Anxiety-like behavior and whole-body cortisol responses to components of energy drinks in zebrafish (*Danio rerio*)

Alia O Alia, Maureen L Petrunich-Rutherford

1 Department of Psychology, Indiana University Northwest, Gary, IN, United States

Corresponding Author: Maureen L Petrunich-Rutherford

Email address: mlpetrun@iun.edu

The current study investigated the independent and combined effects of caffeine and taurine on anxiety-like behavior and neuroendocrine responses in adult zebrafish (*Danio rerio*). Caffeine (1,3,7-trimethylpurine-2,6-dione), the world’s most commonly used psychoactive drug, acts as an adenosine receptor blocker and a mild central nervous system stimulant. However, excessive use of caffeine is associated with heightened anxiety levels. Taurine (2-aminoethanesulfonic acid), a semi-essential amino acid synthesized within the human brain, has been hypothesized to play a role in regulating anxiolytic behavior. Caffeine and taurine are two common additives in energy drinks and are often found in high concentrations in these beverages. However, few studies have investigated the interaction of these two chemicals with regards to anxiety measures. A suitable vertebrate to examine anxiety-like behavior and physiological stress responses is the zebrafish, which has shown promise due to substantial physiological and genetic homology with humans. Anxiety-like behavior in zebrafish can be determined by analyzing habituation to novelty when fish are placed into a novel tank and scototaxis (light avoidance) behavior in the light-dark test. Stress-related neuroendocrine responses can be measured in zebrafish by analyzing whole-body cortisol levels. The goal of this study was to determine if exposure to caffeine, taurine, or a combination of the two compounds altered anxiety-like behavior and whole-body cortisol levels in zebrafish relative to control. Zebrafish were individually exposed to either caffeine (100 mg/L), taurine (400 mg/L), or both for fifteen minutes. Zebrafish in the control group were handled in the same manner but were only exposed to system tank water. After treatment, fish were transferred to the novel tank test or the light-dark test. Behavior was tracked for the first six minutes in the novel tank and fifteen minutes in the light-dark test. Fifteen minutes after introduction to the behavioral task, fish were euthanized for the analysis of whole-body cortisol levels. The results demonstrate that caffeine treatment decreased the amount of exploration in the top of the novel tank and increased scototaxis behavior in the light-dark test, which supports the established anxiogenic effect of acute exposure to caffeine. Taurine alone did
not alter basal levels of anxiety-like behavioral responses nor ameliorated the anxiogenic effects of caffeine on behavior when the two compounds were administered concurrently. None of the drug treatments altered basal levels of whole-body cortisol. The current results of this study suggest that, at least at this dose and time of exposure, taurine does not mitigate the anxiety-producing effects of caffeine when administered in combination, such as with energy drink consumption.
Anxiety-like behavior and whole-body cortisol responses to components of energy drinks in zebrafish (*Danio rerio*)

Alia O. Alia¹ and Maureen L. Petrunich-Rutherford¹

¹Department of Psychology, Indiana University Northwest, Gary, Indiana, USA

Corresponding Author:

Maureen L. Petrunich-Rutherford

Email address: mlpetrun@iun.edu
The current study investigated the independent and combined effects of caffeine and taurine on anxiety-like behavior and neuroendocrine responses in adult zebrafish (*Danio rerio*). Caffeine (1,3,7-trimethylpurine-2,6-dione), the world’s most commonly used psychoactive drug, acts as an adenosine receptor blocker and a mild central nervous system stimulant. However, excessive use of caffeine is associated with heightened anxiety levels. Taurine (2-aminoethanesulfonic acid), a semi-essential amino acid synthesized within the human brain, has been hypothesized to play a role in regulating anxiolytic behavior. Caffeine and taurine are two common additives in energy drinks and are often found in high concentrations in these beverages. However, few studies have investigated the interaction of these two chemicals with regards to anxiety measures. A suitable vertebrate to examine anxiety-like behavior and physiological stress responses is the zebrafish, which has shown promise due to substantial physiological and genetic homology with humans. Anxiety-like behavior in zebrafish can be determined by analyzing habituation to novelty when fish are placed into a novel tank and scototaxis (light avoidance) behavior in the light-dark test. Stress-related neuroendocrine responses can be measured in zebrafish by analyzing whole-body cortisol levels. The goal of this study was to determine if exposure to caffeine, taurine, or a combination of the two compounds altered anxiety-like behavior and whole-body cortisol levels in zebrafish relative to control. Zebrafish were individually exposed to either caffeine (100 mg/L), taurine (400 mg/L), or both for fifteen minutes. Zebrafish in the control group were handled in the same manner but were
only exposed to system tank water. After treatment, fish were transferred to the novel tank test or
the light-dark test. Behavior was tracked for the first six minutes in the novel tank and fifteen
minutes in the light-dark test. Fifteen minutes after introduction to the behavioral task, fish were
euthanized for the analysis of whole-body cortisol levels. The results demonstrate that caffeine
treatment decreased the amount of exploration in the top of the novel tank and increased
scototaxis behavior in the light-dark test, which supports the established anxiogenic effect of
acute exposure to caffeine. Taurine alone did not alter basal levels of anxiety-like behavioral
responses nor ameliorated the anxiogenic effects of caffeine on behavior when the two
compounds were administered concurrently. None of the drug treatments altered basal levels of
whole-body cortisol. The current results of this study suggest that, at least at this dose and time
of exposure, taurine does not mitigate the anxiety-producing effects of caffeine when
administered in combination, such as with energy drink consumption.

Introduction

Caffeine (1,3,7-trimethylpurine-2,6-dione), the world’s most widely consumed
psychoactive drug, has stimulant-like effects on the central nervous system and overall behavior
(Evans & Battisti, 2018). Widespread use of caffeine is likely due to the positive effects it has on
increasing mental alertness and physical endurance as well as reducing fatigue and overall
tiredness (Heckman, Weil, & Gonzalez de Mejia, 2010). However, caffeinated beverages may
also be associated with increasing anxiety and other negative health outcomes (Richards &
Smith, 2016), particularly in youth (De Sanctis et al., 2017) and with individuals with certain
genetic variants associated with caffeine pharmacokinetics and pharmacodynamics (Nehlig,
2018). The consumption of energy drinks has increased significantly over the last decade in all
age groups surveyed in a recent study (Vercammen, Koma, & Bleich, 2019). It is likely that
ergy drinks are popular due to the stimulant-like effects produced by caffeine; thus, energy
drinks are commonly consumed by populations such as young adults in college settings for
supporting academic demands (Trunzo et al., 2014) and by military service members (Attipoe,
Delahanty, Stephens, & Deuster, 2018). However, as with beverages containing caffeine,
excessive consumption of energy drinks is associated with negative health outcomes like adverse
cardiac events (for review, see Higgins, Babu, Deuster, & Shearer, 2018). Adolescents are more
at risk to experiencing ill effects of energy drink consumption (Curran & Marczinski, 2017). In
addition, energy drinks are frequently consumed with alcohol, a practice which is associated with
increased risky decision making (Manchester, Eshel, & Marion, 2017) and increased risk for
negative health consequences in young adults (Caviness, Anderson, & Stein, 2017).

