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A Methodological Discussion of Caffeine Research and Animal Avoidance Behavior

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Introduction: We present a review of the methodological aspects of caffeine research within animal tests of escape and avoidance behavior in the presence of aversive stimuli.
Method: We highlight species, methods of caffeine administration, dosage, dependent measures, and research designs commonly used in this research.
Results: Typical subjects were rodents and zebrafish, with species-specific vehicles of caffeine administration and dependent measures. Behavioral tests for escape and avoidance as a function of caffeine consumption were conceptually similar across species, although the arrangement of measures was necessarily adapted to the physiological contingencies of the different species.
Discussion and Conclusions: Caffeine administration preceding the presentation of aversive stimuli generally, but not exclusively, enhanced the effect of escape and avoidance of aversive stimuli. The many commonalities in methods and results across species suggest similar methods may be relevant to human subjects as well.

Introduction

Caffeine is a popular central nervous system stimulant commonly available in tea, coffee, and soda, as well as some foods.[1] The effects of caffeine on behavior have been widely studied with both humans and animals, the latter offering experimental and procedural latitude to investigators while maintaining potential extrapolation of findings to humans. The effects of caffeine on escape and avoidance behavior have comprised a major element of caffeine research in the animal literature. In this regard, caffeine has been found to both increase the anxiogenic effect of a stimulus (i.e., increasing the likelihood of escape and avoidance)[2–4] and to have an anxiolytic effect (i.e., decreasing the likelihood of escape and avoidance, and increasing the likelihood of approach),[5] subject to dosage and timing of caffeine administration.[6] To help elucidate these issues, we illustrate methods of caffeine research in the animal literature and exemplify modes of administration and dependent measures across frequently used species.

Significant avoidance of a stimulus (i.e., a reduction in exploratory behavior or time spent in the presence of the stimulus)[7,8] has been considered indicative of ''anxiety.'' Stimuli, events, or conditions (e.g., caffeine) that result in an increase in such behavior have been considered anxiogenic.[2,7] In contrast, an event is considered anxiolytic (i.e., anxiety-reducing) when there is an increase in exploratory behavior or time spent in the presence of a known aversive stimulus.[9] The use of approach and escape/avoidance behavior as dependent measures in animal tests of anxiety is methodologically similar to the behavioral approach test (BAT) used with ''anxious'' human participants. The BAT presents participants with a series of graduated steps, with each successive step closer to the previously avoided stimulus.[10]

In contrast to the BAT, however, animal tests typically measure approach and avoidance by changes in the time spent in the presence of known aversive versus nonaversive stimuli and the number of entries into the spaces where aversive stimuli are located rather than the number of successive approximation steps completed.[11,12] The approach/avoidance paradigm permits measurements of changes in both behaviors at once, in that an increase in approach necessarily results in a reduction in avoidance. Authors in the caffeine literature typically report results in terms of changes in avoidance.

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TABLE 1. METHODOLOGICAL DESCRIPTION OF REVIEWED CAFFEINE STUDIES

Authors	*Subjects*	*Design*	*Timing of administration*	*Method of administration*	*Dosage*	*Behavioral test*
Baldwin and File[26]	Male hooded Listar rats; $n = 7–15$	Between-groups, controlled design	30 min before testing	IP	20, 40 mg/kg[a]	Social interaction test
Baldwin *et al.*[8]	Male hooded Listar rats; $n = NA$	Between-groups, controlled design	30 min before testing	IP	40 mg/kg[a]	Social interaction testElevated plus mazePunished-drinking test
Bert *et al.*[13]	Male, female Sprague Dawley and Wistar rats; $n = 9–10$	Controlled, mixed research design	30 min before testing	IP	50 mg/kg[a]	Free exploration testElevated plus maze
Bradley *et al.*[34]	Male, female Mongolian gerbils, seizure-resistant strain; $n = 10–12$	Between-groups, controlled design	30 min before testing	IP	0.5–30 mg/kg[a]	Black-white box
Braun *et al.*[47]	Male, female Sprague Dawley rats; $n = 7$	Between-groups, controlled design	30 min before testing	Subcutaneous injection	100 mg[a]	Elevated plus mazeElevated zero maze
Cachat *et al.*[19]	Male, female wild-type zebrafish; $n = NA$	Between-groups, controlled design	1-week exposure, 12-h washout before testing	Tank water exposure	50 mg/L[a]	Novel tank test
Carvalho-Netto and Nunes-de-Souza[33]	Male Swiss mice; $n = 10–12$	Controlled, mixed research design	30 min before testing	IP	10, 30 mg/kg[a]	Elevated T-maze
Celec and Behuliak[28]	Male Wistar rats; $n = NA$	Between-groups, controlled design	3-month exposure before testing	Free access to caffeinated drinking water	30–60 mg/kg/day (Approx.)	Light/dark box
Egan *et al.*[7]	Male, female wild-type, long-fin, leopard, and albino zebrafish; $n = 21$	Between-groups, controlled design	5-min exposure before testing	Exposure in beaker water	100 mg/L in 3-L beaker[a]	Novel tank diving test
El Yacoubi *et al.*[16]	Male Swiss albino CD1 mice, A_{2A} receptor knockout mice and wild-type controls; $n = 8–16$	Between-groups, controlled design	Acute: 30 min before testingChronic: 1-, 8-, 60-day exposure before testing	Acute: IPChronic: free access to caffeinated drinking water	Acute: 12.5–100 mg/kg Chronic: 4.1–5.9 mL/mouse day (Approx.)[a]	Elevated plus mazeLight/dark test
File[37]	Male hooded Listar rats; $n = 23$	Controlled, mixed research design	Daily injections on days 1–7; testing occurred on days 35–36	Subcutaneous injection	15–30 mg/kg[a]	Social interaction testElevated plus maze

