

Effect of Feeding Frequency on Anxiety-Like Behavior and Cortisol Levels in Group-Housed Zebrafish (*Danio rerio*)

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ABSTRACT. As the popularity of the zebrafish (*Danio rerio*) model increases, it is critically important to establish standardized care (husbandry) procedures. Standardization ensures that subjects are treated similarly, eliminating some variability between laboratories, as has been observed in pharmacological and environmental stress studies. Previous research demonstrated that the feeding regimen of zebrafish modulates stress responses. The current experimental study was designed to clarify the impact of feeding regimen in group-housed, adult zebrafish by assessing anxiety-like behavior and basal (baseline) cortisol levels after fish ($N = 36$, housed at a density of five fish/liter) were fed either once or twice per day for two weeks. Based on previous research, we hypothesized that zebrafish fed twice daily would demonstrate lower levels of anxiety-like behavior (i.e., increased exploration in the novel tank test) and have decreased basal levels of cortisol compared to zebrafish fed once daily. However, there were no significant differences ($p > .05$) in anxiety-like behavior or basal levels of cortisol between the two groups. This suggests that feeding once vs. twice per day in group-housed fish, at least in the short-term, had no effect on basal stress responses. It is possible that feeding frequency could impact stress responses with longer habitation in a research facility, although further studies are necessary to clarify. The current findings emphasize the need for a full understanding of how care, including feeding, influences neuroendocrine and behavioral endpoints in zebrafish subjects used in biobehavioral research, an important research area critical for understanding human conditions, such as anxiety-related disorders.

Keywords: zebrafish, feeding, behavior, stress, cortisol, husbandry, standardization

As of this writing, there are over 57,000 publications indexed in PubMed with the keyword “zebrafish” published since 1948. Nearly 65% of these reports were published in the last decade alone. These figures reflect the fact that the number of zebrafish labs have grown exponentially in recent years, including large facilities maintained by the U.S. National Institutes of Health (Kalueff et al., 2014) and the Mayo Clinic (Leveque et al., 2016), among others. The zebrafish (*Danio rerio*) model is popular for a variety of applicational and practical reasons. There is some similarity between zebrafish and humans in terms of morphological, physiological, and genetic factors (Kachanov et al., 2023; Kalueff et al., 2014). For example, zebrafish have 70 percent of the nucleotide sequence found in humans, sharing 82 percent of disease-related genes (Howe et al., 2013). The zebrafish can serve as a model for a variety of human health-related conditions

(Adhish & Manjubala, 2023; Cheresiz et al., 2020) as well as in drug and toxicity studies (Bauer et al., 2021; Lachowicz et al., 2021; Rosa et al., 2022).

With regards to studying stress responses, zebrafish can be exposed to a variety of stressors to elicit specific behavioral and physiological responses (Eachus et al., 2021; Egan et al., 2009; Steenbergen et al., 2011). Zebrafish exhibit similar anxiety-like behavior patterns as rodents when exposed to a new environment (Champagne et al., 2010), which serves as a basis for some popular zebrafish behavior tests, such as the novel tank test or the light-dark test (Blaser & Rosemberg, 2012; Blaser & Gerlai, 2006; Kysil et al., 2017; Wong et al., 2010). Introduction of zebrafish into a novel environment, such as with the novel tank test, initially elicits an instinctual anxiety-like behavioral response, which includes freezing or immobility and diving to the bottom compartment of the tank. After habituation, the

subject exhibits less freezing and increased exploration of the rest of the tank (Cachat et al., 2010). Zebrafish also release cortisol in response to stress via a conserved physiological stress response system, the hypothalamic-pituitary-interrenal (HPI) axis, which is homologous to the mammalian hypothalamic-pituitary-adrenal (HPA) axis (Alsop & Vijayan, 2009; Wendelaar Bonga, 1997).

