ゼブラフィッシュの不安，うつ様遊泳行動の定量的解析法の開発

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〔Research Paper〕

Development of a quantitative analysis method for anxiety- and depression-like swimming behavior of zebrafish

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Abstract

Anxiety and depressive disorders, which account for a large proportion of mental disorders, cause significant losses to the sufferers themselves as well as to their social activities. The identification of compounds that exhibit anxiolytic and antidepressant effects may be useful in the treatment of these disorders. However, screening for compounds with anxiolytic and antidepressant activities in animal models is challenging. This is because screening requires the use of many individuals. Once an individual is used, the effect remains in its memory; therefore, new individuals must always be used for screening. Recently, zebrafish have been used as a model to analyze anxiety and depressive disorders. Zebrafish are ideal for screening because they lay approximately 200 eggs at a time and hatch within 3 d. Zebrafish also responded to anxiolytic and antidepressant drugs. In this study, we developed a method for setting thresholds for anxiety- and depression-like swimming behaviors and found that alarm substance- and social isolation-induced stress stimuli produced different quantitative results. The results showed that the thresholds of bottom dwelling and freezing behavior were effective in detecting alarm substance-induced swimming behavior, but were not effective against the stress caused by social isolation, and that the use of the thresholds of erratic behavior was necessary. Because anxiety and depression are multifactorial disorders, differences in the results may reflect different molecular mechanisms, suggesting that this method is an effective tool for screening compounds.

Keywords: zebrafish, swimming behavior, video tracking, alarm substance, stress

Introduction

In 2019, one in eight people worldwide were reported to have mental disorders, with anxiety and depressive disorders being the most common mental disorders (World Health Organization 2022). Anxiety disorders cause physical symptoms, such as panic attacks and hyperventilation, whereas depression causes physical symptoms, such as insomnia and anorexia, as well as suicidal thoughts and suicide attempts. Therefore, a key to solving these problems is the discovery of new compounds with anxiolytic and antidepressant activities.

Monoamine, inflammation, and neuroplasticity hypotheses have been proposed to explain the molecular mechanisms underlying anxiety and depressive disorders (Gold 2015). However, the underlying mechanism remains unclear. Psychotropic drugs,

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such as antidepressants and anxiolytics, are used to treat anxiety and depression. However, the effects of these drugs have been observed to vary depending on the individual and the type of disease, as well as the side effects of the drugs, such as headaches, nausea, insomnia, decreased libido, and dependence (Usami 2017). Similar to humans, animals, such as livestock, exhibit anxiety, depressive disorder-like behaviors, and physiological responses. Treatment may include drug therapy similar to that used in humans; however, as in humans, the above-mentioned problems can arise (Takeuchi 2018).

Currently, several animal models are used to study anxiety and depressive disorders (Nagai *et al.* 2021). Among these animal models, zebrafish have characteristics that make them suitable for screening compounds with anxiolytic and antidepressant effects, such as the fact that they lay approximately 200 eggs at a time and fertilized eggs hatch in 3 d. Zebrafish has also been demonstrated to respond to anxiolytic and antidepressant drugs (Hawkey *et al.* 2021; Jaka *et al.* 2021). In animal models, a large number of animals are required to screen compounds with anxiolytic and antidepressant activities. Screening requires the use of many individuals because once an individual is used, the effect remains in its memory; therefore, new individuals must always be used for screening.

Stress stimuli that induce anxiety- and depression-like behaviors in zebrafish include stress (Onarheim *et al.* 2022), which is caused by the separation of one fish from a school of fish; stress from being placed in a new environment caused by being transported to an unfamiliar aquarium environment; and stress that a fish experiences when stimulated with the alarm substance collected from the scales of other zebrafish (Egan *et al.* 2009). Although stimuli that induce anxiety- and depression-like behaviors in zebrafish are well established, the measurement criteria for observing anxiety- and depression-like behaviors in zebrafish differ from report to report. Specifically, the measurement environment, such as the size, shape, and lighting intensity of the aquarium used, differs among reports (Chia *et al.* 2019; Onarheim *et al* 2022; Pinheiro *et al.* 2023). This variation in anxiety- and depression-like swimming behaviors within these measurement environments (Shishis *et al.* 2022) remains a barrier to quantitatively measuring changes in zebrafish swimming behavior in various anxiety and depressive disorder models in response to stress stimuli.