(*continued*)

Table 1. (Continued)

Authors	*Subjects*	*Design*	*Timing of administration*	*Method of administration*	*Dosage*	*Behavioral test*
Garcia *et al.*[5]	Male Wistar-derived rats; $n = 11–12$	Between-groups, controlled design	30 min before testing	IP	10, 30 mg/kg[a]	Elevated plus maze
Gulick and Gould (2009)[43]	Male C57BL/6J mice; $n = 6–10$	Between-groups, controlled design	30 min before training; testing occurred 24 h later	IP	5–40 mg/kg[a]	Plus maze discrimination avoidance task
Hughes[11]	Male, female Piebald Virol Glaxo/C rats; $n = 10$	Between-groups, controlled design	10-day exposure day 45–55; testing occurred day 120	Free access to caffeinated drinking water	20–30 mg/kg (Approx.)	Light/dark boxOpen field test
Jain *et al.* (1995)[44]	Male ICR or MF1 rats; $n = 10$	Between-groups, controlled design	15 min before testing	IP	15–60 mg/kg[a]	Elevated plus maze
Jain *et al.*[14]	Male Sprague Dawley rats; $n = 6$	Between-groups, controlled design	30 min before testing	IP	5–100 mg/kg[a]	Elevated plus maze
Khor *et al.*[22]	Male wild-type zebrafish; $n = 12–20$	Between-groups, controlled design	24-h exposure followed by 24 h before testing	Exposure in Petri dish	5, 50 mg/L[a]	Novel tank diving test
Kulkarni *et al.*[6]	Male, female Laca mice; $n = 6$	Between-groups, controlled design	30 min before testing	NA	8–60 mg/kg[a]	Elevated plus mazeElevated zero maze
Kurt *et al.*[41]	Male, female ICR rats; $n = 8$	Between-groups, controlled design	30 min before testing	IP	30 mg/kg[a]	Elevated plus maze
Lapin (1998)[45]	Male Albino SHR mice; $n = 32$	Between-groups, controlled design	15 min before testing	IP	50 mg/kg[a]	Dark/light chamber
Lister[12]	Male NIH Swiss mice; $n = 7–11$	Between-groups, controlled design	25 min before testing	IP	15–60 mg/kg[a]	Elevated plus maze
Maximino *et al.*[23]	Unsexed wild-type zebrafish; $n = 10$	Between-groups, controlled design	15 min before testing	Injection	100 mg/kg[a]	Scototaxis test
Maximino *et al.*[2]	Unsexed wild-type zebrafish; $n = 10$	Between-groups, controlled design	15 min before testing	Injection	1–100 mg/kg[a]	Scototaxis test
Noschang *et al.*[3]	Male, female Wistar rats; $n = 7–13$	Between-groups, controlled design	50–60-day exposure before testing	Free access to caffeinated drinking water	40, 108 mg/kg/day (Approx.)	Elevated plus mazeOpen field test
Oettinger *et al.*[21]	Female Wistar; rats; $n = 15$	Controlled, mixed research design	15 min before testing	IP	8–32 mg/kg	Tunnel maze with open field
Park *et al.*[4]	Male ICR mice, male Wistar rats; $n = 5–9$	Between-groups, controlled design	30 min before testing	IP	25 mg/kg[a]	Elevated plus mazeOpen field test
Pechlivanova *et al.*[27]	Male Wistar rats; $n = 10$	Between-groups, controlled design	4-week exposure before testing	Oral gavage	8 mg/kg/day[a]	Elevated plus mazeOpen field test