The ease with which stress can be induced in this model, however, implies that special care must be taken to avoid introducing stressors with the care and husbandry practices used to maintain zebrafish colonies. The validity and reproducibility of zebrafish studies have been somewhat troubled by the fact that guidelines for the care and use of zebrafish vary between laboratories, institutions, and countries (Varga et al., 2018). Although typical lab practices have been shared in publications (Aleström et al., 2019; Lawrence, 2011; Matthews et al., 2002; Varga, 2016), video protocols (Avdesh et al., 2012; Paige et al., 2014), and in open-access databases like the Zebrafish Information Network, a standardized diet for laboratory zebrafish has not been established (Fowler et al., 2019), nor has a standardized feeding regimen (i.e., the frequency and timing of feeding) been accepted (Licitra et al., 2024).

Dietary contaminants influence the health and survival of adult and developing zebrafish (Tye & Masino, 2019; Tye et al., 2018); similarly, improper diet may cause significant alterations in zebrafish physical health and behavior. The specific protein and lipid content of the diet may also need to be specifically tailored according to developmental stage (Collins et al., 2021; Fernandes et al., 2016; O'Brine et al., 2015). For example, in one study, two different brands of processed food elicited differential effects on survival rate, embryo viability, and growth in both developing and adult zebrafish (Monteiro et al., 2018). In another example, the effects of two different diets (varying in biotin and avidin content) were compared over 12 weeks. Zebrafish given the control diet (containing no biotin or avidin) had the highest mortality and lowest weight gain, but zebrafish fed a diet containing supplemental biotin but no avidin showed the highest weight gain and lowest mortality (Yossa et al., 2011). Fewer studies have examined whether the frequency of feeding can directly influence specific behavioral measures. Standardization of zebrafish husbandry conditions, such as feeding practices, will help researchers gain a better understanding of behavior regulation and minimize any experimental design issues that increase variability and threaten the reproducibility of behavioral studies (Gerlai, 2019; de Abreu et al., 2024).

One recent study examined both the acute effects of the time lapse between feeding on behavior and the chronic effects of daily feeding frequency on anxiety-like

behavior in zebrafish (Dametto et al., 2018). In the acute feeding experiment, zebrafish fed three hours prior to behavioral assessment in the novel tank test demonstrated decreased locomotor activity and a trend toward anxiolysis compared to fish fed 0.5, 6, 12, 24, or 48 hours prior to behavioral testing. In the chronic feeding experiment (fifteen days), fish fed once per day had increased anxiety-like behavior compared to fish fed twice per day. Fish fed four or six times per day or only once every other day did not differ in anxiety-like behavioral measurements compared to fish fed twice per day. Zebrafish fed once a day also had decreased levels of glucose and glycogen and increased lactate when compared to fish fed twice a day, which indicated that carbohydrate metabolism may be related to behavioral changes observed in the novel tank test (Dametto et al., 2018). Because activity levels and exploratory behavior are linked to energy usage, metabolic changes caused by feeding regimen may impact fish performance in behavioral testing procedures. It is important to note that the zebrafish in chronic feeding study were physically isolated by a mesh barrier in the home tank in order to track the food consumption and body weight of each subject. Thus, although fish were exposed to visual and chemical cues from conspecifics, individual fish were still physically isolated from tank mates. Potentially, isolation could have exacerbated the effect of the feeding regimen on anxiety-like behavior. Social isolation has been shown to influence behavior, brain levels of neurotransmitters and metabolites, and cortisol levels, depending on developmental stage and the duration of isolation (Shams et al., 2015, 2017, 2018). As zebrafish are typically housed socially, it is important to discern the impact of feeding frequency on group-housed fish to better understand the impact of feeding regimen on stress responses.

The purpose of the current study was to investigate the impact of feeding frequency on anxiety-like behavior and basal cortisol levels in adult zebrafish housed in standard laboratory housing conditions. There are many methods used to assess anxiety-like behavior in the zebrafish model; the novel tank test chosen for the current study is a well-validated option (Blaser & Rosemberg, 2012; Kysil et al., 2017; Raymond et al., 2012; Wong et al., 2010). Based on the literature (Dametto et al., 2018), we hypothesized that adult zebrafish fed twice daily would demonstrate lower levels of anxiety-like behavior (i.e. increased exploratory behavior in the novel tank test) and have decreased basal levels of cortisol compared to adult zebrafish fed once daily. The results of this study will add to the current body of knowledge regarding the optimum feeding frequency in socially housed fish and may further support the need of a standardized feeding regimen across laboratories.