Although caffeine is one of the primary chemicals present in energy drinks, another
additive found in high concentrations is taurine. Taurine (2-aminoethanesulfonic acid) is
considered a semi-essential amino acid, as is it not used in protein synthesis (Ripps & Shen,
2012). However, taurine does play other critical roles, particularly in the central nervous system,
such as by helping to regulate cell volume and modulate neurotransmission in the brain (Oja &
Saransaari, 2017). In addition, taurine has been hypothesized to play a role in anxiolytic
behavior in rodents (Chen et al., 2004; El Idrissi et al., 2009; Francisco & Guedes, 2015; Kong et
al., 2006; McCool & Chappell, 2007; Wu et al., 2017; Zhang & Kim, 2007). The effects of
caffeine and taurine in combination have mainly been studied in the context of physical
performance and cardiovascular function. Administration of caffeine and taurine in combination
altered measures of cardiovascular function (Bichler, Swenson, & Harris, 2006) and elevated
mental performance and mood (Seidl, Peyrl, Nicham, & Hauser, 2000) over placebo in human
participants. When these two components were studied alone and in combination, taurine counteracted the effects of caffeine on cardiovascular function (Schaffer et al., 2014), mitigated some of the effects of caffeine on cognitive measures (Giles et al., 2012), reduced caffeine-induced physiological alterations associated with cycling performance (Warnock, Jeffries, Patterson, & Waldron, 2017), and attenuated the effects of caffeine on specific parameters of reaction time (Peacock, Martin, & Carr, 2013) in human subjects. However, in vitro, taurine did not alter caffeine-induced effects in human cardiac muscle tissue (Chaban et al., 2017) or mouse skeletal muscle tissue (Tallis, Higgins, Cox, Duncan, & James, 2014). In other measures in a variety of animal models, caffeine and taurine have synergistic effects, such as on sleep parameters in Drosophila (Lin et al., 2010), plasma calcium levels in rats (Owoyele, Oyewole, Biliaminu, & Alashi, 2015), locomotor activity in mice (Kimura, Ushijima, Hiraki, Kimura, & Ono, 2009), and memory and attention in rats (Valle et al., 2018). Thus, the specific impact of caffeine and taurine appears to be dependent on the physiological and behavioral parameters under investigation. Although caffeine and taurine can modulate anxiety-like states on their own, little is known regarding the impact of these two popular energy drink components in combination.

The zebrafish (Danio rerio) animal model is rapidly becoming an attractive model organism in neuropharmacology research due to its low cost and ease of maintenance; in addition, the nervous and endocrine systems regulating biological and behavioral responses to stress are highly conserved (Stewart et al., 2012). Stress and anxiety-like states can be inferred in the zebrafish model by measuring various behavioral responses such as shoaling, immobility, erratic movements, and the detection of jumping in response to various stimuli such as the introduction of pharmaceuticals, visual stimuli, and alarm pheromones (Egan et al., 2009;
Maximino et al., 2014; K. Wong et al., 2010). The novel tank test is a well-validated measure of anxiety-like behavior in zebrafish and involves measuring freezing and exploratory behavior upon introduction to a new tank (Kysil et al., 2017; Mezzomo, Silveira, Giuliani, Quadros, & Rosenberg, 2016; Raymond et al., 2012). Another behavioral paradigm, the light-dark test, measures anxiety-like behavior in the form of scototaxis, or light avoidance, in response to pharmacological or behavioral manipulation (Maximino, Marques de Brito, Dias, Gouveia, & Morato, 2010; Stewart et al., 2011). Stress responses can also be assessed through measuring neuroendocrine responses, namely cortisol, elicited by specific stimuli (Cachat et al., 2010; Canavello et al., 2011). The hypothalamic-pituitary-interrenal (HPI) axis of teleost species, such as zebrafish, is homologous to the hypothalamic-pituitary adrenal (HPA) axis of mammals (Nesan & Vijayan, 2013; Wendelaar Bonga, 1997). Thus, exposure to pharmacological compounds will elicit behavioral and neuroendocrine effects in the fish that may generally model the effects these compounds have in humans.

Consistent with findings in rodent models and human subjects, zebrafish exposed to caffeine at a variety of ages demonstrate anxiety-like behavior (Egan et al., 2009; Richendrfer, Pelkowski, Colwill, & Creton, 2012; Rosa et al., 2018; Schnörr, Steenbergen, Richardson, & Champagne, 2012; Steenbergen et al., 2011; K. Wong et al., 2010). The specific anxiogenic effect of caffeine is possibly due to antagonism at A₁ adenosine receptors (Maximino, Lima, Olivera, Picanço-Diniz, & Herculano, 2011). Acute exposure to taurine, on the other hand, is associated with anxiolytic effects on behavior (Mezzomo, Silveira, Giuliani, Quadros, & Rosemberg, 2016). The modulation of anxiety-like behavior by taurine in zebrafish may be induced by blunting neuroendocrine cortisol responses to stress (as observed in Mezzomo et al., 2019). Similarly, glucocorticoid hormone regulation has been proposed as a possible mechanism
by which taurine modulates anxiety-like behavior in unpredictable chronic stress exposure in rodents (Wu et al., 2017). However, whether taurine mitigates the anxiogenic effects of caffeine via HPA/HPI regulation is currently not known.

Thus, in this study, the effects of caffeine and taurine on anxiety-like behavior and neuroendocrine responses were explored. The purpose of this study was to determine if acute exposure to caffeine, taurine, or both altered anxiety-like behavior and whole-body cortisol levels in zebrafish. If caffeine operates as an anxiogenic in zebrafish as expected, then the fish will exhibit more anxiety-like behavior and display increased whole-body cortisol levels relative to control. If taurine operates as an anxiolytic in zebrafish, then the fish exposed to taurine should have decreased cortisol levels and exhibit decreased anxiety-like behavior, such as spending more time in the upper portion of the tank during the novel tank test or entering the light zone more frequently in the light-dark test. If taurine modulates the effects of caffeine, taurine should mitigate any caffeine-induced anxiety-like effects on behavior and increases in whole-body cortisol when the fish are acutely exposed to both drugs simultaneously. This study will potentially aid in elucidating the effects of caffeine and taurine when co-administered acutely. In addition, the findings from this study will provide insight on the interaction between chemicals commonly found in energy drinks and whether the modulation of anxiety-like behavior is related to the activity of the neuroendocrine stress axis.

Methods and Materials

Animals and Housing

Wild-type, adult zebrafish (N = 139) were purchased from Carolina Biological Supply (Burlington, NC). Upon arrival to the facility, the fish were maintained on a circulating system
on a 14:10 light:dark cycle at a density of approximately 5-6 fish per liter. Fish were fed flake food once per day and dried brine shrimp once per day. The internal environment of the housing tanks was maintained at a temperature of 26 ± 2°C. Animals were housed and maintained in accordance with ethical guidelines (Harper & Lawrence, 2016; National Research Council, 2011; Westerfield, 2000). All fish were allowed to acclimate to the facility for at least a week before any experimental procedures were conducted (Dhanasiri, Fernandes, & Kiron, 2013). All experimental procedures involving animals were performed between 9:00 a.m. and 1:00 p.m.

**Drug Administration**

Drugs were purchased from Santa Cruz Biotechnology (Dallas, TX). On the day of the experiment, housing tanks were removed from the system and placed in the experimentation room thirty minutes prior to treatment to allow for habituation. Individual fish were selected at random, carefully netted from the tank, and placed in a tank containing 1L of either a drug or control solution for a duration of 15 minutes. There were four independent conditions: control (system water), caffeine (1,3,7-trimethylpurine-2,6-dione), taurine (2-aminoethanesulfonic acid), or a combination of caffeine/taurine. Fish were either immersed in a solution of either caffeine (N = 29) at 100 mg/L (Maximino et al., 2014), taurine (N = 30) at 400 mg/L (Mezzomo et al., 2016), or caffeine and taurine combined (N = 29) at 100 mg/L and 400 mg/L, respectively. Subjects in the control group (N = 51) were simply immersed in system water for 15 minutes. Treatment solutions were replaced after every subject. Immediately following treatment, each subject was transferred to either a novel tank (Experiment 1) or light-dark tank (Experiment 2) for behavioral analysis.

**Novel Tank Test (Experiment 1)**
After drug treatment, individual fish (N = 100 total) were placed in a trapezoidal tank (15.2 cm height × 27.9 cm top × 22.5 cm bottom × 7.1 cm width). The tank was positioned to allow for recording of behavior from the wide side of the tank, using a camera placed on a tripod. The first six minutes of behavior was recorded and subsequently analyzed by Ethovision XT software (Noldus, Leesburg, VA), which was generously provided as part of the Faculty for Undergraduate Neuroscience (FUN) Equipment Loan Program. Behavioral measures included the total distance traveled (cm), mean speed during ambulation (cm/s), immobility duration (s), number of times fish were immobile, latency to first top entry (s), total time in top (s), distance in top (cm), and number of entries to top. The percentage of fish from each group that did not re-enter the top zone after being introduced to the novel tank (N = 18 total) was also calculated. These samples were not included in the analysis of the latency to top measure but were included in all other behavioral measures. One subject was not included in behavioral analyses due to corruption of the video file.