(*continued*)

Table 1. (Continued)

Authors	*Subjects*	*Design*	*Timing of administration*	*Method of administration*	*Dosage*	*Behavioral test*
Prediger *et al.*[17]	Male Swiss albino mice; $n=8$	Between-groups, controlled design	30 min before testing	IP	10, 30 mg/kg[a]	Elevated plus maze
Richendrfer *et al.*[24]	Wild-type zebrafish (Sex NA); $n=35$–48	Controlled, mixed research design	2 h before testing and 1 h during testing	Caffeinated egg water exposure	10–100 mg/L[a]	Thigmotaxis test with five-fish behavioral assay
Ruiz-Medina *et al.*[39]	Male CD1 mice; $n=10$	Controlled, mixed research design	7–21-day exposure before testing	IP	10–30 mg/kg[a]	Dark/light box test
Sanday *et al.*[40]	Swiss male mice; $n=10$	Controlled, mixed research design	30 min before either pretraining, testing or both	IP	20 mg/kg	Plus maze discrimination avoidance taskInhibitory avoidance task
Silva and Frussa-Filho[46]	Male Swiss EPM-M1 mice; $n=10$	Controlled, mixed research design	30 min before conditioning, testing occurred 24 h later	IP	20 mg/kg[a]	Plus maze discrimination task
Schnörr *et al.*[9]	Wild-type zebrafish (sex NA); $n=25$	Between-groups, controlled design	10-min exposure during testing	Caffeinated egg water exposure	50 mg/L[a]	Thigmotxis test with 24-well assay
Steenbergen *et al.*[25]	Wild-type zebrafish (sex NA); $n=27$	Between-groups, controlled design	7 min exposure, followed by 3-s washout before testing	Caffeinated egg water exposure	85 mg/L[a]	Light/dark preference test
Vila-Luna *et al.*[20]	Male Wistar rats; $n=13$	Controlled, mixed research design	6-month exposure, followed by 2-week washout before testing	Free access to caffeinated water	5 mg/kg/day	Elevated plus maze
Vitale *et al.*[15]	Male Wistar rat; $n=8$	Between-groups, controlled design	30 min before testing	IP	20 mg/kg[a]	Defensive burying test

Studies are presented in the alphabetical order of authorship. Doses listed as ranges if more than two were used.
[a]Other drugs evaluated in study in addition to caffeine.
IP, intraperitoneal injection; *n*, number of subjects in caffeine group; NA, information not available.

Within this context, we describe methods to assess caffeine's functional relationship to approach and avoidance behaviors in the presence of known species-specific aversive stimuli. In these studies, it is common to evaluate additional drugs within the same study using the same methods used to evaluate caffeine (Table 1). In this way, the methods described here may be relevant to researchers of other drugs beyond caffeine; however, our purpose is to describe methods as they specifically have pertained to caffeine. Studies are described in terms of subjects, means of caffeine administration, dose levels, experimental designs, and dependent measures.

Method

PubMed literature searches, conducted with the assistance of a university librarian, used ''caffeine,'' ''anxiety'' ''approach,'' and ''avoidance'' as search terms. Studies with Medical Subject Headings (MeSH) terms before 2008, and with and without MeSH terms between 2008 and 2013, were included. MeSH terms were used to narrow the search in a way that would include a broad selection of past and recent studies. A total of 37 studies were included. Table 1 provides a complete list of reviewed studies.

Subjects

A significant advantage of animal research is the degree of control over subjects' histories resulting in greater assurance to researchers that the independent variable is responsible for changes in behavior. In addition, caffeine can be precisely titrated across subjects and performance measures can be readily observed, uncontaminated by faulty retrospective data sometimes found when using human subjects. Rodents (i.e., rats, mice, and gerbils) were the most common subjects, included in over three-quarters of the studies reviewed. Typical rat strains were Sprague-Dawley[13,14] and Wistar,[4,15] and for mice, the Swiss albino.[16,17] Rodents have many attributes that make them appealing for animal research. Specifically, they have a short gestation period and produce large numbers of offspring, which make them economically feasible in laboratory settings.[18] Also, as both rats and humans are mammals, there are similarities in physiological structures and functional responses.

Recently, zebrafish have become increasingly used as animal models in neurobehavioral and biological psychiatry research.[19] Approximately one-quarter of studies reviewed included zebrafish as subjects. As a vertebrate species, the zebrafish also shares physiological similarity to humans, as well as the practical advantages of being relatively low cost and quite prolific in producing offspring.[7] Wild-type strains of zebrafish, a strain of zebrafish that occurs in natural populations in the wild, are most commonly used and are considered the standard (Table 1).

