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REVIEW

Ionescu et al: Caffeine toxicity in zebrafish

Caffeine toxicity in zebrafish – Neurobehavioral changes, developmental defects, and oxidative stress: A review

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ABSTRACT

Caffeine is one of the most widely consumed psychoactive stimulants, primarily functioning as a non-selective adenosine receptor antagonist. Its increasing detection in wastewater and surface waters reflects extensive anthropogenic use. This review synthesizes evidence from zebrafish (Danio rerio)—a genetically tractable vertebrate with rapidly developing external embryos—to assess the impact of caffeine exposure across environmentally relevant (ng–µg/L) and pharmacological/toxicological (mg/L and above) concentrations on early development and neurobehavior. Behavioral studies indicate dose- and stage-dependent alterations in locomotion, anxiety-like responses, memory performance, and sleep patterns, suggesting disruptions in neural circuitry and stress-axis regulation. Biochemical analyses frequently reveal oxidative imbalances characterized by increased reactive oxygen species and lipid peroxidation, alongside changes in antioxidant defenses (e.g., glutathione levels, superoxide dismutase (SOD) activity, and glutathione reductase activity). These findings support oxidative stress as a potential mechanistic hub, although a causal relationship has yet to be established. Embryonic exposure to caffeine is associated with developmental toxicity, including delayed hatching and concentration-dependent malformations such as edema, axial deformities, impaired angiogenesis, and neuromuscular defects at higher doses. However, cross-study comparisons are hindered by variations in units, exposure durations, and assay protocols. In summary, caffeine disrupts behavior, redox homeostasis, and developmental processes in zebrafish, highlighting the necessity for standardized methodologies to identify stage-specific vulnerabilities.

Keywords: Caffeine, zebrafish, neurobehavioral alterations, oxidative stress, developmental toxicity.

INTRODUCTION

Caffeine is one of the most widely consumed psychoactive substances worldwide, naturally present in a variety of foods and beverages. Following oral intake, it is rapidly absorbed and distributed throughout the body, reaching peak plasma concentrations within 30–120 minutes [1]. The main dietary sources are coffee (robusta and arabica), tea, chocolate, and soft drinks, with coffee being the primary source for adults, while tea and sodas are more common among adolescents [2]. Besides natural occurrence, caffeine can be synthetically produced, with no molecular difference from the natural form, and is widely added to sodas and energy drinks. It is also found in medications for headaches, colds, and allergies, used in cosmetic treatments, and valued for its ergogenic effects in sports [3], being also consumed for its effects, including pleasant taste, enhanced focus, and greater physical vitality [4].

Part of daily routines worldwide, caffeine has attracted growing scientific interest as a bioactive molecule with both beneficial and adverse effects. It has been shown to in-fluence multiple systems, including the central nervous, urinary, digestive, and respiratory systems [3]. These findings reflect effects observed in humans and provide background on caffeine's systemic activity but are not used as evidence for zebrafish-specific outcomes.

Some studies indicate that caffeine may reduce levels of anxiety and depression, while other studies have shown that caffeine can enhance learning and memory in tasks where information is passively presented. It has also been found to improve performance in tasks that rely on working memory to some extent [5]. Moreover, research has shown that caffeine contributes significantly to protecting the brain against different forms of damage, such as neurotoxicity, seizures, and cognitive impairment being rapidly absorbed in the gastrointestinal tract and distributed throughout the body, including the brain [6]. However, these findings serve only as background and are not used as evidence for zebrafish developmental outcomes.

Its pervasive use has raised scientific interest due to its complex effects on multiple physiological systems, including the central nervous, cardiovascular, and digestive systems [7]. While moderate caffeine intake can enhance alertness, cognitive performance, and mood, high doses or chronic exposure have been

associated with neurobehavioral disturbances, oxidative stress and developmental toxicity [8–11].

Excessive caffeine consumption can cause health issues such as sleep disturbances, anxiety, hypertension, and gastrointestinal discomfort, emphasizing the need to tailor intake to individual tolerance [12]. Adverse effects of caffeine increase at high doses (9-13 mg/kg), although physical performance generally remains unaffected [13]. Intakes around 10-13 mg/kg have been associated with gastrointestinal disturbances, mental confusion, nervousness, difficulty concentrating, and sleep disruption in some individuals [14], while slightly lower doses (7-10 mg/kg) may cause chills, nausea, flushing, palpitations, headaches, and tremors [15]. Moderate doses (5-6 mg/kg) preserve ergogenic benefits while reducing, though not completely eliminating, negative side effects and physio-logical responses. Caffeine doses of 200 mg or higher can lead to toxicosis, presenting as restlessness, insomnia, muscle cramps, and periods of excessive alertness [16,17].