Light-Dark Test (Experiment 2)

After the drug treatment, individual fish (N = 39 total) were placed in a rectangular tank (approximately 15 cm x 30 cm x 20 cm) with a water depth of 3 cm. The dark side of the tank (sides and bottom) was covered with black plastic aquarium background and the other side was left uncovered (Magno, Fontes, Gonçalves, & Gouveia, 2015; Maximino et al., 2010). The behavior of the fish was recorded from above the tank for fifteen minutes with a Logitech C922x Pro Stream Webcam. Video files were uploaded to and analyzed with BehaviorCloud motion-tracking software (https://www.behaviorcloud.com/, San Diego, CA). Six behavioral measures were quantified for each fish: total distance traveled (cm), mean speed during ambulation (cm/s),...
immobility duration (s), number of entries to the light zone, total time spent in light zone (min),
and total distance traveled in the light zone (cm).

**Euthanasia**

Fifteen minutes after each subject was introduced to the respective behavioral task, the fish were netted from the tank and placed into 30 ml euthanasia solution (0.1% clove oil in system water) for approximately 60 seconds (Davis et al., 2015; D. Wong, von Keyserlingk, Richards, & Weary, 2014). Once the subjects displayed no movement or responsiveness, the bodies were gently dried, placed in a microcentrifuge tube, and were stored at -20°C until the cortisol extraction was performed.

**Cortisol Extraction/Assay**

Cortisol extraction and analysis procedures were adapted from previously published methods (Cachat et al., 2010; Canavello et al., 2011). Briefly, whole-body samples were thawed and individually weighed. Samples were cut into smaller pieces with a scalpel and placed in 1 ml of 25 mM of ice-cold phosphate buffer solution (PBS). Each sample was homogenized for 30-60 seconds and placed back on ice. Diethyl ether (5 ml) was added to each sample and thoroughly vortexed, then centrifuged at 2500 rpm for 15 minutes. Following the centrifugation, the organic layer containing the cortisol was removed from the sample and placed in a separate tube. The addition of ether, vortexing, centrifugation, and organic layer removal was repeated two more times to maximize the amount of cortisol extracted from each sample. The samples from Experiment 1 were allowed to dry at room temperature under a fume hood until the ether layer was fully evaporated; samples from Experiment 2 were dried with a light stream of air. In both procedures, samples were dried until a yellow oil containing cortisol remained.
After the samples were dry, 1 ml of ice-cold PBS was added to each tube, and a commercially-available enzyme-linked immunosorbent assay (ELISA) kit (Salimetrics, Carlsbad, CA) was used to assess cortisol levels. ELISA procedures were conducted according to manufacturer instructions. Binding values for each sample was compared to a standard curve generated by My Curve Fit software (https://mycurvefit.com/). Cortisol levels were normalized to body weights of each sample and are displayed in ng cortisol/g body weight. Four samples from Experiment 1 were excluded from the cortisol analysis due to methodological errors incurred during the extraction procedure.

**Data Analysis**

Behavioral and cortisol dependent measures were expressed as the mean ± standard error of the mean and were analyzed using a two-way analysis of variance (ANOVA) with caffeine (levels: yes, no) and taurine (levels: yes, no) as the independent variables. Group means were compared by a Tukey post-hoc test when appropriate. The percentage of each group that did not explore the top zone of the novel tank test was analyzed with a chi-squared test. All analyses were conducted using JASP software (https://jasp-stats.org/). Results were considered statistically significant if p < 0.05.

**Results**

Anxiety-like responses of subjects were determined by assessing behavioral measures exhibited within the novel tank test and light-dark test. In addition, whether neurochemical measures of anxiety were altered by drug treatment was assessed by measuring whole-body cortisol levels after each of the behavioral tasks. Measures of each behavioral test were broken down into three discrete domains: motor activity, immobility, and exploration. In the novel tank
test (Experiment 1), motor activity was assessed by examining the total distance moved overall and the mean speed of the subjects (while mobile) within the tank. The second domain assessed the number of times the subjects were immobile and the total duration of immobility (s). The third domain included the activity in the top zone of the novel tank; this included assessing the distance moved in the top zone (cm), the number of times the subjects entered the top zone, the time spent in the top zone (s), and the latency to first top entry (s). The percentage of subjects in each group that did not explore the top zone was also calculated. In the light-dark test (Experiment 2), motor activity was represented by the total distance moved overall and the mean speed of the subjects (while mobile) within the tank. The second domain assessed freezing by measuring the duration of immobility (s). The third domain included the activity in the light zone of the tank; this included assessing the number of times the subjects entered the light zone, the distance moved in the light zone (cm), the time spent in the light zone (s), the number of crossings from one compartment to the other, and the latency to re-enter the light zone after the first visit to the dark zone (s).

Experiment 1

Motor activity in the novel tank test. The total distance traveled and the mean ambulatory speed in the novel tank test is illustrated in Figure 1. A two-factor ANOVA revealed no significant main effect of caffeine (F(1,95) = 2.898, p = 0.092), no significant main effect of taurine (F(1,95) = 1.101, p = 0.297), and no significant interaction between caffeine and taurine (F(1,95) = 2.263, p = 0.136) on the total distance traveled in the novel tank test (Figure 1A). A two-factor ANOVA indicated a marginally significant main effect of caffeine (F(1,95) = 3.214, p = 0.076), no significant main effect of taurine (F(1,95) = 0.061, p = 0.806), but no significant interaction between caffeine and taurine (F(1,95) = 0.047, p = 0.829) on the mean ambulatory
speed traveled in the novel tank test (Figure 1B). Thus, it appears that caffeine and taurine, either
alone or in combination, did not significantly affect general motor activity of adult zebrafish in
the novel tank test.

**Freezing behavior in the novel tank test.** Freezing behavior (Figure 2) displayed in
zebrafish in the novel tank test can be used as an indication of anxiety-like behavior induced by
treatment. As the number of freezing bouts or time spent immobile increases, it can be inferred
that the subjects are experiencing higher levels of anxiety. A two-factor ANOVA revealed no
significant main effect of caffeine (F(1,95) = 1.674, p = 0.199), no significant main effect of
taurine (F(1,95) = 0.534, p = 0.467), and no significant interaction between caffeine and taurine
(F(1,95) = 0.339, p = 0.562) on the number of immobility bouts in the novel tank test (Figure
2A). A two-factor ANOVA indicated no significant main effect of caffeine (F(1,95) = 1.004, p =
0.319), no significant main effect of taurine (F(1,95) = 0.062, p = 0.805), and no significant
interaction between caffeine and taurine (F(1,95) = 0.184, p = 0.669) on the total time spent
immobile in the novel tank test (Figure 2B). Thus, it appears that caffeine and taurine, either
alone or in combination, did not significantly affect freezing behavior of adult zebrafish in the
novel tank test.

**Exploratory behavior in the novel tank test.** Figure 3 displays the mean ± SEM for each
group for each of the four exploratory measures of interest in the novel tank test. If the subjects
are less exploratory (e.g., spend less time in the top, enter the top fewer times, etc.), then it can
be inferred that the subjects are experiencing more anxiety. A two-factor ANOVA revealed no
significant main effect of caffeine (F(1,95) = 2.019, p = 0.159), no significant main effect of
taurine (F(1,95) = 2.150, p = 0.146), and no significant interaction between caffeine and taurine
(F(1,95) = 2.701, p = 0.104) on the distance traveled in the top zone of the novel tank test (Figure
A two-factor ANOVA indicated a significant main effect of caffeine ($F(1,95) = 6.379, p = 0.013$, caffeine $<$ no caffeine), no significant main effect of taurine ($F(1,95) = 0.515, p = 0.475$), but no significant interaction between caffeine and taurine ($F(1,95) = 0.021, p = 0.886$) on the number of entries to the top zone of the novel tank test (Figure 3B). A two-factor ANOVA revealed no significant main effect of caffeine ($F(1,95) = 0.361, p = 0.550$), no significant main effect of taurine ($F(1,95) = 1.480, p = 0.227$), and no significant interaction between caffeine and taurine ($F(1,95) = 2.372, p = 0.127$) on the time spent in the top zone of the novel tank test (Figure 3C). A two-factor ANOVA indicated no significant main effect of caffeine ($F(1,77) = 0.786, p = 0.378$), a significant main effect of taurine ($F(1,77) = 4.308, p = 0.041$, taurine $<$ no taurine), but no significant interaction between caffeine and taurine ($F(1,77) = 0.567, p = 0.454$) on the latency to enter the top zone of the novel tank test (Figure 3D). It is of note that, for this behavioral task, not all fish returned to the top (see Table 1) and thus could not be included in this analysis. Table 1 displays the percentage of fish from each group that did not explore the top portion of the novel tank. Almost half (42.1%) of the caffeine-treated group did not explore the top but fewer than 10% of the fish exposed to the control or taurine conditions did not explore the top. About 25% of fish exposed to the mixed drug treatment failed to explore the top zone in the novel tank test. These group differences were significant according to a chi-squared test ($\chi^2(3, N = 99) = 12.02, p = 0.007$). Thus, the pattern of data suggests that caffeine treatment decreased the tendency to explore the top, and that fish that did re-enter the top took more time to do so after treatment with caffeine alone.