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Method

Animals

Wild-type, adult, mixed-sex (approximately 50:50 males:females) zebrafish (total $N = 36$) were purchased from a commercial supplier (Carolina Biological Supply, Burlington, NC) and were randomly assigned to one of four 1.8L housing tanks upon arrival to the facility (stocking density of 5 fish/L). Subjects were allowed to acclimate to the facility standards for care and feeding for approximately four days before any experimental procedures were initiated (Dhanasiri et al., 2013). All zebrafish were maintained in a two-shelf, stand-alone zebrafish housing rack purchased from Aquaneering (San Diego, CA) on a 14 h:10 h light:dark cycle (lights on at 6 a.m.), with water kept at $27 \pm 1^\circ\text{C}$ and pH of approximately 7.2. Other water quality parameters were measured biweekly, such as ammonia, nitrates, nitrites, alkalinity, and hardness, and were kept constant throughout the experiments. All procedures were conducted according to ethical guidelines (Harper & Lawrence, 2011; National Research Council, 2011; U.S. Department of Agriculture, 2015; Westerfield, 2000) and were in accordance with the Indiana University School of Medicine-NW Institutional Animal Care and Use Committee (protocol NW-49).

Experimental Procedure

After fish had acclimated to the facility, two of the four tanks ($n = 18$ total subjects) were chosen at random to be fed twice per day for 14 days. The other two tanks of fish ($n = 18$ total subjects) served as the facility standard controls of feeding once per day for 14 days. Fish were fed with flake food and dried shrimp ground to a powder with a mortar and pestle. For each feeding, the total food weight administered per fish approximated 4% of the average fish body weight. Fish that were fed once

per day were fed at approximately 9 a.m. each day; fish that were fed twice per day were fed around 9 a.m. and 4 p.m. each day. The fish were not fed on the morning of the behavioral testing. Behavioral testing was conducted on the fifteenth day after the regimen of feeding once or twice per day commenced. On the morning of the behavioral test, home tanks were relabeled and coded by the Principal Investigator to mask the Research Assistants collecting the data to the feeding conditions of each tank. Each coded tank was moved from the system to the experimental room, which had the same illumination and temperature as the housing room. Subjects were allowed to acclimate from the move for about 30 minutes prior to behavioral sampling. Fish from each condition were randomly chosen from the housing tanks, assigned a sample number, and were placed in the novel tank test one at a time to assess anxiety-like behavior. Fish were individually euthanized within a minute of completing the six-minute behavioral assay; each was netted from the novel tank and placed in the euthanasia solution without returning to the home tank or any other container. The euthanasia solution consisted of 0.1% clove oil/1% ethanol in system water (American Veterinary Medical Association, 2020; Davis et al., 2015; Wong et al., 2014). Subjects were used for both behavioral and cortisol analyses. Body samples were lightly dried with Kimwipes and stored at -20°C in individual 1.5 ml tubes for whole-body analysis of basal cortisol levels. Behavioral data collection and euthanasia of the subjects occurred between 9:30 a.m. and 12:00 p.m.

Behavioral Assay

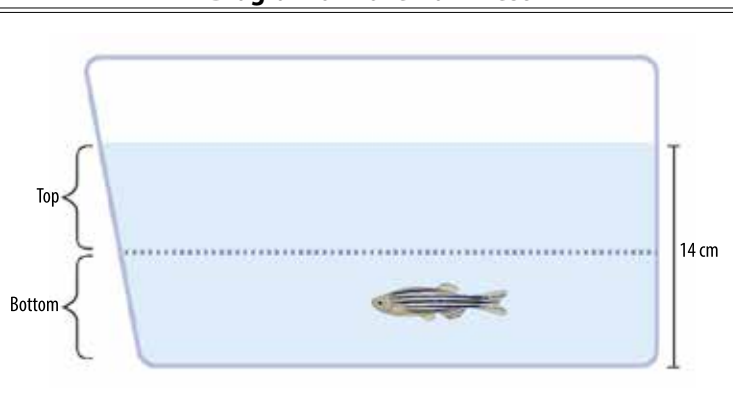
The novel tank test was chosen to assess anxiety-like behavior (Blaser & Rosemberg, 2012; Kysil et al., 2017; Raymond et al., 2012; Wong et al., 2010). Fish were individually netted from the home tank and placed into a novel tank with the same size and dimensions as the home tanks (15.2 cm height \times 27.9 cm top \times 22.5 cm bottom \times 7.1 cm width). The top of the tank was defined as the top 50% (approximately 7 cm) of the water column (total approximately 14 cm). See Figure 1 for diagram of novel tank. The first six minutes of behavior of each fish was recorded. Subsequently, BehaviorCloud motion-tracking software (Columbus, OH) was used to analyze the following dependent measures: distance in top zone (cm), time in top zone (sec), numbers of entries to top zone, total distance traveled (cm), mean ambulatory speed (cm/s), and immobility (sec).