In the present study, we developed a method for setting thresholds for anxiety- and depression-like swimming behaviors and found that different stress stimuli produced different quantitative results. This study provides useful information for the screening of new compounds with anxiolytic and antidepressant activities.

Materials and Methods

Setting up the housing environment

Male zebrafish (Tübingen strain) aged 6–8 months were used. This strain was kindly provided by Dr. Nishina of the Tokyo Medical and Dental University. Zebrafish were divided into groups of four fish each and kept in a housing tank (6 cm × 25 cm × 13.5 cm). The water temperature in the housing tanks was maintained at 26–28℃. The light cycle in the facility was 14 h light:10 h dark, with lights turned on at 6:00 am and off at 10:00 pm. All fish were fed twice daily with a combination of freshly hatched brine shrimp (Japan Pet Design Co., Ltd., Tokyo, Japan) and dried micropellets (Otohime B2, Marubeni Nissin, Feed, Co., Ltd., Tokyo, Japan). All animals were naïve and had not been previously used in experiments.

Acquisition of the alarm substance

The alarm substance is stored in the club cells of zebrafish skin tissue and is released upon physical injury (Chia *et al.* 2019). After the adult zebrafish were euthanized in ice water and drained with paper, several fine incisions were made on both sides of the abdomen using a razor. The fish were then placed in 50 mL of distilled water for 10 min to elute the alarm substance.

Recording of swimming behavior

A swimming behavior observation tank (28 cm × 15 cm × 15 cm) was constructed with white walls on three sides and the bottom and a transparent acrylic plate on the front. This tank (observation tank) could be divided into left and right areas by a white partition, and the swimming behaviors of two zebrafish could be observed simultaneously. The lighting conditions of the observation tank were 2400–2500 lux. The tank was covered with a white curtain to minimize the influence of human shadows on swimming behavior. Swimming behavior was observed and recorded from the transparent wall using a commercially available web camera connected to a computer. The recording was performed at 640 × 480 pixels at 30 fps.

Acclimation

To record the swimming behavior of one zebrafish per arena in the observation tank, acclimation was performed to minimize the stress caused by the transfer from the housing tank to the observation tank, and the anxiety and depression-like responses caused by the injection procedure performed to expose the zebrafish to the alarm substance. First, the zebrafish were transferred from the housing tank to the observation tank using a handheld net and kept in the observation tank for 20 min. Subsequently, instead of the alarm substance, housing water was injected from a tube placed near the water surface, and the zebrafish were maintained for an additional 10 min. The zebrafish were then returned to their housing tank. This process was continued for five consecutive days to acclimatize the zebrafish to this series of operations. This acclimation process was performed between 10:00 and 14:00, which was the actual time of the experiment. To evaluate the degree of acclimation during the 5-d acclimation process, we recorded a 10-min period from the 10th to the 20th min after the zebrafish were transferred to the observation tank and the subsequent 10 min when the alarm substance was to be injected.

Alarm substance stimulus

Recordings began 10 min after the zebrafish were transferred from the housing tank to the observation tank. Two sessions of 10 min of recording each (before and after injection) were conducted when the zebrafish tank was injected with the alarm substance. The control group tank was injected with housing water instead of the alarm substance.

Social isolation experiment

The housing tanks were changed from one tank per four zebrafish to one tank per zebrafish. Each zebrafish was kept alone for 5 d, and the acclimation process of each individual was recorded and analyzed.

Behavioral analysis

The swimming behavior of each zebrafish was recorded and analyzed using a SMART 3.0 video image behavior analyzer (Bio Research Centre Co., Ltd., Nagoya, Japan).