Although some of the exploratory measures did not reach the criterion for statistical significance, in general, caffeine-treated fish appeared to demonstrate less exploratory behavior in the top zone, whereas taurine generally did not alter overall exploration besides shortening the
latency to explore the top zone. The data loosely suggest that when caffeine and taurine were co-
administered, taurine may have mitigated some of the effects of caffeine on exploration (e.g.,
increased distance and time spent in the top zone and decreased the latency to enter the top zone
of the novel tank test); however, a higher dose and/or longer course of exposure to taurine is
likely necessary to elicit any significant effect on caffeine-induced anxiety-like behavior in the
novel tank test.

Whole-body cortisol levels post-novel tank test. A neurochemical marker of anxiety was
determined by analyzing whole-body cortisol levels of each subject (Figure 4). A two-factor
ANOVA revealed no significant main effect of caffeine (F(1,92) = 0.189, p = 0.665), no
significant main effect of taurine (F(1,92) = 0.283, p = 0.596), and no significant interaction
between caffeine and taurine (F(1,92) = 0.660, p = 0.419) on whole body cortisol levels (Figure
4). Thus, it does not appear that acute exposure to different components of energy drinks altered
stress hormone responses, at least when cortisol was assessed fifteen minutes after introduction
to the novel tank test.

Experiment 2

Motor activity in the light-dark test. Similar to the novel tank test, the total distance
traveled and the mean ambulatory speed (Figure 5) can be used as markers for general motor
activity in the light-dark test. A two-factor ANOVA revealed a significant main effect of caffeine
(F(1,35) = 17.791, p < 0.001, caffeine < no caffeine), no significant main effect of taurine
(F(1,35) = 0.336, p = 0.566), but no significant interaction between caffeine and taurine (F(1,35)
= 0.343, p = 0.562) on the total distance traveled in the light-dark test (Figure 5A). A two-factor
ANOVA indicated no significant main effect of caffeine (F(1,35) = 2.074, p = 0.159), no
significant main effect of taurine (F(1,35) = 0.245, p = 0.624), and no significant interaction
between caffeine and taurine (F(1,35) = 0.013, p = 0.911) on the mean speed traveled (while ambulatory) in the light-dark test (Figure 5B). Thus, it appears that caffeine, but not taurine, significantly decreased the total distance traveled by adult zebrafish in the light-dark test. None of the treatments altered mean swimming speed of the subjects.

Freezing behavior in the light-dark test. Freezing behavior (Figure 6) displayed in zebrafish can be used as an indication of anxiety-like behavior induced by treatment. A two-factor ANOVA indicated a significant main effect of caffeine (F(1,35) = 27.792, p < 0.001, caffeine > no caffeine), no significant main effect of taurine (F(1,35) = 0.764, p = 0.388), but no significant interaction between caffeine and taurine (F(1,35) = 0.699, p = 0.409) on the total time spent immobile in the novel tank test (Figure 6). Thus, it appears that caffeine, but not taurine, significantly increased the time spent immobile by adult zebrafish in the light-dark test. This may explain why the total distance traveled was less for caffeine-treated fish (see Figure 5A).

Exploratory behavior in the light-dark test. Figure 7 displays the mean ± SEM for each group for each of the three exploratory measures of interest in the light-dark test. If the subjects are less exploratory (e.g., spend less time in the light zone, enter the light zone fewer times, etc.) in the light-dark test, then it can be inferred that the subjects are experiencing more anxiety. A two-factor ANOVA revealed a significant main effect of caffeine (F(1,35) = 19.033, p < 0.001, caffeine < no caffeine), no significant main effect of taurine (F(1,35) = 0.020, p = 0.887), but no significant interaction between caffeine and taurine (F(1,35) = 0.261, p = 0.613) on the distance traveled in the light zone of the light-dark test (Figure 7A). A two-factor ANOVA indicated a significant main effect of caffeine (F(1,35) = 30.364, p < 0.001, caffeine < no caffeine), no significant main effect of taurine (F(1,35) = 0.122, p = 0.729), but no significant interaction between caffeine and taurine (F(1,35) = 0.639, p = 0.430) on the number of entries to the light
zone of the light-dark test (Figure 7B). A two-factor ANOVA indicated a significant main effect of caffeine ($F(1,35) = 20.088, p < 0.001$, caffeine < no caffeine), no significant main effect of taurine ($F(1,35) = 0.024, p = 0.877$), but no significant interaction between caffeine and taurine ($F(1,35) = 0.137, p = 0.714$) on the number of crossings in the tank from one compartment to the other in the light-dark test (Figure 7C). A two-factor ANOVA revealed no significant main effect of caffeine ($F(1,35) = 1.188, p = 0.283$), no significant main effect of taurine ($F(1,35) = 2.107, p = 0.155$), and no significant interaction between caffeine and taurine ($F(1,35) = 2.961, p = 0.094$) on the time spent in the light zone of the light-dark test (Figure 7D). A two-factor ANOVA revealed no significant main effect of caffeine ($F(1,35) = 2.426, p = 0.128$), no significant main effect of taurine ($F(1,35) = 1.411, p = 0.243$), and no significant interaction between caffeine and taurine ($F(1,35) = 1.320, p = 0.258$) on the latency of the fish to re-enter the light zone after the first time visiting the dark zone in the light-dark test (Figure 7E).

Similar to the results found in the novel tank test in Experiment 1, caffeine-treated fish generally appeared to be less exploratory, as caffeine-treated fish traveled less in the light zone, made fewer crossings, and entered the light zone fewer times. Taurine generally did not alter overall exploration, and it does not appear that taurine has any mitigating or additive effects on caffeine-induced alterations in exploratory behavior in the light-dark test when both drugs were administered at the same time.

*Whole-body cortisol levels post-light-dark test.* A neurochemical marker of anxiety was determined by analyzing whole-body cortisol levels of each subject (Figure 8). A two-factor ANOVA revealed no significant main effect of caffeine ($F(1,35) = 0.243, p = 0.625$), no significant main effect of taurine ($F(1,35) = 0.274, p = 0.604$), and no significant interaction between caffeine and taurine ($F(1,35) = 0.024, p = 0.879$) on whole body cortisol levels (Figure
Thus, it does not appear that acute exposure to different components of energy drinks altered stress hormone responses, at least when cortisol was sampled immediately after behavioral measures were assessed in the light-dark test.

Discussion

The purpose of this study was to identify, in a zebrafish model, the various behavioral and neurochemical changes elicited in response to three treatments compared to control: caffeine, taurine, and caffeine and taurine in combination. Based on previous studies, it was expected that caffeine treatment would elicit anxiogenic effects, taurine treatment would elicit anxiolytic effects, and exposure to both caffeine and taurine would result in mixed effects on cortisol levels and behavioral measures associated with anxiety.

The results of this study indicate there are mixed effects of drug treatment on the various behavioral measures; however, in general, it appears that caffeine is anxiogenic in both the novel tank and light-dark tests. Taurine does not appear to have anxiolytic effects on its own, nor does it significantly impact the effects of caffeine when the two drugs are administered simultaneously, at least at the dose and time tested in the current study. In addition, there is no effect of drug treatment on whole-body cortisol levels in zebrafish.