Cortisol Assay

The cortisol extraction and assay were conducted by modifying previously published procedures (Cachet et al., 2010; Canavello et al., 2011; Egan et al., 2009). In brief, frozen whole-body samples were thawed and then

FIGURE 1

Diagram of Novel Tank Test



Note. This diagram indicates the top and bottom zones of the novel tank used in the current study. Figure was created with BioRender.com.

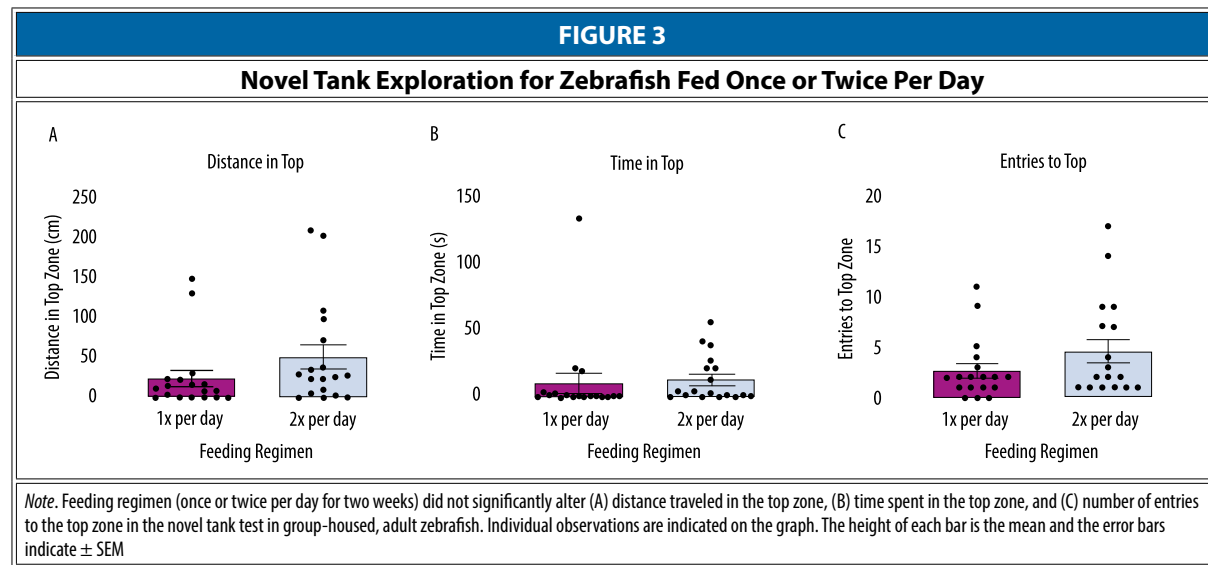
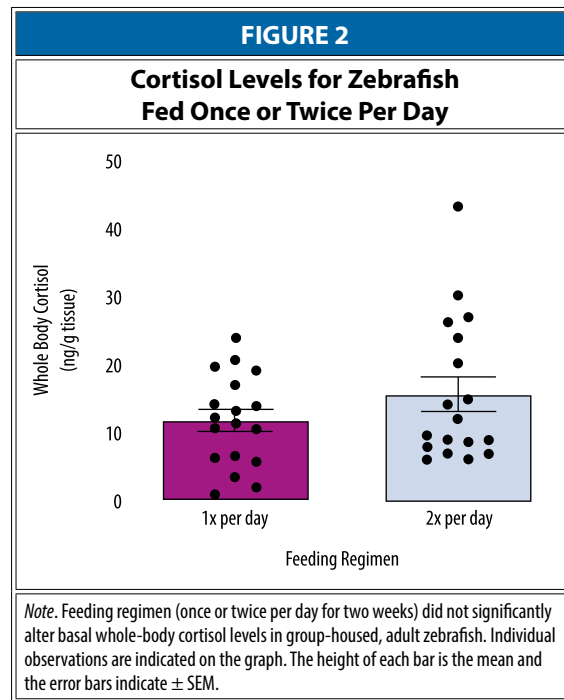
weighed. Ice-cold 25 mM phosphate-buffered saline (PBS) buffer was added to each tube; subsequently, each sample was blended using a handheld homogenizer. Diethyl ether was added to the homogenates and were vortexed and centrifuged. After centrifugation, the organic layer containing the cortisol was transferred by a glass pipet to a new test tube. After removal, the ether extraction was done twice more. All ether layers from each sample were collected in a single tube. After, all tubes containing the extracted material were dried with a light stream of air under the fume hood until a yellow oil containing cortisol remained. After the evaporation, PBS was added to the lipid-containing extract in each tube. Cortisol was quantified via an enzyme-linked immunosorbent assay (ELISA) as per the manufacturer's instructions (Salimetrics, State College, PA) and normalized to the whole-body weight of each subject. Thus, cortisol values are shown in ng cortisol/g tissue.