All but one rodent study in our sample included male subjects; 28% of rodent studies also included female subjects. Few studies provided a rationale for the inclusion of male or female subjects. Vila-Luna *et al.*[20] stated only males were used because of prior evidence suggesting that estrogen may interfere with neuroprotective actions of caffeine in rodents. Oettinger *et al.*,[21] the one rodent study that included only female subjects, stated that female subjects were selected because they had been more active (and hence exhibited more variability) than males in prior research, using the specific behavioral test used in their research (i.e., tunnel maze with open field). Given the consumption of caffeine across male and female humans, further investigation of male and female animal counterparts seems warranted.

We reviewed eight zebrafish studies; three used male zebrafish,[7,19,22] two included both male and female zebrafish,[7,19] and two included unsexed zebrafish.[2,23] The sex of subjects was unstated in three studies,[9,24,25] in which experimenters collected embryos from tanks that housed adult male and female zebrafish. This procedure suggests that both male and female larvae were included, although this was never specified.

Dependent measures

The effect of caffeine on approach/avoidance behavior has been examined extensively with rodents and increasingly with zebrafish. In general, these studies present the animal with a known, species-specific aversive stimulus or condition following caffeine administration and changes in approach/avoidance behavior are measured using operationally defined measures. Measures used to evaluate the effects of caffeine on zebrafish have been developed for the novel tank diving test,[22] the scototaxis test[21,23] and the thigmotaxis test.[9,24] Common measures of rodent approach/avoidance behavior have been obtained for the social interaction test[26]; the elevated plus maze[6]; the light/dark box[11]; and the open field test.[21]

One of the most prevalent tests of changes in rodent behavior in the presence of an aversive stimulus is the elevated plus maze. This task places subjects, 30 minutes postcaffeine, in the center of an elevated apparatus with four narrow tracks or arms crossed at right angles at the center forming a plus shape. The ends of two opposed arms are open, in that they are not enclosed by walls or have clear walls. The other two opposed arms are enclosed by walls and usually painted black. This apparatus is designed to capture changes to a subject's aversion to bright, open spaces and preference for dark, enclosed spaces.[24] Change in avoidance behavior is measured by the number of entries into the open arms of the maze and the duration of time spent in the open arms. In other words, a decrease in time spent in the open arms is considered to be indicative of an increase in anxiogenic behavior.[5] In contrast, an increase in the amount of

time in the open arms is considered to be reflective of an anxiolytic response to caffeine.[8]

For example, Baldwin *et al.*[8] examined the effect of 40 mg/kg caffeine and 2.5 mg/kg of yohimbine (an $\alpha 2$-adrenoceptor antagonist) on rat behavior in the elevated plus maze. Male hooded Listar rats received one of the following combinations: (1) two administrations of distilled water (i.e., water with impurities removed), (2) one administration of either yohimbine or caffeine and an administration of distilled water, or (3) an administration of yohimbine and caffeine, before the start of behavioral tests. The results showed that both yohimbine and caffeine significantly decreased the percentage of time spent in the open arms of the elevated plus maze, with yohimbine producing a greater reduction, and thus, both compounds contributed to the rats' avoidance of open spaces.

Another common dependent measure of rodent behavior is the open field test, which takes advantage of the tendency of rodents to avoid open spaces. The open field test arena consists of a square box with a floor demarcated into an inner and outer square. Time spent in the inner square is considered an aversive experience for rodents.[11] Several authors[3,4] examined the effect of caffeine on rat and mouse behavior in the open field. For example, Pechlivanova *et al.*[27] evaluated how chronic caffeine administration affected the behavior of male Wistar rats exposed to chronic and unpredictable stress, which involved foot shocks, food deprivation, and restraint, compared to saline-treated control rats. Eight mg/kg of caffeine was administered daily by an oral gavage 30 minutes before the rats underwent the chronic stress procedure. The chronic stress condition began 2 weeks before the start of caffeine treatment and continued throughout 4 weeks of caffeine exposure. Behavioral tests took place during the fifth and sixth week of the chronic stress exposure.

In the open field test, Pechlivanova *et al.*[27] recorded the number of squares crossed and the amount of exploratory activity (e.g., rears) that took place in the inner square. The authors found groups exposed to caffeine only or chronic stress and caffeine showed a lower activity in the inner square compared to the saline-only treated group, suggesting that caffeine produced a reduction in the open space activity (i.e., an increase in avoidance behavior).