The neurochemical analysis performed after each of the behavioral tests suggests that acute exposure to caffeine, taurine, or both does not affect basal levels of whole-body cortisol. Although previous studies have indicated that caffeine can alter basal levels of cortisol, the modulating effects of taurine on cortisol responses are only evident in response to acute stress (Mezzomo et al., 2019). Thus, the differences between the current and previously published studies are likely to be an outcome of methodological differences between experiments, such as...
varying durations of treatment and timing of behavioral measurements. Although behavioral alterations persist in response to acute caffeine exposure (Tran et al., 2017), the time course of the cortisol response may not necessarily parallel behavioral alterations induced by pharmacological agents or stressors. In adult zebrafish, whole-body cortisol levels peak at 15 minutes in response to acute stressors (Ramsay et al., 2009; Tran, Chatterjee, & Gerlai, 2014). In the current study, cortisol was assessed 15 minutes after introduction to the behavioral task; thus, it is possible that any perturbations in the cortisol levels elicited by the drug treatments may have returned back to basal levels by the time of the assessment. One previous study assessed cortisol levels immediately after recording behavioral measurements and demonstrated that a dose and exposure time to caffeine comparable to the one used in the current study (100 mg for 15 minutes) significantly elevated whole-body cortisol levels compared to zebrafish not exposed to caffeine (Rosa et al., 2018). Further studies should investigate whether caffeine, taurine, and a mixture of these two drugs alter basal levels of cortisol immediately after drug exposure or if these compounds alter peak cortisol responses after exposure to an acute stressor, such as a two-minute net chase or exposure to conspecific alarm pheromone. The current results indicate that acute exposure to the different treatments do not appear to elicit longer-term alterations (i.e. 30 minutes after the beginning of drug exposure) in basal levels of cortisol. Alternatively, these treatments may not significantly affect basal cortisol at all, as has been observed with salivary cortisol levels in human participants (Giles et al., 2012).

With regards to the behavioral assays, the only treatment to significantly impact behavior was caffeine alone. Caffeine treatment significantly decreased the distance traveled, increased resting time, and increased scototaxis (light avoidance) in the light-dark test and decreased exploration of the top zone in the novel tank test. These findings support previous studies that
indicate caffeine induces a heightened anxiety-like state (Egan et al., 2009; Richendrfer et al., 2012; Rosa et al., 2018; Schnörr et al., 2012; Steenbergen et al., 2011). Taurine treatment alone did not appear to influence anxiety-like behavior, as measures on the different behavioral parameters did not reach statistical significance. These findings are similar to the results from a previously published study that demonstrated that taurine treatment alone had no measurable effect on anxiety-like behavior in the novel tank test (Mezzomo et al., 2016). In that same study, however, one hour of exposure to taurine did alter scototaxis in the light-dark task, as subjects spent more time in the lit portion of the tank, suggesting an anxiolytic effect in this behavioral test with longer exposure to treatment (Mezzomo et al., 2016). In the current study, a shorter exposure time of fifteen minutes was used to keep the taurine treatment time equivalent to the caffeine treatment. Thus, taurine alone may directly modulate anxiolytic behavior, but only in certain behavioral paradigms with a minimum exposure time of greater than 15 minutes. To the best of our knowledge, a full time course of the anxiolytic effects of taurine in either behavioral task has yet to be investigated. Perhaps an even longer exposure time (> one hour) would be sufficient to elicit behavioral effects in the novel tank test as well. A potential confound to this study may be the feeding regimen of the zebrafish. Currently, there is no standardized diet or feeding regimen across zebrafish colonies (Watts, Lawrence, Powell, & D’Abramo, 2016). However, a recent study suggests that feeding zebrafish once per day is associated with decreased exploratory behavior compared to fish that were fed twice per day (Dametto et al., 2018). Although all of the fish in the current study were fed similarly, it is possible that any potential effects of treatment may have been masked by anxiety induced by the feeding regimen. The current study is the first to examine the potential interaction of taurine and caffeine on anxiety-like measures. At least at the dose and time course of taurine exposure used in the
current study, it does not appear that taurine mitigates the anxiogenic effects of caffeine when subjects are exposed to both drugs simultaneously. Further studies should investigate whether a longer exposure time to taurine would mitigate the effects of caffeine on anxiety-like behavior, and, if so, what neural mechanism would best explain these effects. As taurine exposure has been shown to be anxiolytic in other studies in the literature, potentially, caffeine and taurine could modulate anxiety-like behavior via similar neural targets. Some shared molecular targets include adenosine and $\gamma$-amino butyric acid (GABA). Adenosine receptors are involved with modulating anxiety in humans, rodents, and zebrafish (López-Cruz et al., 2017; Maximino et al., 2011; Prediger, Batista, & Takahashi, 2004; Prediger, da Silva, Batista, Bittencourt, & Takahashi, 2006; Vincenzi, Borea, & Varani, 2017; Yamada, Kobayashi, & Kanda, 2014). Caffeine antagonizes adenosine receptors (Ribeiro and Sebastião, 2010). Specifically, blockade of adenosine $A_1$ receptors in zebrafish attenuates the anxiogenic effect of caffeine (Maximino et al., 2011). Higher doses or longer exposure to taurine could potentially elicit an increase in brain levels of adenosine (Rosemberg et al., 2010), which may partially overcome the caffeine antagonism of adenosine receptors. Alternatively, modulation of GABA transmission may be a possible mechanism by which caffeine and taurine could regulate anxiety-like behavior. The inhibitory activity of GABA is directly associated with anxiety-like responses (for review, see Nuss, 2015). Both caffeine and taurine may be moderating the activity of GABAergic cells, as administration of caffeine blocks GABAergic inhibitory postsynaptic potentials (IPSPs) (Isokawa, 2016) and taurine enhances the activity of GABAergic interneurons (Sava, Chen, Sun, Luhmann, & Kilb, 2014). Although there is evidence to suggest that both caffeine and taurine can mediate opposing functions within the same neurotransmitter system (e.g., adenosine or GABA), and thus have the potential to modulate anxiety by a shared circuit, it is entirely possible
that each of these compounds could be modulating separate systems to alter anxiety-like behavior. Future studies should investigate whether caffeine and taurine are working on either (or both) of these putative regulators of anxiety-like behavior, or if one or both of these compounds modulate some other system entirely, such taurine modulating the glycine system (Zhang & Kim, 2007). Further studies should also investigate the impact of this acute regulation of neural targets on downstream effects of the HPA/HPI system.

Future studies should also address the impact of caffeine, taurine, and mixed drug exposure on different strains of zebrafish; some strains, such as the leopard strain, appear to have higher baseline levels of anxiety (Egan et al., 2009). Future studies should also investigate whether these compounds affect behavioral and neuroendocrine responses differently in male and female fish. Stress-related behavior may vary in zebrafish depending on sex, as has been observed in many other species (Donner & Lowry, 2013). Sex differences in exploratory and other behavioral responses in zebrafish have been studied less than other species (Ampatzis & Dermon, 2016), but indicate that sex may be a major factor in responsiveness to pharmacological manipulations (Singer, Oreschak, Rhinehart, & Robison, 2016).

Although the current study did not demonstrate that fifteen minutes of exposure to taurine modulated caffeine-induced anxiety-like behavior, it is the first to study this question. More studies are required to fully elucidate any synergistic effect of caffeine and taurine, as has been observed in other measures, and whether activity of the HPA/HPI axis is involved with the regulation of anxiety-like behavior. In addition, many more studies are needed to determine whether anxiety-like states are altered by human consumption of highly caffeinated beverages with significant taurine concentrations (e.g., energy drinks). It is also important to note that energy drinks often contain many more additives that may or may not alter the properties of the
two compounds under investigation of the current study. Also, given the previous literature, whether taurine supplementation would be a viable avenue for treating anxiety conditions in humans should be a focus of future investigations.

Conclusions

The current study is the first to investigate a possible interaction between caffeine and taurine on anxiety-like behavior and neuroendocrine measures in zebrafish. Although caffeine elicited anxiogenic effects in two different behavioral paradigms, taurine treatment alone or in combination with caffeine did not significantly affect anxiety-like behavior. None of the treatments in the current study altered whole-body cortisol levels in zebrafish. However, other studies in the literature suggest that taurine may have the potential to modulate anxiety-like states with longer exposure times. Further studies are necessary to investigate the involvement of the hypothalamic-pituitary-adrenal/interrenal axis and neurotransmitter systems such as adenosine and GABA in regulating anxiety-like behavior altered by caffeine and taurine. In addition, supplemental products commonly consumed by humans should be investigated in more detail, particularly for those individuals at higher risk for stress or anxiety-related disorders.