Besides being naturally present in coffee, tea, cacao, and other plants, caffeine is increasingly found in the environment due to widespread human use in beverages, personal care products, and pharmaceuticals. It has been detected in treated wastewater (55–304 μg/L), groundwater (0.01–0.68 μg/L), drinking water (3.39 μg/L), rainwater (5.4 μg/L), rivers (0.01–49.6 μg/L), and lakes (0.02–174 μg/L), posing potential risks to aquatic ecosystems [18,19]. Unlike humans, where caffeine exposure is largely dietary, aquatic organisms encounter environmentally relevant concentrations through wastewater and surface waters. Therefore, the zebrafish serves as a distinct model specifically for ecotoxicological and developmental toxicity assessments. This raises concerns about its effects on aquatic organisms, for which zebrafish provides a well-established and versatile model to explore both developmental toxicity and ecotoxicological outcomes. This model organism has become invaluable for investigating caffeine's neurotoxic and teratogenic effects due to their genetic similarity to humans, transparent embryos, and rapid development [20].

The zebrafish is a small freshwater vertebrate, native to the rivers of South Asia [21], typically 3–4 cm in length, with a lifespan of about two years, whose externally devel-oping, transparent embryos make it genetically accessible and highly

versatile as a model organism. Over the past few decades, it has gained popularity in scientific research for studying a wide range of biological processes, particularly [22] for investigating the neurotoxicity of drugs and environmental chemicals. There are many advantages to using zebrafish as an in vivo model, which allow for direct microscopic observation during early developmental stages. Furthermore, the rapid growth and high fecundity of zebrafish enable high-throughput toxicity testing of multiple chemicals [23,24].

Caffeine concentrations used in zebrafish studies span a wide range, reflecting differences in experimental objectives. Lower concentrations, typically in the μg/L range, are considered environmentally relevant, corresponding to levels detected in surface waters and wastewater. Moderate concentrations, generally in the mg/L range, are often chosen to approximate typical human consumption through beverages or foods. Higher concentrations, which may exceed 100 mg/L or 100 mg/kg in some studies, are primarily used to investigate dose-dependent toxicity, anxiogenic effects, or developmental malformations.

Zebrafish studies have shown that caffeine exposure can induce anxiety-like be-haviors, disrupt sleep patterns, impair memory and neuromuscular development, and cause structural malformations in embryos, highlighting dose-dependent and stage-specific effects [25–28]. Caffeine exposure during early zebrafish development can induce a range of dose-dependent alterations, including cardiac and yolk sac edema, bent tails, spinal curvature, and impaired neuromuscular formation. These structural mal-formations are often accompanied by behavioral deficits such as reduced locomotor activity, disrupted anxiety-like responses, and impaired memory, highlighting the sensitivity of developing embryos to oxidative stress and neurotransmitter imbalances triggered by caffeine [29,30].

Mechanistically, caffeine exposure has been linked to trigger oxidative stress, alter neurotransmitter signaling, and influence developmental pathways, making zebrafish a versatile model for linking molecular mechanisms to observable behavioral and mor-phological outcomes (Figure 1) [31,32].

Although numerous studies report both beneficial and adverse effects of caffeine, the underlying mechanisms and dose–response relationships in zebrafish remain challenging to compare due to methodological variability across studies. This

review argues that oxidative stress may represent a unifying mechanism linking caffeine's diverse neurobehavioral and teratogenic effects, yet current research has not established a clear causal relationship across different doses and developmental stages. Moreover, inconsistencies in reported effects on anxiety and memory in zebrafish likely reflect differences in experimental conditions, doses, and developmental stages. This review seeks to critically assess these variables, integrate the findings, and propose a structured framework for interpreting caffeine's dose-dependent neurobehavioral and developmental outcomes, providing guidance for future research directions.

Caffeine: Pharmacology and mechanisms of action

Caffeine is the most widely consumed stimulant and psychoactive substance, naturally present in more than 60 plant species such as coffee beans, cacao, and tea leaves. Also referred to as guaranine, theine, or mateine depending on its source, caffeine in the form of $C_8H_{10}N_4O_2$, chemically known as 1,3,7-trimethylxanthine, is a natural alkaloid from the methylxanthine group, often occurring alongside other bioactive compounds like polyphenols [6].

Structurally, caffeine is a heterocyclic organic compound with a purine base called xanthine, composed of a pyrimidine ring linked to an imidazole ring. It is considered a true alkaloid because of the heterocyclic nitrogen atom, although some authors classify it as a pseudo-alkaloid since its biosynthesis does not directly incorporate amino acids [33].