Statistical Analysis

A priori sample size calculations were conducted using G*Power software (Faul et al., 2007) using the following parameters: $d = 1.0$, $\alpha = .05$, power = .90. Effect size was determined by analyzing the provided open-access raw data on the anxiety-like measures in the novel tank test in fish fed either once per day vs. twice per day in a previously published study (Dametto et al., 2018). For all three zone-related measures in the novel tank test, the provided data indicated an absolute effect size of at least 1.00 (Cohen's d). Thus, the results comparing these two specific feeding schedules indicate that feeding regimen had a strong effect on zone exploration anxiety-related measures.

As mentioned previously, the treatments were masked to the research assistants during the data collection and analysis stages. Treatments were unmasked after

statistical analyses were conducted. All dependent measures (behavioral variables and cortisol levels) were first analyzed using Student's independent sample t tests with feeding regimen as the independent variable. According to Levene's test, one of the behavioral variables (entries to top) violated the equal variance assumption ($p = .028$). Thus, all the variables were reanalyzed using Welch's unequal variance t -tests. All data was processed with JASP software (University of Amsterdam, Amsterdam, The Netherlands). Data are presented as group means \pm standard errors of the mean. The criterion for statistical significance was set at $p < .05$.



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Results

Whole-Body Cortisol Responses

According to Welch's unequal variance *t* test, feeding frequency did not significantly affect whole-body cortisol levels, $t(28.84) = -1.32, p = .20$, see Figure 2. It appears that, at least in the short term (14 days), feeding twice per day does not alter basal levels of cortisol in group-housed zebrafish compared to group-housed zebrafish fed once per day.

Exploratory Activity in the Novel Tank Test

According to Welch's unequal variance *t* test, feeding frequency did not significantly affect distance traveled in the top zone of the novel tank test, $t(29.53) = -1.44, p = .16$, see Figure 3a. Feeding frequency did not significantly alter time spent in the top zone of the novel tank test, $t(26.66) = -0.32, p = .75$, see Figure 3b. Feeding frequency also did not alter the number of entries to the top zone of the novel tank test, $t(27.99) = -1.39, p = .18$, see Figure 3c. Feeding twice per day for two weeks did not significantly alter exploratory measures in the novel tank test in group-housed zebrafish compared to group-housed zebrafish fed once per day for two weeks.