Data analysis

Estimation statistics were used for statistical analyses. Significance tests are widely used in various fields of research; however, the use of p-values to evaluate the results of statistical tests leads to a dichotomous evaluation (e.g., $p < 0.05$, OK; $p > 0.05$, not OK). In contrast, estimation statistics, place more emphasis on the effect size than on the p-value. In other words, it is not about whether there is an effect but how much of an effect exists. Estimation statistics focus on the magnitude of the effect (effect size) and its precision. It complements null hypothesis significance testing by indicating whether an effect is present and providing information about how large the effect is (https://www.estimationstats.com/). The eNeuro journal and the International Society of Physiotherapy Journal recommended the use of estimation methods instead of null hypothesis statistical tests. Data were analyzed using ESTIMATION STATISTICS (https://www.estimationstats.com/) (Ho *et al.* 2019). The confidence interval was set to 0.95.

Ethics

This study was approved by the Animal Experiment Committee of the Meiji University (Approval number: MUIACUC2022-01).

Results

Effect of acclimatization

To confirm the effect of acclimation, we analyzed a 10-min swimming trace using SMART 3.0, a video image behavior analyzer, before (Day 1) and after (Day 5) acclimation (Fig. 1). One of the anxiety- and depression-like swimming behaviors of zebrafish is that they spend more time in the lower area of the tank when stressed (Egan *et al.* 2009). A comparison of swimming behavior before injection (Fig. 1A and 1B) revealed that the zebrafish spent more time swimming near the water surface after acclimation (Day 5) than before acclimation (Day 1). This indicates that acclimation reduces anxiety- and depression-like behaviors. In addition, even when housing water was added as a control instead of the alarm substance, pre-acclimated zebrafish spent more time in the lower area of the tank (Fig. 1A). Conversely, after acclimation, the zebrafish exhibited the same swimming behavior as before the addition of the housing water (Fig. 1B). These results suggest that acclimation reduced anxiety- and depression-like swimming behavior caused by the stress of moving to the observation tank. The acclimated zebrafish were used in the subsequent experiments.

Determination of thresholds for anxiety- and depression-like swimming behaviors induced by alarm substance stimulus

To establish a method to quantify anxiety- and

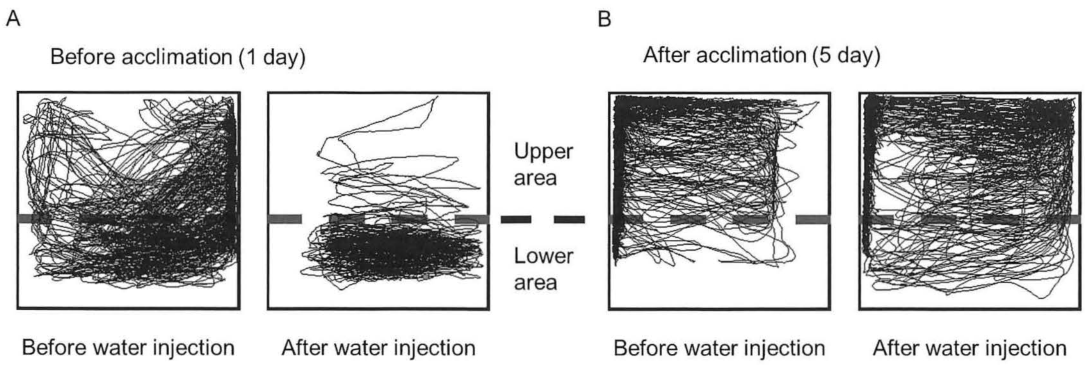


Fig. 1 Representative examples of zebrafish swimming traces before and after acclimation (A) Swimming traces of zebrafish before acclimation. The left panel shows the trace from the 10th to the 20th min after the fish were transferred from the housing tank to the observation tank, and the right panel shows the trace for an additional 10 min after injection of housing water at the 20th min. (B) Trace of zebrafish swimming behavior on the fifth day after acclimation. The left and right panels show the results of the same process as in (A). The lower area of the tank is below the dashed line. The swimming traces were analyzed using SMART 3.0 Video Image Behavior Analyzer.