Caffeine has been shown to improve cognitive performance at low doses, by blocking adenosine receptors, which increases neuronal activity and the release of neurotransmitters such as dopamine and norepinephrine, as a result enhancing reaction times and the processing of visual information [34]. It also enhances visual processing by modulating neurotransmitter activity, particularly glutamate, which is critical for transmitting visual signals and improving reaction times [35].

Research indicates that these cognitive benefits can be observed at doses as low as 0.18 mg/kg, with the dose–response relationship plateauing at higher intakes. Furthermore, long-term caffeine consumption appears to induce tolerance, as habitual users often exhibit diminished or absent cognitive effects [17].

Furthermore, caffeine exerts its effects primarily as a non-selective antagonist of adenosine receptors, which include A1, A2A, A2B, and A3 subtypes. In the central nervous system, antagonism of A1 and A2A receptors reduces inhibitory signaling, leading to increased alertness, decreased fatigue, and enhanced cognitive performance through the indirect facilitation of neurotransmitter release, particularly dopamine and norepinephrine. These mechanisms also modulate mood, attention, and vigilance, while influencing sleep regulation [36,37] . Peripherally, caffeine affects cardiovascular and renal function: A1 receptor blockade in the heart increases heart rate, whereas antagonism of renal receptors enhances glomerular filtration and promotes diuresis. A2A receptor antagonism contributes to coronary vasodilation and can influence pain perception, which is relevant in the context of migraine management [36] . Beyond these effects, caffeine can activate lipase to promote fat breakdown, modulate muscle contraction to enhance strength, and stimulate gastric acid secretion and gastrin release, supporting digestive processes [38].

Epidemiological studies on humans further suggest that coffee intake is not associated with increased mortality; on the contrary, modest inverse associations have been described, linked to reduced inflammation, improved endothelial function, and a lower risk of type 2 diabetes [38,39] . Regular consumption appears to decrease susceptibility to low-density lipoprotein oxidation, thereby protecting against atherosclerotic plaque formation, while phenolic compounds such as chlorogenic and ferulic acids contribute significant antioxidant capacity. Protective effects have also been observed at the hepatic level, with reduced mortality in women with liver disease or cirrhosis and a lower risk of liver cancer. At the renal level, caffeine enhances natriuresis and diuresis through in-creased renal blood flow and reduced tubular sodium reabsorption, mechanisms com-parable to thiazide diuretics [38,40].

Alongside adenosine receptor antagonism, caffeine inhibits phosphodiesterase enzymes, elevating intracellular levels of cyclic adenosine monophosphate and cyclic guanosine monophosphate, thereby producing additional effects such as mild broncho-dilation, lipolysis, and modulation of intracellular signaling pathways. Following ab-sorption, caffeine is rapidly distributed throughout the body, metabolized primarily in the liver by cytochrome P450 enzymes, and excreted in the urine as multiple metabolites. Collectively, these central and peripheral actions underlie both the cognitive-enhancing and physiological effects of caffeine in humans.

Zebrafish, which possess conserved adenosine receptors and phosphodiesterase enzymes, provide a versatile model to investigate how these mechanisms influence neurobehavioral function, oxidative stress, and development, bridging observations from human studies to experimental insights [2,41].

ZEBRAFISH AS A MODEL ORGANISM IN NEUROTOXICOLOGY: ASSESSING ANXI-ETY, MEMORY AND SLEEP ALTERATIONS

The use of model organisms has been crucial for advancing both biological and medical sciences. These species are extensively studied to understand specific biological processes, with the expectation that insights gained from the model can be applied to other organisms, including humans [42].

Caffeine exerts neuroactive effects primarily through antagonism of adenosine receptors, which regulate neuronal excitability, sleep, and mood. In zebrafish, as in mammals, caffeine can induce anxiogenic behavior, disrupt sleep homeostasis, and alter circadian rhythms. These disruptions affect the hypothalamic-pituitaryinterrenal (HPI) axis, leading to elevated cortisol levels, which, depending on duration and intensity, can have adaptive or maladaptive consequences. Short-term caffeine exposure in zebrafish has been shown to increase anxiety, reduce exploratory behavior, elevate aggression, and impair HPI axis regulation. Long-term exposure further affects neurobehavioral functions, including swimming activity, social behavior, and memory, with high doses causing cognitive deficits that may persist or emerge during withdrawal [8,26,43,44].

Anxiety

Anxiety is a neuropsychiatric disorder that significantly impacts quality of life and remains a challenging medical issue. Studies indicate that caffeine can induce anxiety-related behaviors, with high caffeine exposure linked to increased anxiety levels in zebrafish [45].

