fish is consistent with previous reports showing that caffeine at a dose of 100 mg/L suppresses habituation to a novel environment, with the occurrence of prominent erratic movements [14,40]. This explains in part the higher swimming velocity in the caffeine group in periods 1 and 2. However, caffeine-treated fish also showed clear behavioral habituation during the test period, even under the condition of heightened anxiety, suggesting that the cognitive process underlying habituation to a novel environment is not impaired by caffeine treatment.

4.2. Physiologic effects of ethanol and caffeine on habituation to the novel environment

Incorporating physiologic measures (i.e., ventilation in the present case) into the quantification of the effects of a drug on fish in a novel tank test enabled more-accurate evaluation of the differences in the effects of ethanol and caffeine. Before habituation, there were no significant differences between groups in terms of ventilation interval. However, although the control and ethanol groups showed significant increases in ventilation interval as habituation proceeded in periods 2 and 3, no change in this measure was observed in the caffeine group, and the ventilation interval was significantly smaller than that of control fish.

Although ventilation interval, or ventilation rate, can be a useful measure of emotional state, nRMSSD has been proposed as a better additional measure for evaluating the emotional state of zebrafish [32], as ventilation interval alone provides limited information regarding emotion-related physiologic responses [32,48].

When introduced into a novel environment, the initial response of zebrafish is driven by heightened anxiety/fear, as indicated by shorter inter-ventilation interval and relatively small nRMSSD in the ethanol group. Importantly, the increasing trends in ventilation interval in the ethanol and control groups were almost parallel. Although neither ventilation interval nor nRMSSD in caffeine-treated fish differed from those in control fish in period 1, there was no increase (in contrast to control fish) in these measures in the later periods in the caffeine group; hence, both ventilation interval and nRMSSD in the caffeine group were markedly smaller compared with the control group. These observations, together with behavioral results, further confirm the anxiogenic effect of caffeine at a concentration of 100 mg/L, as shown by previous studies [8,14,21].

SD1 and SD2 of a fitted ellipse of a Poincaré plot are useful measures for describing short- and long-term variability in heart rate and ventilatory interval [46,47]. We applied these measures to further examine the effects of ethanol and caffeine treatment on VRV in zebrafish in the novel-tank test situation. The anxiolytic effect of ethanol treatment was apparent in the larger SD1 and SD2 values in period 1 compared with control fish. On the other hand, except for period 1, during which fear/anxiety responses in control fish were most intense, SD1 and SD2 were smaller in caffeine-treated fish compared with control fish, suggesting that ethanol and caffeine produce opposite short- and long-term effects on VRV.

4.3. Integration of behavioral and physiologic parameters to evaluate drug effects

Fig. 7 summarizes the behavioral and physiologic effects of ethanol and caffeine treatment on zebrafish before (period 1) and after (period 3) habituation to the novel tank. The most important finding was that the directions of the behavioral responses, except for outer area
preference, in both ethanol- and caffeine-treated fish were substantially the same and qualitatively indistinguishable. As described in Section 4.1, the lower preference for the outer area in the caffeine group was probably due to disorganized swimming activity rather than “location preference”, and thus, this measure might be inadequate as a parameter for comparing behavioral responses between groups. This finding suggests caution in terms of relying solely on behavioral observations that could be misleading regarding the effects of drugs, which could potentially have opposite effects on emotional state. Although behavioral tests for zebrafish are accepted as a powerful tool for evaluating responses to various environmental challenges and emotional states [4,10,11,17,50–53], care should be taken to ensure precise evaluation of the biological and psychological background of the behavioral responses.

The present study suggests that this potential problem can be overcome by combining physiologic and behavioral measures. It was found that the modes of the action of ethanol and caffeine could be differentiated based on physiologic, or ventilatory, characteristics of the responses to these drugs in the novel tank test situation. VRV was larger in ethanol-treated fish than control fish before habituation to the novel environment, whereas the effect of caffeine was not apparent in this respect (Fig. 7). In contrast, as habituation to the new environment proceeded, caffeine-treated fish exhibited smaller VRV compared with control fish, whereas the measure in ethanol-treated fish was almost the same as that in control fish (Fig. 7).