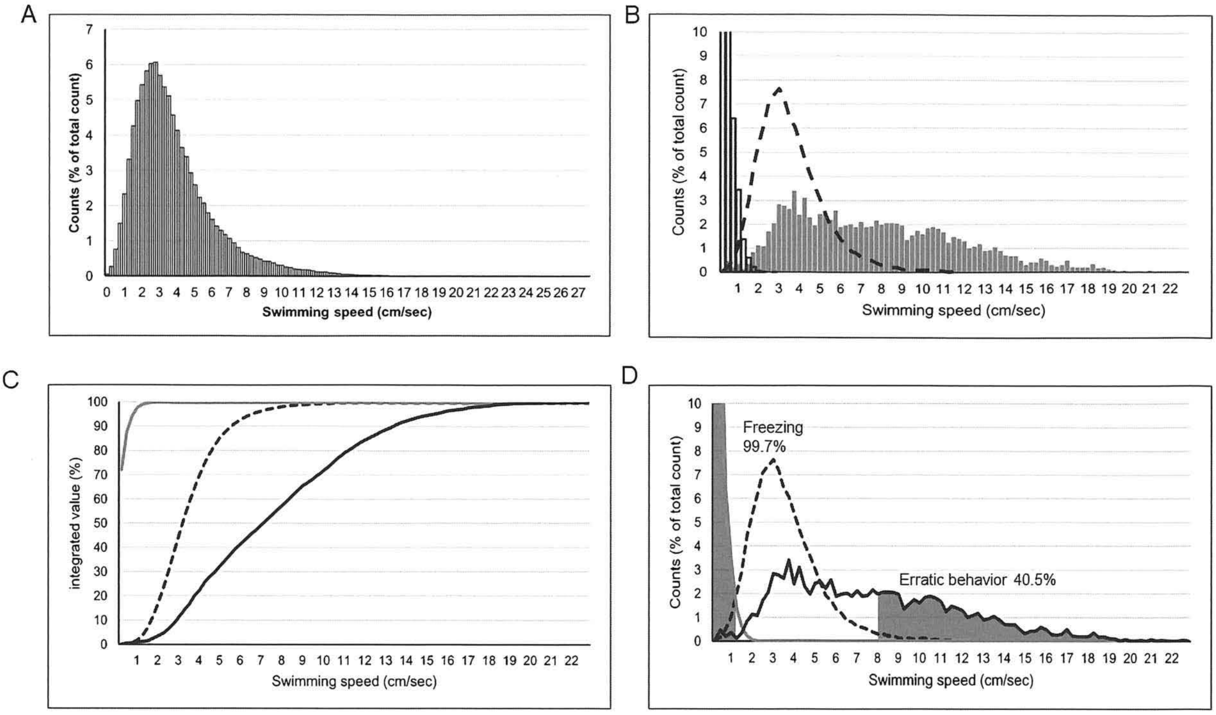


Fig. 2 Determination of swimming speed threshold for anxiety and depression using the alarm substance. (A) Normal swimming speed distribution in zebrafish after acclimation and injection of housing water (n=16). (B) Distribution of anxiety- and depression-like swimming behaviors after the addition of the alarm substance (n=4). The white and black columns represent the swimming speed distributions of freezing and erratic behavior, respectively. The dashed line represents the normal swimming speed distribution, as shown in (A). (C) Integrated values of (A) and (B) at each speed. The gray lines represent the integrated values of freezing behavior induced by the alarm substance, dashed lines represent the integrated values of (A), and black lines represent the integrated values of erratic behavior induced by the alarm substance. (D) The gray area represents the range of anxiety- and depression-like swimming speeds set by the thresholds.