Acknowledgements

The authors would like to thank Amy Aponte, Beatriz Castro, Emma DiPasquo, Tye Dominguez, Alyssa Fassoth, Brianna Henning, Aleesa Parker, Summer Pattison, Adeel Shafiq, Jessica Singh, Horace Townsend, Elijah Weathersby, and Jennifer Wright for their technical
assistance with some aspects of this study. The use of Ethovision XT software (Noldus) was made possible by the Faculty for Undergraduate Neuroscience (FUN) Equipment Loan program.

References

Attipoe, S., Delahanty, L., Stephens, M., & Deuster, P. A. (2018). Energy Beverage Use Among U.S. Service Members. *Military Medicine*. https://doi.org/10.1093/milmed/usy169

Bichler, A., Swenson, A., & Harris, M. A. (2006). A combination of caffeine and taurine has no effect on short term memory but induces changes in heart rate and mean arterial blood pressure. *Amino Acids, 31*(4), 471–476. https://doi.org/10.1007/s00726-005-0302-x

Cachat, J., Stewart, A., Grossman, L., Gaikwad, S., Kadri, F., Chung, K. M., … Kalueff, A. V. (2010). Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. *Nature Protocols*, 5(11), 1786–1799. https://doi.org/10.1038/nprot.2010.140

Canavello, P. R., Cachat, J. M., Beeson, E. C., Laffoon, A. L., Grimes, C., Haymore, W. A. M., … Kalueff, A. V. (2011). Measuring Endocrine (Cortisol) Responses of Zebrafish to Stress. In *Neuromethods: Vol. 51. Zebrafish Neurobehavioral Protocols* (pp. 135–142).

Caviness, C. M., Anderson, B. J., & Stein, M. D. (2017). Energy drinks and alcohol-related risk among young adults. *Substance Abuse, 38*(4), 376–381.
Chaban, R., Kornberger, A., Branski, N., Buschmann, K., Stumpf, N., Beiras-Fernandez, A., & Vahl, C. F. (2017). In-vitro examination of the positive inotropic effect of caffeine and taurine, the two most frequent active ingredients of energy drinks. *BMC Cardiovascular Disorders, 17*(1), 220. https://doi.org/10.1186/s12872-017-0625-z

Chen, S. W., Kong, W. X., Zhang, Y. J., Li, Y. L., Mi, X. J., & Mu, X. S. (2004). Possible anxiolytic effects of taurine in the mouse elevated plus-maze. *Life Sciences, 75*(12), 1503–1511. https://doi.org/10.1016/j.lfs.2004.03.010

Curran, C. P., & Marczinski, C. A. (2017). Taurine, caffeine, and energy drinks: Reviewing the risks to the adolescent brain. *Birth Defects Research, 109*(20), 1640–1648. https://doi.org/10.1002/bdr2.1177

Dametto, F. S., Fior, D., Idalencio, R., Rosa, J. G. S., Fagundes, M., Marqueze, A., … Barcellos, L. J. G. (2018). Feeding regimen modulates zebrafish behavior. *PeerJ, 6*, e5343. https://doi.org/10.7717/peerj.5343

Davis, D. J., Klug, J., Hankins, M., Doerr, H. M., Monticelli, S. R., Song, A., … Bryda, E. C. (2015). Effects of Clove Oil as a Euthanasia Agent on Blood Collection Efficiency and Serum Cortisol Levels in Danio rerio. *Journal of the American Association for Laboratory Animal Science: JAALAS, 54*(5), 564–567.

De Sanctis, V., Soliman, N., Soliman, A. T., Elsedfy, H., Di Maio, S., El Kholy, M., & Fiscina, B. (2017). Caffeinated energy drink consumption among adolescents and potential health consequences associated with their use: a significant public health hazard. *Acta Bio-Medica: Atenei Parmensis, 88*(2), 222–231. https://doi.org/10.23750/abm.v88i2.6664

Dhanasiri, A. K. S., Fernandes, J. M. O., & Kiron, V. (2013). Acclimation of zebrafish to
transport stress. *Zebrafish*, 10(1), 87–98. https://doi.org/10.1089/zeb.2012.0843

Donner, N. C., & Lowry, C. A. (2013). Sex differences in anxiety and emotional behavior. *Pflugers Archiv: European Journal of Physiology*, 465(5), 601–626. https://doi.org/10.1007/s00424-013-1271-7

Egan, R. J., Bergner, C. L., Hart, P. C., Cachat, J. M., Canavello, P. R., Elegante, M. F., … Kalueff, A. V. (2009). Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behavioural Brain Research*, 205(1), 38–44. https://doi.org/10.1016/j.bbr.2009.06.022

El Idrissi, A., Boukarrou, L., Heany, W., Malliaros, G., Sangdee, C., & Neuwirth, L. (2009). Effects of taurine on anxiety-like and locomotor behavior of mice. *Advances in Experimental Medicine and Biology*, 643, 207–215.

Evans, J., & Battisti, A. S. (2018). Caffeine. In *StatPearls*. Retrieved from http://www.ncbi.nlm.nih.gov/books/NBK519490/

Francisco, E. da S., & Guedes, R. C. A. (2015). Neonatal taurine and alanine modulate anxiety-like behavior and decelerate cortical spreading depression in rats previously suckled under different litter sizes. *Amino Acids*, 47(11), 2437–2445. https://doi.org/10.1007/s00726-015-2036-8

Giles, G. E., Mahoney, C. R., Brunyé, T. T., Gardony, A. L., Taylor, H. A., & Kanarek, R. B. (2012). Differential cognitive effects of energy drink ingredients: caffeine, taurine, and glucose. *Pharmacology, Biochemistry, and Behavior*, 102(4), 569–577. https://doi.org/10.1016/j.pbb.2012.07.004

Harper, C., & Lawrence, C. (2016). *The Laboratory Zebrafish*. Boca Raton: CRC Press.

Heckman, M. A., Weil, J., & Gonzalez de Mejia, E. (2010). Caffeine (1, 3, 7-trimethylxanthine)
in foods: a comprehensive review on consumption, functionality, safety, and regulatory matters. *Journal of Food Science*, 75(3), R77-87. https://doi.org/10.1111/j.1750-3841.2010.01561.x

Higgins, J. P., Babu, K., Deuster, P. A., & Shearer, J. (2018). Energy Drinks: A Contemporary Issues Paper. *Current Sports Medicine Reports*, 17(2), 65–72. https://doi.org/10.1249/JSR.0000000000000454

Isokawa, M. (2016). Caffeine-Induced Suppression of GABAergic Inhibition and Calcium-Independent Metaplasticity. *Neural Plasticity*, 2016, 1239629. https://doi.org/10.1155/2016/1239629

Kimura, M., Ushijima, I., Hiraki, M., Kimura, M., & Ono, N. (2009). Enhancement of caffeine-induced locomotor hyperactivity produced by the combination with L-arginine or taurine in mice: Possible involvement of nitric oxide. *Methods and Findings in Experimental and Clinical Pharmacology*, 31(9), 585–589. https://doi.org/10.1358/mf.2009.31.9.1435462

Kong, W. X., Chen, S. W., Li, Y. L., Zhang, Y. J., Wang, R., Min, L., & Mi, X. (2006). Effects of taurine on rat behaviors in three anxiety models. *Pharmacology, Biochemistry, and Behavior*, 83(2), 271–276. https://doi.org/10.1016/j.pbb.2006.02.007

Kysil, E. V., Meshalkina, D. A., Frick, E. E., Echevarria, D. J., Rosemberg, D. B., Maximino, C., … Kalueff, A. V. (2017). Comparative Analyses of Zebrafish Anxiety-Like Behavior Using Conflict-Based Novelty Tests. *Zebrafish*, 14(3), 197–208. https://doi.org/10.1089/zeb.2016.1415

López-Cruz, L., Carbó-Gas, M., Pardo, M., Bayarri, P., Valverde, O., Ledent, C., … Correa, M. (2017). Adenosine A2A receptor deletion affects social behaviors and anxiety in mice: Involvement of anterior cingulate cortex and amygdala. *Behavioural Brain Research*,
Magno, L. D. P., Fontes, A., Gonçalves, B. M. N., & Gouveia, A. (2015). Pharmacological study of the light/dark preference test in zebrafish (Danio rerio): Waterborne administration. *Pharmacology, Biochemistry, and Behavior, 135*, 169–176. https://doi.org/10.1016/j.pbb.2015.05.014