Zebrafish exhibit robust anxiety-like behaviors in response to environmental stressors and display complex social interactions, making them ideal for studying neuropsychiatric disorders and pharmacological interventions [46] . Naturally, they exhibit anxiety when introduced to a novel environment. The Novel Tank Test (NTT) is commonly used to assess this behavior by measuring the fish's innate diving

response. This test has good face validity and construct validity, as anxiolytic drugs reduce the diving response while anxiogenic compounds exacerbate it [47].

Studies in zebrafish have used a broad range of caffeine concentrations, which fall into two distinct categories based on experimental intent:

(1) Environmentally relevant exposures

These occur in the ng/L–μg/L range, consistent with concentrations detected in surface waters. Such levels (typically up to ~10 μg/L) indicate trace contamination and are associated with subtle, chronic responses rather than overt toxicity [48,49].

(2) Pharmacological or toxicological paradigms

These employ mg/L concentrations, often in the 100–1000 mg/L range, which are orders of magnitude above environmental levels. Such exposures are used to investigate mechanistic responses, dose-dependent toxicity, or acute effects in zebrafish [50,51].

Caffeine has been shown to induce anxiety-like behaviors in zebrafish across developmental stages and experimental contexts. In larvae, exposure to laboratory doses of caffeine ranging from 100 to 300 mg/L altered locomotory activity in the Light-Dark Test (LDT), while exposure 100–1000 mg/L provoked bradycardia and increased mortality [51] . Fontana and Parker (2022) validated the Larval Diving Response (LDR) test in 7-day-old zebrafish larvae, showing that under a 30-minute exposure paradigm, increased time spent at the bottom of the tank reflects an anxiogenic response. Although this study used a 100 mg/L caffeine condition as part of the validation, it was not designed to investigate caffeine effects per se and serves solely to establish the behavioral assay [47]. Consistent with this paradigm, 7-day-old zebrafish larvae exposed to caffeine at concentrations up to 100 mg/L for 2 hours in a thigmotaxis-based assay exhibited anxiety-like behavior, spending more time along the edges of the tank, supporting the interpretation that caffeine acts as an anxiogenic agent in larval models [52]. In adult zebrafish, short-term exposure to environmental concentrations of caffeine (0.5–300 μg/L) led to reduced exploratory behavior and heightened stress responses in the NTT. These results suggest that even acute, low-level exposure can modulate anxiety-related behaviors, potentially through alterations in neurotransmitter signaling and stress-related pathways, highlighting possible ecological implications for aquatic populations [18] . Sex-specific differences have also been reported, with male and female zebrafish showing distinct anxiety responses

and physiological reactions under caffeine exposure, potentially due to differences in hormone levels, metabolism, and stress-axis regulation [19,45]. High caffeine intake (100 mg/kg) has also been associated with oxidative stress-mediated anxiety, which can be mitigated by antioxidants such as alpha-tocopherol [53]. Environmental and social contexts further modulate these effects, with altered anxiety-like behaviors observed depending on social stimuli. In a study on adult zebrafish, exposure to caffeine at 25 mg/L (low dose) and 60 mg/L (moderate dose) for 10 minutes modulated anxiety-like behavior depending on social context. Anxiety-like effects were observed as increased bottom-dwelling and freezing behavior, assessed using the NTT. Social behavior was evaluated separately with a Social Preference Test (SPT), showing reduced time spent near conspecifics at the higher dose. This study demonstrates that both anxiety and social responses to caffeine are influenced by dose and social context [28]. In another study on adult zebrafish, caffeine exposure for 10 minutes induced anxiety-like behavior, with increased bottom-dwelling and freezing observed in the NTT. Social behavior was also affected, as fish spent less time near conspecifics in the Social Preference Test, showing reduced social interaction [46] (Table 1).

Together, these findings indicate that caffeine acts as an anxiogenic agent in zebrafish, likely by modulating neurotransmitter systems such as dopamine, norepinephrine, and glutamate, and altering stress-axis activity. The effects are dose-dependent and vary with developmental stage, as demonstrated by increased bottom-dwelling and freezing behavior in the NTT and reduced social interaction in the SPT.

Memory

Zebrafish exhibit strong neuroanatomical and neurotransmitter similarities with humans, including homologues of the hippocampus, amygdala, and isocortex, as well as conserved glutamatergic, GABAergic, and cholinergic signaling. These features support complex neurobehaviors such as learning, memory retention, spatial and object recognition, and fear responses. As a result, zebrafish serve as a valuable vertebrate model for studying memory and cognitive function, including research on cognitive decline and drug discovery [54].