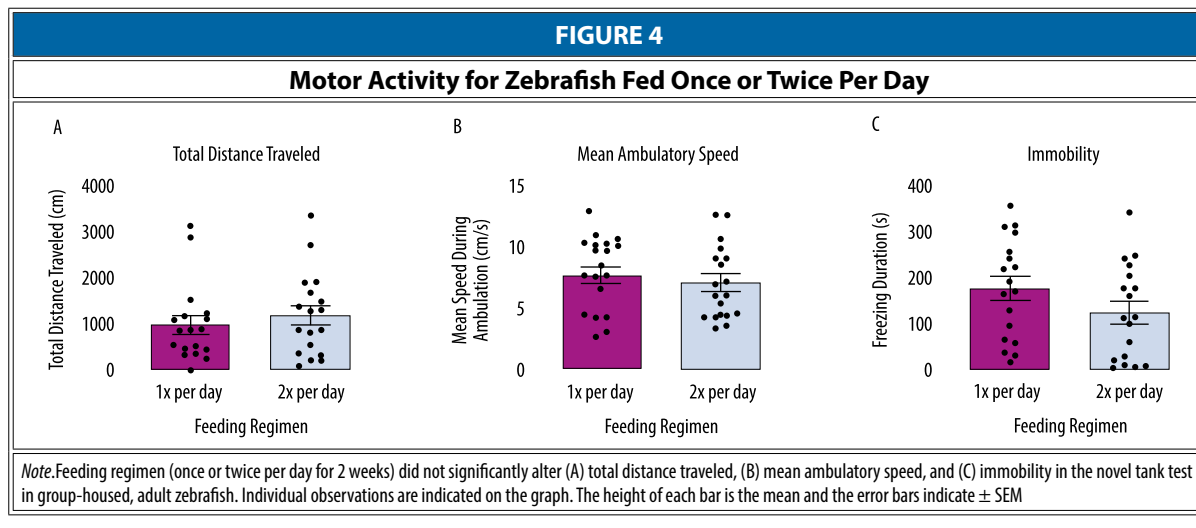
Locomotor Activity in the Novel Tank Test

According to Welch's unequal variance *t* test, feeding frequency did not significantly affect the total distance traveled, $t(33.85) = -0.70, p = .49$, and the mean ambulatory speed, $t(33.88) = 0.56, p = .58$, in the novel tank test, see Figures 4a and 4b, respectively. Fish fed twice per day did not demonstrate any significant differences in the time spent immobile or frozen in the novel tank test, $t(33.91) = 1.48, p = .15$, see Figure 4c. There does not appear to be any differences between feeding fish once or twice per day on locomotor measures in the novel tank test in group-housed zebrafish.

Discussion

A need for standardization of husbandry practices has become apparent in zebrafish research (Varga et al., 2018), such as with breeding, feeding, and housing procedures (Tsang et al., 2017; Watts et al., 2016). Stress responses in particular are affected by husbandry practices (Pavlidis et al., 2013), such as prior social history of zebrafish test subjects (Shams et al., 2017) or color of the housing tanks (de Abreu et al., 2020). Different feeding frequencies and housing conditions may create irregular basal stress levels amongst subjects between laboratories, which may markedly affect the impact of acute and chronic stress on a variety of measures. Dysregulated stress responses can ultimately have a number of undesirable downstream effects on health and disease states. Thus, feeding regimen may be a particularly important factor to consider in both the regular care of experimental subjects and in the interpretation of results of stress studies. Developing a uniform or standardized protocol regarding the feeding regimen of zebrafish may help to decrease variability between laboratories, thus producing more reliable and reproducible results in stress research. Contrary to previous research suggesting that feeding once per day was anxiogenic compared to other feeding frequencies (Dametto et al., 2018), the results of the current study suggest that subjects housed and cared for under our typical laboratory once-a-day feeding practice did not differ significantly with regards to anxiety-like behavior or whole-body cortisol levels compared to fish housed under identical conditions but fed twice per day. Although our results did not support our original hypothesis, the current study helps further elucidate the effects of feeding practices for zebrafish on behavioral and physiological markers of stress. The results here suggest that feeding subjects once per day, at least in the short term, does not produce effects on group-housed zebrafish that would indicate stress.