depression-like swimming behaviors in zebrafish, we examined the distribution of swimming speeds after acclimation. The speed distribution of acclimated zebrafish is shown in Fig. 2A. Next, we examined how this speed distribution changed under stimuli that induced anxiety- and depression-like behaviors. In this study, we used the alarm substance, which is a stress stimulus that induces strong anxiety- and depression-like swimming behaviors in zebrafish under various experimental conditions (Speedie & Gerlai 2008; Egan *et al.* 2009). The results showed that under stimulation with the alarm substance, zebrafish exhibited freezing and erratic behaviors, as shown in Fig. 2B, which were not observed in the unstimulated condition. To compare and quantify these two types of anxiety- and depression-like swimming behaviors (Fig. 2B) with normal swimming behavior (Fig. 2A), we attempted to determine the thresholds under these conditions. As shown in Fig. 2C, all freezing behaviors derived from alarm substance stimulation were within a speed of 1.2 cm/s, and 99.7% of the freezing behaviors were derived from the alarm substance below this speed (Fig. 2D). Therefore, 1.2 cm/s was set as the threshold for freezing behavior. In contrast, erratic behavior tended to occur at a higher speed than the unstimulated speed; however, a clear difference was absent between the two (Fig. 2B and 2C). Therefore, we set a threshold that included as much erratic behavior as possible caused by the alarm substance. Within the range of speeds at which erratic behavior was observed, 8.0 cm/s was chosen as the value at which the proportion of non-stimuli behavior was small, whereas the proportion of erratic behavior was as large as possible. The results indicated that 40.5% of the erratic behavior at speeds above 8.0 cm/s was owing to alarm substance stimulation (Fig. 2D). Based on these results, we set anxiety- and depression-like behaviors below 1.2 cm/s and above 8.0 cm/s as the thresholds for anxiety- and depression-like behaviors, respectively.

Changes in anxiety and depression-like swimming behavior in response to different stress stimuli

Using these thresholds, we examined how different stress stimuli affected the freezing and erratic behaviors of zebrafish. The results are shown using estimation statistics. As the number of samples at each time point was eight, we performed several bootstrap resamples from this set. The mean of the resampled set was calculated, and the resampled mean of the control was subtracted to obtain the "Data Distribution of Mean Difference."

This histogram is the "mountain" in the bottom panel of Fig. 3, and the area of this distribution that contains 95% of the elements is shown as a black bar. Therefore, the expression "subtract control" is used to illustrate the difference in means. As shown in Fig. 3B and C, the freezing and erratic behaviors were increased after alarm substance stimulation (AS) compared to acclimatization (Acc), indicating that the thresholds were effective (freezing AS minus Acc, mean difference 36.9 [95.0% CI 10.2, 67.1]; erratic AS minus Acc, mean difference 18.9 [95.0% CI 4.5, 33.5]). Under these conditions, the time spent by zebrafish in the lower area of the tank was also predominantly increased by alarm substance stimulation, which is another indicator of anxiety- and depression-like behavior (bottom-dwelling AS minus Acc, mean difference 49.0 [95.0% CI 31.4, 58.8]). We tested whether these thresholds were also effective for another stressful stimulus, social isolation, which causes anxiety- and depression-like behaviors in zebrafish. Zebrafish live in schools, and social isolation is stressful for them (Daniel & Bhat 2022). For this experiment, we used zebrafish that had been acclimated for 5 d after isolation and measured their swimming behavior. In contrast to the results of alarm substance stimulation, a significant difference was absent in the bottom dwelling and freezing behaviors (bottom dwelling isolation minus Acc, mean difference −11.0 [95.0% CI −23.9, 3.82], freezing isolation minus Acc, mean difference −2.83 [95.0% CI −6.68, −0.561]) (Fig. 3A and B); however, a clear difference in their erratic behavior was observed between isolated and non-isolated zebrafish (erratic isolation minus Acc, mean difference 56.1 [95.0% CI 29.8, 102]) (Fig. 3C).

For reference, the results of an analysis of variance are included as Fig. 4; the estimation statistics provide more visual information. By comparing Fig. 3 with Fig. 4, we can visually obtain information that is not shown in Fig. 4, such as the number of samples and the degree of variation among the samples.

Discussion

In this study, we attempted to establish a method for determining the threshold for quantifying anxiety- and depression-like swimming behaviors in zebrafish. The results showed that the threshold was effective not only in detecting anxiety- and depression-like swimming behaviors induced by alarm substance stimuli but also by other stressful stimuli such as social isolation. Bottom-dwelling and freezing behaviors, which were used as indices in this study, appear to be similar to thigmotaxis and freezing in mice. Erratic behavior may be a form of panic behavior.