A final example of a common test for escape and avoidance behavior in rodents is the light/dark box. This test is based on rodents' tendency to spend time in dark places and avoid bright places.[28] El Yacoubi *et al.*[16] used behavior in the light/dark box as a dependent measure when comparing the effect of caffeine and two selective A_{2A} antagonists on avoidance behavior. Swiss albino CD1 mice (a strain derived from Swiss mice and originally produced by Charles Rivers laboratories)[29] were injected with 25, 50, or 100 mg/kg caffeine or the vehicle (a water-based solution containing 10 mg/mL of sodium benzoate) 30 minutes before being placed in the dark compartment of the light/dark box. The light/dark box apparatus consisted of two adjoining boxes, one painted black and covered with a lid, and the other painted white. The white compartment was not covered and was lit by a 100-W light bulb. Mice were able to travel freely between the two compartments using a small, centrally located hole in the panel that connected the two compartments.

The authors assessed changes in avoidance, as a function of caffeine, using several behavioral measures; specifically, the latency to make the first entry into the lit compartment, the time spent in the lit compartment, the number of entries into the lit compartment, and the number of attempted entries into the lit compartment that were followed by an avoidance response (i.e., not followed by an entry into the lit compartment). El Yacoubi *et al.*[16] found a significant reduction in the number of transitions between compartments and the total time spent in the lit box for mice treated with 50 and 100 mg/kg caffeine, compared to vehicle-treated controls. The authors also found a significant reduction in the number of attempted entries into the lit compartment followed by an avoidance response for mice treated with 100 mg/kg of caffeine. These reductions across several behaviors associated with the lit compartment following caffeine treatment are consistent with an increase in avoidance behavior.

Dependent measures used in studies with zebrafish share many similarities with those used with rodents. In particular, tests for escape and avoidance measure zebrafish avoidance of an aversive stimulus, usually an open or unknown space.[19,23,24] Several studies evaluated the effect of caffeine using the novel tank diving test, which is considered to be conceptually similar to the elevated plus maze and the open field test[30] often used to measure rodent behavior.

The novel tank diving test is designed to take advantage of zebrafish's natural tendency to initially spend time at the bottom of a novel tank before increasing the range of movement to the upper portions.[7] An increase in the latency to move into the higher portion of the tank and a reduction in the total amount of time spent in the higher portion of the tank represent avoidance of the upper portion of the tank. As an example, Egan *et al.*[7] administered the novel tank diving test to adult zebrafish following exposure to 100 mg/L of caffeine. The authors found that caffeine-treated fish showed an increase in avoidance behavior, as evidenced by a significantly greater latency to move into the upper half of the tank and an increase in the number of erratic movements, as well as a decrease in the number of transitions into the upper half of the tank.

Two studies by Maximino *et al.* examined the effect of caffeine on zebrafish behavior within the scototaxis test.[2,23] In contrast to the novel tank diving test, which is analogous to the elevated plus maze for rats and mice,

the scototaxis test assesses preference for darkness similar to the light/dark box used in rodent research. In the scototaxis test, zebrafish are placed in a tank, in which one horizontal portion is white and the other is black. A reduction in the time spent in the white portion of the tank is considered avoidance of an aversive stimulus. In Maximino *et al.*,[2] the authors examined the acute effects of caffeine doses ranging from 0 to 100 mg/kg on the time spent in the white portion of the tank and the number of crossings between the two portions of the tank. The authors found that the percentage of time spent in the white compartment was functionally related to the highest dose of caffeine, resulting in significantly less time in the white portion of the tank, suggesting that this level of caffeine was related to avoidance behavior.

Caffeine also has been used in the thigmotaxis test to examine edge preference and avoidance behavior. In Richendrfer *et al.*,[24] zebrafish larvae were exposed to caffeine (range, 10–100 mg/L) for 2 hours before being placed in five-fish assays (i.e., five fish per well). For the first 30 minutes of the assessment, plates holding multiple wells were placed on top of a plain white laptop screen. The laptop screen provided a background color and a means to present visual stimuli. In the second 30 minutes of the assessment, the laptop screen displayed two red balls, one bouncing and one stationary, intended to serve as aversive stimuli similar to the shadow of predators. Moving to the edge of the well suggested the fish were attempting to avoid the shapes. The authors found caffeine administration increased the percentage of intervals on the edge during the visual stimulus presentations, compared to control fish that were not administered caffeine.