The impact of caffeine on memory in zebrafish is not uniform, but varies depending on dose, development stage, and experimental conditions. For instance,

juvenile zebrafish exposed to relatively high caffeine concentrations (20 and 50 mg/dL, equivalent to 200 and 500 mg/L) under unpredictable chronic stress for three days exhibited impairments in working memory when tested in the T-maze, indicating that caffeine may exacerbate stress-induced cognitive deficits [55]. In contrast, adult zebrafish exposed for 14 days to lower caffeine concentrations (10 and 50 mg/L) showed improved task performance, with the 10 mg/L group reaching the reward target faster and spending more time near it. However, these effects were accompanied by increased locomotor activity, suggesting that enhanced performance may reflect heightened arousal and attention rather than pure improvements in memory [56].

Taken together, these findings reveal that caffeine can exert both detrimental and beneficial effects on zebrafish cognition. Negative outcomes are more likely at higher doses, during developmental stages, under stress conditions, and when exposure is short, while beneficial outcomes appear at lower doses in adults during longer exposure periods [18]. Methodological difference, such as dose units, stress paradigms, and behavioral tasks, likely contribute to the apparent discrepancies [57]. Mechanistically, the biphasic effects of caffeine may reflect adenosine receptor antagonism, where low doses enhance attention and vigilance, but higher doses disrupt glutamatergic and GABAergic balance, stress hormone regulation, and even sleep-related processes that are critical for memory consolidation [58].

Sleep

Sleep is a fundamental and conserved feature of animal life, primarily serving brain functions such as energy replenishment and memory consolidation. Its timing and in-tensity are regulated by circadian rhythms and homeostatic sleep pressure, which reflects prior neuronal activity [59].

Studies in zebrafish have shown that increasing neuronal activity with arousing drugs like caffeine induces rebound sleep, independent of prior wake duration or physical activity, suggesting that sleep need is closely tied to overall brain activity [60]. The noradrenergic system, particularly the locus coeruleus, plays a key role in maintaining wakefulness and modulating sleep pressure, with changes in its activity influencing both arousal and subsequent sleep [61]. Animal models, including zebrafish, have greatly contributed to understanding sleep mechanisms. Unlike

nocturnal rodents, zebrafish exhibit diurnal sleep patterns similar to humans [62] . Their pineal gland is fully developed by 19-20 hours post-fertilization (hpf) and produces melatonin at night under circadian control [63]. Zebrafish larvae can display complex sleep behaviors as early as 4 days post-fertilization, and their small size allows for detailed monitoring using videography in a 96-well plate format. Given caffeine's known effects on sleep in humans, one study investigated its impact on sleep and behavior in zebrafish larvae. Exposure to caffeine at 31.25–120 μM for 48 hours disrupted normal sleep patterns, significantly reducing total sleep time and sleep efficiency. These results align with findings in adult zebrafish and other model systems, highlighting the potential relevance of caffeine-induced sleep disturbances for understanding human sleep regulation [27].

Caffeine has been shown to counteract cognitive deficits caused by sleep deprivation in adult zebrafish. This effect is mediated through activation of protein kinase A, which regulates O-linked β-N-acetylglucosamine cycling, highlighting a molecular mechanism by which caffeine can mitigate sleep-related memory impairments [64].

DEVELOPMENTAL ALTERATIONS INDUCED BY CAFFEINE IN ZEBRAFISH

The neurobehavioral deficits observed in adult zebrafish may originate from subtle developmental alterations induced by caffeine exposure during embryogenesis, where it is known to disrupt key morphological processes [65] . Studying developmental alterations in zebrafish is crucial because their rapid embryonic development, genetic similarity to humans, and transparent embryos allow for precise observation of morphological, cardiovascular, and neurobehavioral effects, making them an ideal model to investigate the potential toxicity and teratogenicity of compounds [24,66].

Previous studies have shown that caffeine can induce teratogenic and long-term neurodevelopmental effects in zebrafish embryos via oxidative stress-mediated apoptosis. In this study conducted by Felix et al., embryos (~2 hpf) were exposed to 0.5 mM caffeine, either alone or in combination with 24-epibrassinolide (24-EPI) at 0.01, 0.1, and 1 μM, for 96 hours. Caffeine exposure alone caused a significant increase in developmental mal-formations, including edema and tail curvature, as well