Here, we propose that combining physiologic measures such as ventilation rate and its variability with behavioral measures enables characterization of the effect of environmental challenges on zebrafish in a multi-dimensional and more-detailed manner. This approach can contribute to evaluations of the emotional states of zebrafish as well as drug assessment and discovery.

Declaration of Competing Interest

The author declares no competing interests.

Acknowledgements

This work was supported in part by Grants-in-Aid from KAKENHI (grant nos. 19K06767, 22K06316).

References

[1] A.J. Hill, H. Teraoka, W. Heideman, R.E. Peterson, Zebrafish as a model vertebrate for comparing behavioral responses between groups. This finding suggests caution in terms of relying solely on behavioral observations that could be misleading regarding the effects of drugs, which could potentially have opposite effects on emotional state. Although behavioral tests for zebrafish are accepted as a powerful tool for evaluating responses to various environmental challenges and emotional states [4,10,11,17,50–53], care should be taken to ensure precise evaluation of the biological and psychological background of the behavioral responses.

The present study suggests that this potential problem can be overcome by combining physiologic and behavioral measures. It was found that the modes of the action of ethanol and caffeine could be differentiated based on physiologic, or ventilatory, characteristics of the responses to these drugs in the novel tank test situation. VRV was larger in ethanol-treated fish than control fish before habituation to the novel environment, whereas the effect of caffeine was not apparent in this respect (Fig. 7). In contrast, as habituation to the new environment proceeded, caffeine-treated fish exhibited smaller VRV compared with control fish, whereas the measure in ethanol-treated fish was almost the same as that in control fish (Fig. 7).

Here, we propose that combining physiologic measures such as ventilation rate and its variability with behavioral measures enables characterization of the effect of environmental challenges on zebrafish in a multi-dimensional and more-detailed manner. This approach can contribute to evaluations of the emotional states of zebrafish as well as drug assessment and discovery.

Declaration of Competing Interest

The author declares no competing interests.

Acknowledgements

This work was supported in part by Grants-in-Aid from KAKENHI (grant nos. 19K06767, 22K06316).

References

[1] A.J. Hill, H. Teraoka, W. Heideman, R.E. Peterson, Zebrafish as a model vertebrate for comparing behavioral responses between groups. This finding suggests caution in terms of relying solely on behavioral observations that could be misleading regarding the effects of drugs, which could potentially have opposite effects on emotional state. Although behavioral tests for zebrafish are accepted as a powerful tool for evaluating responses to various environmental challenges and emotional states [4,10,11,17,50–53], care should be taken to ensure precise evaluation of the biological and psychological background of the behavioral responses.

The present study suggests that this potential problem can be overcome by combining physiologic and behavioral measures. It was found that the modes of the action of ethanol and caffeine could be differentiated based on physiologic, or ventilatory, characteristics of the responses to these drugs in the novel tank test situation. VRV was larger in ethanol-treated fish than control fish before habituation to the novel environment, whereas the effect of caffeine was not apparent in this respect (Fig. 7). In contrast, as habituation to the new environment proceeded, caffeine-treated fish exhibited smaller VRV compared with control fish, whereas the measure in ethanol-treated fish was almost the same as that in control fish (Fig. 7).

Here, we propose that combining physiologic measures such as ventilation rate and its variability with behavioral measures enables characterization of the effect of environmental challenges on zebrafish in a multi-dimensional and more-detailed manner. This approach can contribute to evaluations of the emotional states of zebrafish as well as drug assessment and discovery.

Declaration of Competing Interest

The author declares no competing interests.

Acknowledgements

This work was supported in part by Grants-in-Aid from KAKENHI (grant nos. 19K06767, 22K06316).