It should be noted that caffeine has psychostimulant properties, which promote increased arousal and motor activity.[31,32] Many of the animal tests studied in this review, such as the open field, elevated plus maze, and light/dark box, are influenced by motor activity. To control for motor stimulant effects, many studies reported measurements of locomotion across treatment and control groups in an attempt to detect the influence of increased motor activity on changes in avoidance behavior. Maximino *et al.*[2] measured zebrafish locomotion in the light/dark box by measuring the number of crossings between the light and dark compartments. In this case, caffeine had a stimulant effect on the number of crossings at a lower dose (10 mg/kg), but not at a higher dose (100 mg/kg). In contrast, 100 mg/kg produced a significant increase in the avoidance of the white compartment. Together, the results suggest that caffeine's motor stimulant effect did not interfere with detection of changes in avoidance behavior.

Schnörr *et al.*[9] also reported taking steps to isolate the influence of caffeine on avoidance behavior. The authors evaluated the effect of caffeine on thigmotaxis. In this case, a zebrafish that is largely immobile in the outer portion of the well could be described as high thigmotaxis, or highly avoidant of the central section of the well. However, the authors chose not to report on thigmotaxis for animals that showed a low frequency of movement when fish were acclimating to the wells, because it was possible that the lack of movement was due to factors, such as acclimatization itself, other than caffeine.

Methods of Delivery

Means of administration. Caffeine was administered to rodents using intraperitoneal (IP) and subcutaneous injections, oral gavage, and *ad lib* (i.e., free access) solutions. IP injections are injections of substances directly into the animal's peritoneum (i.e., body cavity). This form of injection has been widely used as a means of administering caffeine to rats, mice, and gerbils.[32] The IP method is considered easy to implement and advantageous because it allows long periods of absorption.[33] Subcutaneous injections are injections into the subcutis, or lowermost, layer of skin, as used by Bradley *et al.*,[34] who examined the effects of caffeine and other drugs on rat behavior. Subcutaneous injections are easily administered and appear to cause the animal less distress than other methods, although the rate of absorption is lower than IP.[35]

Investigators have flexibility in selecting a means of administration when only one administration is required. However, repeated administrations over time present challenges to researchers who attempt to minimize stress and harm to animal subjects.[34] Most rodent studies reviewed (69%) included a single administration of caffeine. Of the 10 studies examining more than a single caffeine administration, five provided free access to caffeinated drinking water.[3,11,16,20,28] Others provided repeated exposures to caffeine through oral gavage,[27] subcutaneous injection,[36] and IP.[37–39]

The oral gavage procedure has been less commonly used. In this method, caffeine is delivered directly through a flexible tube inserted through the mouth into the stomach while the animal is conscious. The oral gavage approach is one of the methods available if oral delivery is desired.[34] One advantage of oral gavage is that it allows precise measurement of caffeine consumption. Pechlivanova *et al.*[27] delivered caffeine through oral gavage to examine the effect of caffeine on the behavior of rats previously exposed to chronic and unpredictable stress.

Another means of oral delivery provides caffeine *ad lib* within a regular source of fluids. In this method, caffeinated water solutions are administered through a bottle attached to the cage.[34] Celec and Behuliak[28] replaced drinking water with one of three different sodas for 3 months to study the effect of chronic caffeine on behavior and endocrine changes of rats. El Yacoubi *et al.*[16] also administered caffeine in drinking water for 1, 8, or 60 days to determine the possible escape and avoidance effect of chronic caffeine consumption on mice. For these

animals, caffeine was provided in a solution of 0.3 g/L water in place of typical drinking water, which was provided to control mice.

The *ad lib* means of administration has many advantages, including serving as a model of consumption of caffeine by humans, as well as avoiding potential harm that may come to animals through repeated injections over periods of weeks and months. That said, providing *ad lib* access to caffeinated water presents measurement challenges. For example, caffeine intake was typically measured by recording how much liquid was consumed for each animal on a daily basis but this procedure does not account for liquid lost from the bottle that was not the result of intake (e.g., bottle or administration malfunction, drips, evaporation).[16]

For zebrafish, caffeine typically has been administered directly through tank water[19] or in egg water, which is distilled water with dissolved sea salts.[25] By providing caffeine through tank or egg water, the caffeine is absorbed through the skin of the fish. Cachat *et al*.,[19] for example, dissolved caffeine into the tank water of adult zebrafish as part of their study to validate a zebrafish model of caffeine withdrawal.