Manchester, J., Eshel, I., & Marion, D. W. (2017). The Benefits and Risks of Energy Drinks in Young Adults and Military Service Members. *Military Medicine, 182*(7), e1726–e1733. https://doi.org/10.7205/MILMED-D-16-00339

Maximino, C., da Silva, A. W. B., Araújo, J., Lima, M. G., Miranda, V., Puty, B., … Herculano, A. M. (2014). Fingerprinting of psychoactive drugs in zebrafish anxiety-like behaviors. *PloS One, 9*(7), e103943. https://doi.org/10.1371/journal.pone.0103943

Maximino, C., Lima, M. G., Olivera, K. R. M., Picanço-Diniz, D. L. W., & Herculano, A. M. (2011). Adenosine A1, but not A2, receptor blockade increases anxiety and arousal in Zebrafish. *Basic & Clinical Pharmacology & Toxicology, 109*(3), 203–207. https://doi.org/10.1111/j.1742-7843.2011.00710.x

Maximino, C., Marques de Brito, T., Dias, C. A. G. de M., Gouveia, A., & Morato, S. (2010). Scototaxis as anxiety-like behavior in fish. *Nature Protocols, 5*(2), 209–216. https://doi.org/10.1038/nprot.2009.225

McCool, B. A., & Chappell, A. (2007). Strychnine and taurine modulation of amygdala-associated anxiety-like behavior is “state” dependent. *Behavioural Brain Research, 178*(1), 70–81. https://doi.org/10.1016/j.bbr.2006.12.002

Mezzomo, N. J., Fontana, B. D., Müller, T. E., Duarte, T., Quadros, V. A., Canzian, J., … Barcellos, L. J. G. (2019). Taurine modulates the stress response in zebrafish. *Hormones*
Mezzomo, N. J., Silveira, A., Giuliani, G. S., Quadros, V. A., & Rosemberg, D. B. (2016). The role of taurine on anxiety-like behaviors in zebrafish: A comparative study using the novel tank and the light-dark tasks. *Neuroscience Letters, 613*, 19–24. https://doi.org/10.1016/j.neulet.2015.12.037

National Research Council. (2011). *Guide for the Care and Use of Laboratory Animals* (8th ed.). Washington DC: The National Academies Press.

Nehlig, A. (2018). Interindividual Differences in Caffeine Metabolism and Factors Driving Caffeine Consumption. *Pharmacological Reviews, 70*(2), 384–411. https://doi.org/10.1124/pr.117.014407

Nesan, D., & Vijayan, M. M. (2013). Role of glucocorticoid in developmental programming: evidence from zebrafish. *General and Comparative Endocrinology, 181*, 35–44. https://doi.org/10.1016/j.ygcen.2012.10.006

Oja, S. S., & Saransaari, P. (2017). Significance of Taurine in the Brain. *Advances in Experimental Medicine and Biology, 975 Pt 1*, 89–94. https://doi.org/10.1007/978-94-024-1079-2_8

Owoyele, B. V., Oyewole, A. L., Biliaminu, S. A., & Alashi, Y. (2015). Effect of taurine and caffeine on plasma c-reactive protein and calcium in Wistar rats. *African Journal of Medicine and Medical Sciences, 44*(3), 229–236.

Peacock, A., Martin, F. H., & Carr, A. (2013). Energy drink ingredients. Contribution of caffeine and taurine to performance outcomes. *Appetite, 64*, 1–4. https://doi.org/10.1016/j.appet.2012.12.021

Prediger, R. D. S., Batista, L. C., & Takahashi, R. N. (2004). Adenosine A1 receptors modulate
the anxiolytic-like effect of ethanol in the elevated plus-maze in mice. *European Journal of Pharmacology*, 499(1–2), 147–154. https://doi.org/10.1016/j.ejphar.2004.07.106

Prediger, R. D. S., da Silva, G. E., Batista, L. C., Bittencourt, A. L., & Takahashi, R. N. (2006). Activation of adenosine A1 receptors reduces anxiety-like behavior during acute ethanol withdrawal (hangover) in mice. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology*, 31(10), 2210–2220. https://doi.org/10.1038/sj.npp.1301001

Raymond, J., Chanin, S., Stewart, A. M., Kyzar, E., Gaikwad, S., Roth, A., … Kalueff, A. V. (2012). Assessing Habituation Phenotypes in Adult Zebrafish: Intra-and Inter-Trial Habituation in the Novel Tank Test. In *Neuromethods: Vol. 66. Zebrafish Protocols for Neurobehavioral Research* (pp. 273–285). Totowa NJ: Humana Press.

Richards, G., & Smith, A. P. (2016). A Review of Energy Drinks and Mental Health, with a Focus on Stress, Anxiety, and Depression. *Journal of Caffeine Research*, 6(2), 49–63. https://doi.org/10.1089/jcr.2015.0033

Richendrfer, H., Pelkowski, S. D., Colwill, R. M., & Creton, R. (2012). On the edge: pharmacological evidence for anxiety-related behavior in zebrafish larvae. *Behavioural Brain Research*, 228(1), 99–106. https://doi.org/10.1016/j.bbr.2011.11.041

Ripps, H., & Shen, W. (2012). Review: taurine: a “very essential” amino acid. *Molecular Vision*, 18, 2673–2686.

Rosa, L. V., Ardais, A. P., Costa, F. V., Fontana, B. D., Quadros, V. A., Porciúncula, L. O., & Rosemberg, D. B. (2018). Different effects of caffeine on behavioral neurophenotypes of two zebrafish populations. *Pharmacology, Biochemistry, and Behavior*, 165, 1–8. https://doi.org/10.1016/j.pbb.2017.12.002
Sava, B. A., Chen, R., Sun, H., Luhmann, H. J., & Kilb, W. (2014). Taurine activates GABAergic networks in the neocortex of immature mice. *Frontiers in Cellular Neuroscience, 8*, 26. https://doi.org/10.3389/fncel.2014.00026

Schaffer, S. W., Shimada, K., Jong, C. J., Ito, T., Azuma, J., & Takahashi, K. (2014). Effect of taurine and potential interactions with caffeine on cardiovascular function. *Amino Acids, 46*(5), 1147–1157. https://doi.org/10.1007/s00726-014-1708-0

Schnörr, S. J., Steenbergen, P. J., Richardson, M. K., & Champagne, D. L. (2012). Measuring thigmotaxis in larval zebrafish. *Behavioural Brain Research, 228*(2), 367–374. https://doi.org/10.1016/j.bbr.2011.12.016

Seidl, R., Peyrl, A., Nicham, R., & Hauser, E. (2000). A taurine and caffeine-containing drink stimulates cognitive performance and well-being. *Amino Acids, 19*(3–4), 635–642.

Steenbergen, P. J., Richardson, M. K., & Champagne, D. L. (2011). Patterns of avoidance behaviours in the light/dark preference test in young juvenile zebrafish: a pharmacological study. *Behavioural Brain Research, 222*(1), 15–25. https://doi.org/10.1016/j.bbr.2011.03.025

Stewart, A., Gaikwad, S., Kyzar, E., Green, J., Roth, A., & Kalueff, A. V. (2012). Modeling anxiety using adult zebrafish: a conceptual review. *Neuropharmacology, 62*(1), 135–143. https://doi.org/10.1016/j.neuropharm.2011.07.037

Stewart, A., Maximino, C., de Brito, T. M., Herculano, A. M., Gouveia, A., Morato, S., … Kalueff, A. V. (2011). Neurophenotyping of Adult Zebrafish Using the Light/Dark Box Paradigm. In *Neuromethods: Vol. 51. Zebrafish Neurobehavioral Protocols*. Springer Science+Business Media, LLC.