Anxiety and depression are multifactorial disorders involving multiple genetic and environmental factors. Twin studies have suggested that genetic factors are involved in approximately 40% of these disorders (Smoller 2016). This may be one of the reasons why the effects of antidepressants and anxiolytics vary de-

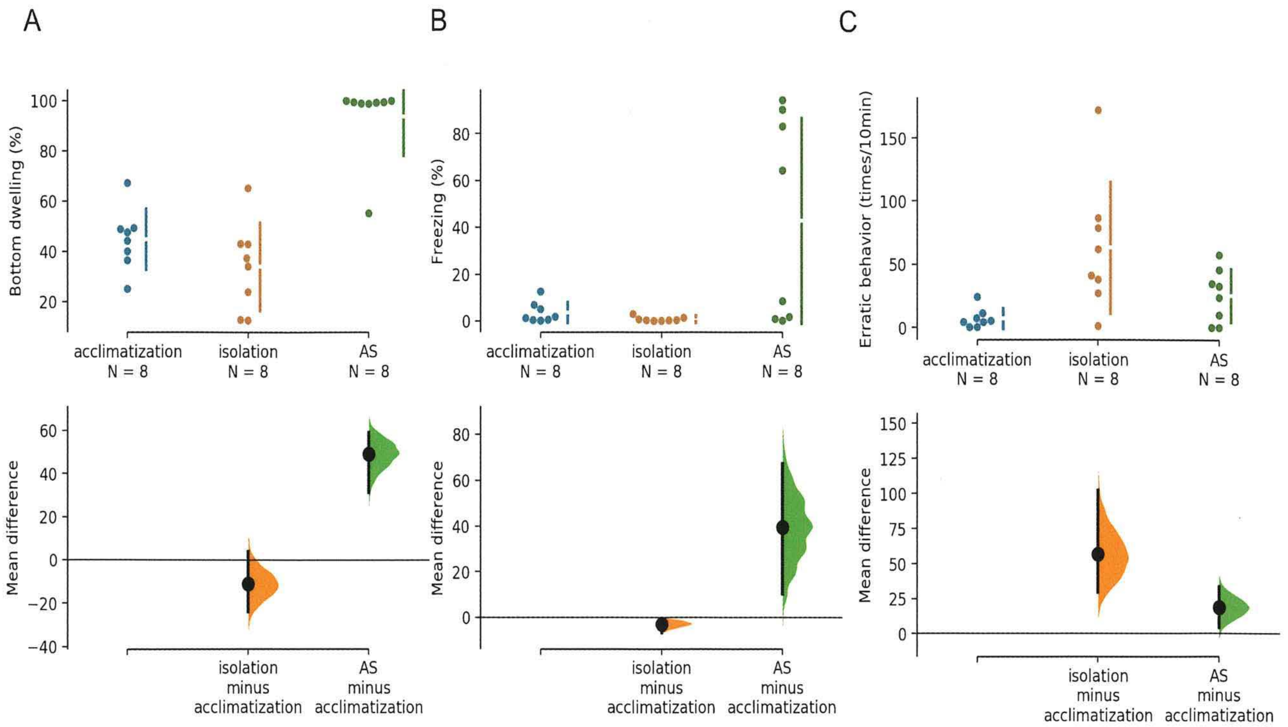
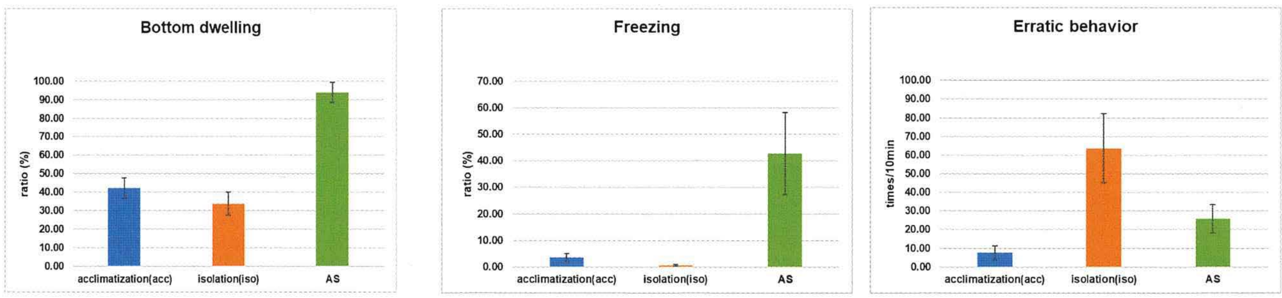