FIGURE 4



Methodological differences may contribute to differences between Dametto and colleagues' work and the current study, namely housing conditions. In the current study, zebrafish were group housed at a density of 5 fish/L in 1.8L tanks. In the previously published study, fish were housed in large (147L) tanks divided into 6L units (1 fish/6L) by a mesh barrier (Dametto et al., 2018). Although the fish had access to visual and chemosecretory cues from conspecifics, it is possible that this housing design elicited isolation-like effects in the subjects. Social isolation has been shown to modulate glucocorticoid levels and the expression of anxiety-like behavior in non-human primates (Cinini et al., 2014), rodents (Harvey et al., 2019), and zebrafish (Shams et al., 2017). Overcrowding, another stressor associated with housing, can be modulated by feeding schedule (Ramsay et al., 2006). Thus, it is possible that feeding frequency may exacerbate effects of housing conditions on cortisol levels and anxiety-like behavior.

In the current study, the sample size used was necessary to detect strong effects elicited by the feeding manipulation, as indicated by a power analysis based on the effect size on the aforementioned published research (Dametto et al., 2018). Anecdotally, the sample size used in the current study ($n = 18$ per group) meets or exceeds typical sample sizes used in zebrafish stress research. Basal cortisol levels of both groups were within the range of cortisol levels of control groups of studies previously published by our lab. However, the size of the sample might not have been sufficient to identify small effect sizes. Another possible limitation of this study is that subjects may not have been exposed to the different feeding frequencies for a sufficient time period to produce significant differences in physiological and behavioral measures. Zebrafish were subjected to a relatively short-term alteration in feeding frequency of two weeks. Although fifteen days was adequate time to produce behavioral alterations in physically isolated fish (Dametto et al., 2018), alterations in cortisol levels and anxiety-like behavior may be more obvious when feeding schedules are prolonged in socially housed fish. In the current study, subjects fed twice daily demonstrated a non-significant but possible trend towards an increase in the top exploration of the novel tank (increased distance traveled and more entries to the top zone compared to fish fed once per day) and less immobility or freezing, indicating a possible trend toward anxiolysis that may be more evident after a longer time held under similar feeding conditions. If the effects of feeding frequency or duration are subtler in socially housed zebrafish than what was observed in this study, a larger sample size would be necessary to detect statistically significant results with a smaller effect size.

This study investigated basal levels of cortisol and anxiety-like behavior changes in socially-housed zebrafish following feeding frequency of once or twice a day.

Further studies are necessary to determine if feeding frequency modulates responses to acute stress exposure in zebrafish, such as stress induced by acute confinement (Ghisleni et al., 2012), or to chronic stress exposure, such as chronic unpredictable stress exposure (Fulcher et al., 2017; Piato et al., 2011). As it appears that there may be important differences in basal stress responses to feeding frequency between physically isolated vs. socially housed zebrafish, it will be critical moving forward to consider whether feeding frequency can modulate zebrafish responses, especially in combination with other stressors.

This study only investigated two types of feeding schedules (once or twice per day); future studies should investigate alternate feeding schedules (e.g., once every other day or more than two times per day) in socially-housed zebrafish, different types of zebrafish diets, sex-dependent differences (Fontana et al., 2020; Genario et al., 2020), and different zebrafish strains (Egan et al., 2009) to determine whether these factors contribute to variability in anxiety-like behavior and stress hormone responses. The timing of regular zebrafish feeding on stress responses should also be addressed in future studies, as the timing of feeding in zebrafish indicated a possible trend toward anxiety-like behavior when subjects were assessed in the novel tank test three hours post-feeding (Dametto et al., 2018). In other fish species, timing of feeding influenced cortisol responses by modulating the synchronization of circadian rhythms (Gómez-Boronat et al., 2018; Montoya et al., 2010). The possibility of self-regulation of feeding also appears to be another area worthy of further investigation in the zebrafish model, as voluntary feeding decreases basal cortisol levels in the common carp (Klaren et al., 2013).

Further research is needed to standardize zebrafish colony practices across laboratories to better understand the lack of replicability that is sometimes indicated in the literature. A standardized protocol regarding the care, maintenance, and feeding of subjects is important to ensure validity and replicability, particularly in studies investigating stress. Alternatively, if adherence to a standardized protocol is not possible, all details about the care, housing, and feeding of zebrafish should be described in detail in published reports. Given that the zebrafish model is rapidly gaining in popularity for examining variety of human health-related conditions (Adhish & Manjubala, 2023; Cheresiz et al., 2020), it is critical that the impact of husbandry factors on behavioral and physiological stress responses are elucidated.

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