Tallis, J., Higgins, M. F., Cox, V. M., Duncan, M. J., & James, R. S. (2014). Does a
physiological concentration of taurine increase acute muscle power output, time to
fatigue, and recovery in isolated mouse soleus (slow) muscle with or without the presence
of caffeine? Canadian Journal of Physiology and Pharmacology, 92(1), 42–49.
https://doi.org/10.1139/cjpp-2013-0195

Trunzo, J. J., Samter, W., Morse, C., McClure, K., Kohn, C., Volkman, J. E., & O’Brien, K.
(2014). College students’ use of energy drinks, social problem-solving, and academic
performance. Journal of Psychoactive Drugs, 46(5), 396–401.
https://doi.org/10.1080/02791072.2014.965291

Valle, M. T. C., Couto-Pereira, N. S., Lampert, C., Arcego, D. M., Toniazzo, A. P., Limberger,
R. P., … Leal, M. B. (2018). Energy drinks and their component modulate attention,
memory, and antioxidant defences in rats. European Journal of Nutrition, 57(7), 2501–
2511. https://doi.org/10.1007/s00394-017-1522-z

Vercammen, K. A., Koma, J. W., & Bleich, S. N. (2019). Trends in Energy Drink Consumption
Among U.S. Adolescents and Adults, 2003–2016. American Journal of Preventive
Medicine, 56(6), 827–833. https://doi.org/10.1016/j.amepre.2018.12.007

Vincenzi, F., Borea, P. A., & Varani, K. (2017). Anxiolytic properties of A1 adenosine receptor
PAMs. Oncotarget, 8(5), 7216–7217. https://doi.org/10.18632/oncotarget.13802

Warnock, R., Jeffries, O., Patterson, S., & Waldron, M. (2017). The Effects of Caffeine, Taurine,
or Caffeine-Taurine Coingestion on Repeat-Sprint Cycling Performance and
Physiological Responses. International Journal of Sports Physiology and Performance,
12(10), 1341–1347. https://doi.org/10.1123/ijspp.2016-0570

Watts, S. A., Lawrence, C., Powell, M., & D’Abramo, L. R. (2016). The Vital Relationship
Between Nutrition and Health in Zebrafish. Zebrafish, 13 Suppl 1, S72-76.
Wendelaar Bonga, S. E. (1997). The stress response in fish. *Physiological Reviews, 77*(3), 591–625. https://doi.org/10.1152/physrev.1997.77.3.591

Westerfield, M. (2000). *The zebrafish book: A guide for the laboratory use of zebrafish (Danio rerio)* (4th ed.). Eugene: University of Oregon Press.

Wong, D., von Keyserlingk, M. A. G., Richards, J. G., & Weary, D. M. (2014). Conditioned place avoidance of zebrafish (Danio rerio) to three chemicals used for euthanasia and anaesthesia. *PloS One, 9*(2), e88030. https://doi.org/10.1371/journal.pone.0088030

Wong, K., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Roy, S., … Kalueff, A. V. (2010). Analyzing habituation responses to novelty in zebrafish (Danio rerio). *Behavioural Brain Research, 208*(2), 450–457. https://doi.org/10.1016/j.bbr.2009.12.023

Wu, G.-F., Ren, S., Tang, R.-Y., Xu, C., Zhou, J.-Q., Lin, S.-M., … Yang, J.-C. (2017). Antidepressant effect of taurine in chronic unpredictable mild stress-induced depressive rats. *Scientific Reports, 7*(1), 4989. https://doi.org/10.1038/s41598-017-05051-3

Yamada, K., Kobayashi, M., & Kanda, T. (2014). Involvement of adenosine A2A receptors in depression and anxiety. *International Review of Neurobiology, 119*, 373–393. https://doi.org/10.1016/B978-0-12-801022-8.00015-5

Zhang, C. G., & Kim, S.-J. (2007). Taurine induces anti-anxiety by activating strychnine-sensitive glycine receptor in vivo. *Annals of Nutrition & Metabolism, 51*(4), 379–386. https://doi.org/10.1159/000107687
Table 1 (on next page)

Percentage of zebrafish that did not explore top zone in the novel tank test

Acute exposure to energy drink components (caffeine, taurine, or both (CAF+TAU)) did significantly influence the number of fish that failed to explore the top zone in the novel tank test in adult zebrafish.
Table 1: Percentage of zebrafish that did not explore top zone

| Variable                  | Control (N = 41) | Caffeine (N = 19) | Taurine (N = 19) | CAF+TAU (N = 20) | $\chi^2$ | $p$  |
|---------------------------|------------------|-------------------|------------------|------------------|---------|------|
| Did not explore top       | 9.8%             | 42.1%             | 5.3%             | 25.0%            | 12.02   | 0.007|


Figure 1

Measures of zebrafish motor activity in the novel tank test

Acute exposure to energy drink components (caffeine, taurine, or both) did not alter (A) the total distance traveled and (B) the mean speed while ambulatory in the novel tank test in adult zebrafish. Bars indicate means of each group ± SEM.
A  Total Distance Traveled
Novel Tank Test

Caffeine
- No
- Yes

Distance (cm)

0  500  1000  1500  2000

Control  Taurine

B  Mean Speed while Ambulatory
Novel Tank Test

Caffeine
- No
- Yes

Mean speed during ambulation (cm/s)

0  2  4  6  8

Control  Taurine
Figure 2

Measures of zebrafish freezing behavior in the novel tank test

Acute exposure to energy drink components (caffeine, taurine, or both) did not alter (A) the total number of immobility bouts and (B) the total time spent immobile in the novel tank test in adult zebrafish. Bars indicate means of each group ± SEM.
**A**

Bouts of Immobility
Novel Tank Test

**Caffeine**
- No
- Yes

**Number of times immobile**

Control | Taurine
--- | ---
| | |

**B**

Time Immobile
Novel Tank Test

**Caffeine**
- No
- Yes

**Time immobile (s)**

Control | Taurine
--- | ---
| | |
Figure 3

Measures of zebrafish exploratory behavior in the novel tank test

Acute exposure to energy drink components (caffeine, taurine, or both) altered exploratory behavior in the novel tank test in adult zebrafish. Caffeine decreased (A) the distance traveled in the top zone, (B) the number of entries to the top zone, and (C) the time spent in the top zone. Taurine decreased (D) the latency to enter the top zone. Bars indicate means of each group ± SEM.
Figure 4

Measures of zebrafish neuroendocrine function after the novel tank test

Acute exposure to energy drink components (caffeine, taurine, or both) did not alter whole-body cortisol levels of zebrafish of fish in Experiment 1 (fish were sacrificed after the novel tank test). Bars indicate means of each group ± SEM.
Figure 5

Measures of zebrafish motor activity in the light-dark test

Acute exposure to energy drink components (caffeine, taurine, or both) affected some aspects of motor activity in adult zebrafish. Acute caffeine decreased (A) the total distance traveled but not (B) the mean speed during ambulation in the light-dark test. Bars indicate means of each group ± SEM and ** indicates significant (p < 0.01) difference from respective non-caffeine treated control (Tukey post-hoc).
A. Total Distance Traveled
   Light-Dark Test

   Caffeine
   - No
   - Yes

   Distance (cm)
   - Control
   - Taurine

   **

B. Mean Speed while Ambulatory
   Light-Dark Test

   Caffeine
   - No
   - Yes

   Mean speed during ambulation (cm/s)
   - Control
   - Taurine
Figure 6

Measures of zebrafish freezing behavior in the light-dark test

Acute exposure to energy drink components (caffeine, taurine, or both) altered the total time spent immobile in the novel tank test in adult zebrafish. Caffeine increased the total time immobile in the light-dark test. Bars indicate means of each group ± SEM. *, ** indicates significant (p < 0.05 and p < 0.01, respectively) difference from respective non-caffeine treated control (Tukey post-hoc).
Figure 7

Measures of zebrafish exploratory behavior in the light-dark test

Acute exposure to energy drink components (caffeine, taurine, or both) altered exploratory behavior in the novel tank test in adult zebrafish. Caffeine decreased (A) the distance traveled in the light zone, (B) the number of entries to the light zone, and (C) the number of crossings between compartments. The acute drug treatments did not significantly alter (D) the time spent in the light zone nor (E) the latency to re-enter the light zone after the first visit to the dark zone. Bars indicate means of each group ± SEM. *, ** indicates significant (p < 0.05 and p < 0.01, respectively) difference from respective non-caffeine treated control (Tukey post-hoc).
Figure 8

Measures of zebrafish neuroendocrine function after the light-dark test

Acute exposure to energy drink components (caffeine, taurine, or both) did not alter whole-body cortisol levels of zebrafish of fish in Experiment 2 (fish were sacrificed after the light-dark test). Bars indicate means of each group ± SEM.