Vehicle solutions. Caffeine has been administered to mice, rats, and gerbils in solutions that have included one or more substances in addition to caffeine, such as distilled water,[26,34] saline,[40,41] and home cage drinking water.[3,20] Of the 24 reviewed rodent studies that used injection as an administration method, 67% used saline and 25% used distilled water. This tendency to use saline as a vehicle may be due to saline producing less pain behavior than distilled water during subcutaneous injections.[33] A number of vehicles consisted of one of these common liquids plus another excipient (i.e., inactive substance that acts as a medium for other active drugs), such as Cremophor EL,[13] Tween 20, Tween-80, and dimethyl sulfoxide. Substances such as these also have been used when experimental drugs do not dissolve readily in water.[42] Vehicles such as Tween 80 are useful in drug evaluation studies because they do not appear to seriously affect drug effects.[33]

Timing and frequency of administration. The timing and frequency of caffeine administration were similar across rodents and zebrafish but varied depending on whether the research question pertained to acute or chronic exposure to caffeine. Acute effects typically were studied following one administration of caffeine, 5–30 minutes before assessing its impact on behavior.[43–45] Examination of chronic or extended exposure involved more frequent and/or longer durations of exposure (range, 1 week-6 months). Acute and extended exposure was occasionally followed by a washout period or an interval of time between caffeine administration and behavioral testing. Washout periods allowed for the examination of delayed or withdrawal effects. Authors reported washout periods that ranged from very brief (3 seconds)[25] to 2 weeks.[20]

Acute effects of caffeine typically (although not exclusively) increased avoidance behavior in the presence of an aversive stimulus. For example, Vitale *et al*.[15] compared the effect of caffeine to diazepam and neuropeptide S on rat behavior with the defensive burying test. Rats were assessed one time with the defensive burying test after receiving one injection of caffeine 30 minutes before the test. Specifically, rats were placed in a cage filled with sawdust. A wooden dowel that delivered electric shocks upon contact was placed in the cage. Rats could avoid the dowel by burying the dowel in sawdust. The total time spent burying the dowel and height of sawdust around the dowel were dependent measures of avoidance behavior. The authors reported that 20 mg/kg of caffeine administered 30 minutes before the defensive burying test significantly increased time spent burying and the height of burying material.

The methods used to study the effect of extended or repeated exposure to caffeine have varied more widely, as have the effects. For example, to assess the interaction between chronic stress and caffeine intake, Noschang *et al*.[3] provided either caffeinated water (40 or 108 mg/kg/day approximately) or tap water to rats for 50 days. After the 50-day exposure to either caffeine or tap water, rats were tested using the elevated plus maze, and 10 days later, the open field test. Both tests measure behaviors indicative of avoidance. In this case, chronic caffeine produced a significant reduction in the amount of time spent in the central area of the open field test and significantly reduced entries into open arms of the elevated plus maze, both of which suggest an anxiogenic effect.

The effect of chronic or extended exposure may be affected by how closely the behavioral test follows caffeine administration. File[37] manipulated both the frequency of caffeine delivery and the duration between caffeine delivery and obtaining dependent measures. The author tested the acute effects of caffeine administered during the first 7 days of life and the remote effects of early-in-life exposure, as measured during adolescence. Male hooded Listar rats were injected subcutaneously twice daily for 7 days with 15 or 30 mg/kg of caffeine, after which caffeine was discontinued. Rat behavior was observed and recorded on days 1, 3, 5, and 7 to test the immediate effects of daily caffeine intake. A battery of behavioral tests was administered on days 35–40 to test the lasting effects during adolescence. File found no significant delayed effect of the early-in-life caffeine exposure on adolescent rat behavior in the elevated plus maze or social interaction test.

Manipulations of timing, frequency, and duration of caffeine exposure in the zebrafish literature are generally similar to those in the rodent literature, with both acute and remote caffeine effects examined, with one notable

exception. As zebrafish are able to absorb caffeine through egg and tank water, two studies examined fish behavior during caffeine administration, which was not possible during rodent administrations.[9,24] For example, Schnörr *et al.*[9] examined the immediate and ongoing effects of caffeine exposure. To examine zebrafish thigmotaxis or wall-hugging behavior, in response to caffeine and the sudden onset of darkness, individual zebrafish were placed in each well of a 24-well plate. All wells were filled with caffeine-treated egg water throughout the duration of the 10 minutes test, while thigmotaxis was measured under lit and dark conditions.

The timing and frequency of caffeine administration in animals can mimic human caffeine consumption and may be informative regarding the effect of various patterns of consumption on human behavior. Measuring the short-term effects of caffeine on behavior during an aversive experience, as in Vitale *et al.*,[15] may be relevant to humans who drink caffeinated beverages before engaging in stressful work tasks. Furthermore, File's[37] and Noschang *et al.*'s[3] evaluations of the delayed effects of caffeine may reveal important information regarding how caffeine consumed at early stages of development affects mature performance.