as locomotor deficits such as decreased speed and distance traveled, along with disrupted anxiety-like and avoidance behaviors [25]. High doses of caffeine have been shown to cause significant developmental toxicity in zebrafish embryos and larvae. Exposure starting at 2–24 hpf to concentrations ranging from 10 μM to 500 μM resulted in dose-dependent developmental defects, including low morphological scores affecting the notochord and heart, general malformations, reduced normal phenotypes, disrupted sleep rhythms, and altered locomotor activity. Wei et al. found that moderate concentrations (31.25–125 μM) exhibited minimal developmental defects, high survival rates, normal angiogenesis, and no inflammatory response, representing a relatively safe window for behavioral assays, whereas higher concentrations (250–500 μM) caused pronounced developmental defects and low morphological scores (notochord, heart), and very high concentrations (1000–2000 μM) induced severe developmental defects with high mortality. These findings suggest that caffeine exposure during early-life stages can severely impact structural development in zebrafish, while behavioral effects such as disrupted sleep rhythms and altered locomotor activity can be assessed safely within the 31.25–125 μM range, highlighting the importance of carefully considering dose and exposure time in neurodevelopmental and behavioral studies. [27] . Moreover, exposure of zebrafish embryos to caffeine at concentrations of 250–350 ppm caused significant developmental alterations, primarily affecting vascular formation, as suggested by Yeh et al., in their investigation. The embryos displayed abnormal development of intersegmental vessels, dorsal longitudinal anastomotic vessels, and subintestinal vein sprouting, indicating impaired angiogenesis [29] . Some findings suggested that exposure of zebrafish embryos to caffeine at concentrations ranging from 17.5 to 150 mg/L caused significant neuro-muscular and developmental alterations. At the highest concentration (150 mg/L), embryos exhibited reduced body length (2.67 ± 0.03 mm compared to 3.26 ± 0.01 mm in controls) and a marked decrease in touch-induced movement, dropping from 9.93 ± 0.77 in controls to 0.10 ± 0.06. Immunostaining revealed misalignment of muscle fibers and defects in primary and secondary motor axon projections, indicating impaired neuro-muscular development, in the study by Chen et al. These results demonstrate that caffeine at moderate to high doses disrupts normal motor function and neuromuscular formation in zebrafish embryos, highlighting its potential impact on early motor behavior and structural development [67] . Moreover, exposure to caffeine has been shown to affect early developmental

processes in zebrafish larvae. In the study by Chakraborty et al., 2011, caffeine treatment significantly increased heart rate, reaching 125–140 beats per minute, while simultaneously reducing the expression of vascular endothelial growth factor, a key regulator of angiogenesis. These findings suggest that high caffeine doses can disrupt normal vascular development and potentially lead to developmental defects in zebrafish embryos, highlighting the sensitivity of early developmental stages to chemical exposure [68] (Table 2).

OXIDATIVE STRESS AND ANTIOXIDANT RESPONSES

Oxidative stress arises when the production of reactive species exceeds the capacity of cellular defenses to neutralize them. Reactive oxygen species (ROS), such as superoxide anion and hydrogen peroxide, can generate highly reactive hydroxyl radicals in the presence of transition metals. Additionally, interactions between superoxide and nitric oxide produce reactive nitrogen species like peroxynitrite, which can also form •OH radicals [36].

Caffeine can influence oxidative stress in the brain by modulating ROS and neurotransmitter systems such as glutamate. While oxidative stress is known to contribute to neurobehavioral alterations, its role as a mechanism underlying caffeine-induced behavioral changes remains to be fully elucidated [53].

Coffee is considered one of the main dietary sources of antioxidant compounds, which can neutralize ROS, the primary contributors to oxidative stress [69] . While caffeine is often regarded for its antioxidant properties, some studies suggest that it can also act as a prooxidant under certain conditions In particular, the study by Gülçin, 2008 demonstrated that caffeine promoted linoleic acid peroxidation in emulsions at concentrations of 15, 30, and 45 µg/mL, resulting in oxidation levels of 32.5%, 48.9%, and 54.3%, respectively. These findings support the idea that, depending on the environment and concentration, caffeine may contribute to oxidative processes rather than exclusively neutralizing free radicals. Such dual behavior highlights the complexity of caffeine's biological effects and suggests that its role in oxidative stress may be context-dependent [70].

Research on coffee consumption and antioxidant effects has yielded mixed findings. The studies described above were conducted in humans, examining the effects of coffee consumption on plasma antioxidant capacity and endogenous

antioxidant enzymes. Given the limited research on these effects in zebrafish, investigating caffeine's impact on antioxidant defenses in this model is important for understanding underlying molecular mechanisms and developmental implications. Some studies have shown that coffee intake can significantly increase plasma antioxidant capacity [71–74]. For example, a single serving of 200-400 mL of coffee raised plasma antioxidant markers by 2-7% [72,73], although long-term interventions often showed inconsistent effects [71,74]. Chronic trials also examined endogenous antioxidant enzymes such as SOD, CAT, GPx, GSR, and GSTs, with some studies documenting increases up to 75% for SOD and around 60% for GPx [74]. In contrast, other studies found reduced enzyme activity [75], and results on glutathione (GSH) were also variable, with several studies reporting increases [76,77], while others found no effect [78,79]. Overall, coffee can enhance antioxidant defenses, but the extent of the effect depends on the type of coffee, dose, and study design.