Fig. 3 Quantification and comparison of thresholds for anxiety- and depression-like swimming behavior induced by social isolation (isolation) and alarm substance (AS). (A) Comparison of the time spent in the lower area of the tank after isolation and AS stimulation compared to that after acclimation (n=8). (B) Comparison of the frequency of freezing after isolation and AS stimulation with that after acclimatization (n=8). (C) Comparison of the frequency of erratic behavior after isolation and AS stimulation with that after acclimatization (n=8). Data are represented as Cumming plots. In the top panel, all observed values are shown. The effect sizes, sampling distribution, and 95% confidence intervals are plotted on separate axes beneath the raw data. For each group, summary measurements (mean ± standard deviation) are drawn as gapped lines. The mean difference represents the amount of change in isolation and AS stimulation relative to acclimation with 95% confidence intervals. Statistical analysis was performed using ESTIMATION STATISTICS (https://www.estimationstats.com/) (Ho *et al.* 2019).



P<0.01 P<0.01 P<0.01

Fig. 4 Data from Fig. 3 were analyzed using analysis of variance.

pending on the individual and the type of disease (Usami 2017). Owing to this heterogeneity, screening for new compounds with anxiolytic and antidepressant effects also requires measuring their effects on a variety of anxiety- and depression-like swimming behaviors. However, the effects of a compound are determined using one type of anxiety or depression-like swimming behavior, in response to one type of stress stimulus (Hawkey *et al.* 2021, Jaka *et al.* 2021). The present experiment also showed that measurement of bottom dwelling and freezing behavior is not an effective measure of stress caused by social isolation, and that measurement of erratic behavior is also necessary. Therefore, screening for compounds that are effective for erratic behavior is necessary. The thresholds we established in the present study will help us determine which changes in swimming behavior can be measured in response to individual stresses when screening compounds. It is useful for each laboratory to first determine the thresholds using the method described in this study to screen the compounds.

In conclusion, the development of a method to determine thresholds as objective indicators of anxiety- and depression-like swimming behavior in zebrafish will be useful for screening new compounds with anxiolytic and antidepressant effects.

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ゼブラフィッシュの不安，うつ様遊泳行動の定量的解析法の開発

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要　旨

精神疾患の中でも大きな割合を占める不安障害やうつ病は，患者本人だけでなく社会活動にも大きな損失をもたらす。抗不安作用や抗うつ作用を示す化合物の探索は，これらの疾患の治療に有用であると考えられる。動物モデルを用いた抗不安・抗うつ作用を有する化合物のスクリーニングは困難である。なぜなら，スクリーニングには多くの個体を使用する必要があり，一度使用した個体は記憶に残るため，常に新しい個体をスクリーニングに使用しなければならないからである。近年，ゼブラフィッシュが不安障害や抑うつ障害の解析モデルとして用いられている。ゼブラフィッシュは一度に約200個の卵を産み，3日で孵化するのでスクリーニングに最適である。また，ゼブラフィッシュは抗不安薬や抗うつ薬にも反応する。本研究では，不安および抑うつ様遊泳行動の閾値を設定する方法を開発し，警告物質と社会的隔離刺激が異なる定量的結果をもたらすことを見出した。すなわち警告物質による遊泳行動の検出には，底棲行動と凍りつき行動の閾値が有効であったが，社会的隔離によるストレスに対しては有効ではなく，不規則行動の閾値を用いる必要があることがわかった。不安と抑うつは多因子疾患であるため，結果の違いは異なる分子メカニズムを反映している可能性がある。この方法が化合物のスクリーニングに有効なツールであることが示唆された

キーワード：ゼブラフィッシュ，遊泳行動，ビデオトラッキング，警告物質，ストレス

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