Dose levels

Within the context of these studies, a number of dosages have been used. Several studies provided a brief rationale for their dose levels. For example, Bradley *et al.*[34] selected the lower end of the expected effective dose range (0.5–30 mg/kg) to reduce the risk of seizures for gerbils. Silva and Frussa-Filho[46] selected their dose level (20 mg/kg) because it had been previously shown to produce an increase in avoidance behavior for mice under similar conditions. Bert *et al.*[13] reported selecting their caffeine dose (50 mg/kg) for rats simply based on doses used in previous research.

Several studies compared caffeine-treated animals to a saline-treated control group. For example, in Sanday *et al.*'s[40] investigation of state-dependent behavior, mice were given either saline or 20 mg/kg of caffeine 30 minutes before training in the plus maze discrimination task. Other studies examined a range of caffeine doses and compared the effect of caffeine to other drugs. For example, Lister[12] compared the effect of several doses of caffeine (15, 30, and 60 mg/kg) to that of various anxiolytic and anxiogenic drugs, including chlordiazepoxide hydrochloride (5 and 10 mg/kg), ethanol (0.8 and 1.6 g/kg) and picrotoxin (1 and 2 mg/kg), on escape and avoidance behavior of mice in the elevated plus maze.

Experimental designs

Studies assessing the effect of caffeine on behavior in the animal literature used between-group, controlled designs (73%) or controlled, mixed research designs (27%). A recent example using a between-groups design compared performance as a function of caffeine on the elevated plus maze and elevated zero maze, which is a variation on the elevated plus maze that features a circle track with two quadrants with black walls and two quadrants with clear walls. Braun *et al.*[47] randomly assigned adult male Sprague Dawley rats to either the plus maze or zero maze groups. Before being placed in the testing arena, animals received one administration of caffeine, nicotine, yohimbine, diazepam, physical restraint (used as a nonpharmacological anxiogenic), or saline. The results compared performance between groups to the control group. The authors found 100 mg/kg of caffeine significantly reduced the percentage of time (i.e., increased avoidance behavior) spent in open spaces in both mazes.

Vila-Luna *et al.*[20] provided an example of a controlled, mixed research design. This design allows comparisons using repeated measures over time from the same group as well as between groups. Vila-Luna *et al.*[20] evaluated how chronic caffeine exposure for male Wistar rats aged 3–10 months affected behavioral and cognitive decline. Animals were assigned to either treatment or control groups. The treatment group received *ad lib* access to caffeine in their only source of drinking water (5 mg/kg per day). The control group received only tap water. Exposure to caffeine occurred for 6 months. Animals were tested in the elevated plus maze twice, once 1 week before caffeine treatment and once again 2 weeks after caffeine treatment was completed. Dependent measures included time spent in the open arms, closed arms, and center zone of the plus maze, as well as the number of entries made to the open and closed arms.

The authors found no significant differences in the amount of time animals spent in the open arms of the elevated plus maze, with animals in both the control and treatment groups spending less time in the open arms at 10 months of age compared to 3 months. In the open field test, animals in both the control and treatment group spent more time in the center of the open field at 10 months of age than at 3 months. These results suggested that chronic exposure to a low dose of caffeine did not produce significant increases in avoidance behavior.

Although other research designs may be used to study the effects of caffeine on avoidance behavior, it appears the most common designs are between-subjects controlled designs and controlled mixed research designs. These designs provide researchers with a controlled method for assessing the effects of several drugs within the same study. They also permit researchers to examine the effects of drugs on animal behavior over time.

Summary

We reviewed the effects of caffeine on avoidance behavior across a number of animal species. Common

species, methods of caffeine administration, dependent measures, and experimental designs were illustrated. Subjects' avoidance responses to known species-specific aversive stimuli, such as bright light, open space, and novel environments, as a function of caffeine, comprised the typical dependent measures. Caffeine administration has varied depending on species (e.g., zebrafish were administered caffeine more often through absorption from egg water, whereas rodents typically received injections or *ad lib* administration). Timing and frequency of administration varied according to whether the research question pertained to acute or chronic exposure. Behavioral tests for escape and avoidance as a function of caffeine consumption were conceptually similar across species, although the arrangement of the tests was necessarily adapted to the physiological needs of the different species. Caffeine administration preceding the presentation of aversive stimuli generally, but not exclusively, enhanced avoidance of aversive stimuli. Further modeling of chronic caffeine exposure and exposure to caffeine during aversive experiences is warranted, considering the parallels to trends in human caffeine consumption.

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