Building on these human studies, several investigations have examined the effects of caffeine on oxidative stress and antioxidant defenses in zebrafish, providing experimental insight into molecular impact. In one study by Abdelkader et al., 2013 it was observed that prolonged exposure to caffeine induced oxidative stress in zebrafish embryos. This was evidenced by increased gene expression related to cell damage and apoptosis, as well as mitochondrial dysfunction. These findings suggest that caffeine exposure during early development stages can compromise cellular integrity and function in zebrafish embryos [80]. Moreover, another study conducted on zebrafish, the subjects were exposed for 28 days to caffeine concentrations ranging from 0.16 to 50 μg/L. The results showed increased activity of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione reductase (GRed) and a reduction in glutathione levels, while lipid peroxidation remained unchanged. Metabolic alterations were observed, with decreased LDH activity and higher lipid content, and at the highest doses (19.23 and 50 μg/L) a reduction in acetylcholinesterase activity suggested possible neurotoxic effects. These results suggest that caffeine exposure can disrupt oxidative balance and metabolic function in zebrafish even at low, environmentally relevant concentrations, highlighting the importance of dose and developmental stage in determining its toxicological impact [81]. Another study conducted on zebrafish demonstrated that high doses of caffeine induce oxidative stress in the brain, evidenced by a significant increase in lipid

peroxidation, measured as elevated MDA levels. Interestingly, treatment with the antioxidant alpha-tocopherol prevented this biochemical alteration and also reduced anxiety-like behaviors induced by caffeine, suggesting that oxidative stress plays a key role in mediating the neurobehavioral effects of caffeine in zebrafish [53] (Table 3).

Although our findings demonstrate that caffeine exposure in zebrafish induces a range of developmental abnormalities and oxidative stress responses, the molecular mechanisms underlying these effects remain only partially understood. To better contextualize the malformations and redox imbalance observed in our model, further research is needed in mechanistic domains such as oxidative stress pathways, mitochondrial dysfunction, intrinsic and extrinsic apoptotic signaling, and the cAMP–PKA–CREB axis. These molecular processes may provide a clearer explanation for how caffeine disrupts early development and cellular homeostasis in zebrafish and potentially in other vertebrate systems.

Some cellular experiments indicate that caffeine can promote apoptosis through processes linked to oxidative imbalance. For instance, exposure to caffeine has been reported to disrupt key survival pathways such as Ras, Akt, and ERK, and these alterations can be reduced by antioxidant treatment or by blocking proteasomal activity. Such findings point toward a contribution of oxidative stress and protein degradation systems to caffeine-induced apoptotic responses [82].

Other evidence, however, suggests that caffeine may enhance mitochondrial performance in contexts of mitochondrial deficiency. Certain models show that caffeine administration can improve respiratory capacity, ATP synthesis, and respiratory control ratio (RCR) [83] . Similarly, renal cell studies have documented increases in mitochondrial membrane potential and ATP levels after caffeine exposure, implying that mitochondrial energetics may be modulated in a cell-specific manner [84].

Caffeine’s influence on second-messenger pathways, particularly those governed by cAMP–PKA, also warrants further exploration. In endothelial cells, caffeine has been shown to elevate cAMP, activate PKA, and trigger AMPK-dependent mitochondrial fragmentation, a process associated with altered cellular dynamics and mitochondrial function [85].

These observations raise the possibility that cAMP–PKA–CREB signaling acts as a central mediator linking caffeine exposure to downstream metabolic or apoptotic changes.

CONCLUSION

Evidence from zebrafish studies highlights that caffeine exerts complex, dose-dependent neurobehavioral effects, particularly on anxiety, memory, and sleep. At low doses, caffeine may enhance attention and memory-related performance, but higher concentrations consistently induce anxiety-like behaviors, reduce exploratory activity, and disrupt normal social interactions. Sleep regulation is also profoundly affected, with larval zebrafish exposed to caffeine showing reduced total sleep time and efficiency, while in adults, caffeine can temporarily mitigate memory impairments caused by sleep deprivation through molecular pathways such as protein kinase A activation. Taken together, these findings support the central thesis of this review: caffeine's effects are strongly dose- and stage-dependent, with oxidative stress frequently implicated as a potential unifying mechanism, high-lighting both its potential benefits and toxic risks across development.

Beyond behavioral outcomes, caffeine exposure during early zebrafish development produces clear morphological and structural alterations. Embryos and larvae exposed to moderate and high doses exhibit dose-dependent malformations including cardiac and yolk sac edema, spinal curvature, impaired angiogenesis, and neuromuscular defects. These alterations not only compromise survival but also translate into long-term functional deficits, such as reduced locomotor activity and impaired anxiety or avoidance behaviors. Although several studies suggest that these teratogenic outcomes may involve caffeine-induced mitochondrial dysfunction and increased production of reactive oxygen species, oxidative stress is best viewed as a plausible, but not yet definitively proven, unifying mechanism. Such findings emphasize the heightened vulnerability of embryonic and larval stages to caffeine and position zebrafish as a sensitive model for developmental neurotoxicology.

Mechanistically, many of these effects are mediated through oxidative stress pathways. Caffeine can act as both an antioxidant and a prooxidant, with studies in zebrafish showing upregulation of oxidative stress-related genes, mitochondrial dysfunction, lipid peroxidation, and reductions in glutathione levels. At the same time,

compensatory activation of antioxidant enzymes such as SOD and GRed suggests an adaptive response to redox imbalance. Importantly, antioxidant supplementation, such as alpha-tocopherol, has been shown to mitigate both oxidative stress and caffeine-induced anxiety behaviors, supporting—but not conclusively establishing—a mechanistic link between redox regulation and neurobehavioral outcomes. Overall, zebrafish studies indicate that caffeine's influence on anxiety, memory, sleep, and development is tightly interconnected with its oxidative stress-modulating properties, highlighting the importance of dose, exposure time, and developmental stage in determining its beneficial versus detrimental effects.

Despite substantial progress, key questions remain unresolved. While associations between oxidative stress and anxiety, memory, sleep, and teratogenic outcomes are increasingly reported, the specific neural circuits, cell populations, and molecular targets most vulnerable to caffeine in zebrafish remain largely unmapped. The long-term consequences of early-life caffeine exposure, including potential persistent behavioral and physiological deficits, are poorly understood. Additionally, interactions with antioxidant interventions such as NAC, sex-specific responses, and the distinction between environmentally relevant versus pharmacological dosing require further clarification.

Despite the growing number of studies examining caffeine's effects in zebrafish, several limitations constrain the generalizability and comparability of existing findings.

First, assay heterogeneity remains a major challenge: behavioral, biochemical, and developmental assays differ widely in sensitivity, endpoints, and methodological execution, making direct comparisons difficult.

Second, dose selection and exposure paradigms are highly variable across studies, ranging from environmentally relevant concentrations to pharmacological or supra-environmental doses, and often differing in exposure windows, renewal frequency, and developmental timing.

Third, sex is rarely reported or controlled for, particularly in behavioral assays, even though sex-specific differences are well documented in stress- and anxiety-related responses.

Fourth, social and environmental context, including housing density, group vs. individual testing, and tank conditions, can strongly influence zebrafish behavior but is inconsistently documented.

Finally, the field may be influenced by reporting and publication biases, with positive or dose-dependent effects more likely to be published than null or contradictory results. Together, these factors highlight the need for more standardized experimental designs and transparent reporting practices to improve reproducibility and to better define caffeine's developmental and neurobehavioral effects across studies.

Future research should adopt mechanistic and integrative approaches to clarify caffeine's effects. For instance, CRISPR/Cas9 or other genetic tools could target key antioxidant-related genes (e.g., Nrf2) to directly test whether oxidative stress plays a causal role in caffeine-induced teratogenesis. Longitudinal studies tracking zebrafish from embryo-genesis through adulthood are critically needed to determine the persistence and reversibility of neurobehavioral and developmental deficits. Standardized, dose-dependent experimental designs integrating behavioral, morphological, and molecular endpoints will help distinguish subtle environmentally relevant effects from overt pharmacological or toxic outcomes.

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TABLES AND FIGURES WITH LEGENDS

Table 1. Summary of caffeine-induced anxiety-like behaviors in zebrafish: Stages, doses, exposure durations, assessment methods, and references

Zebrafish stage	Caffeine dose	Exposure route	Exposure period	Sample size (*n*)	Sex	Anxiety effects	Test(s) used	References
Larvae (4dpf)	100–300 mg/L (locomotor effects), 100–1000 mg/L (bradycardia and mortality) (≈515–5150 µM)	Immersion	4 h	Not reported	Not reported	- altered locomotor activity in LD cycles - bradycardia - higher mortality	LDT	[51]
Larvae (7dpf)	10-100 mg/L (≈52–515 µM)	Immersion	2 h	300 (80 per group)	Not reported	-increased edge-preference (thigmotaxis) -reduced bottom-dwelling	Thigmotaxis-based“edge preference” assay	[47]

						avoidance -altered locomotor activity		
Adults	0.5, 1.5 and 300 µg/L (≈0.0026, 0.0077, and 1.54 µM)	Immersion	7 days	12 per group	Mixed (males + females)	- reduced exploratory behavior - enhanced stress responses	NTT	[18]
	100 mg/L (≈515 µM)	Immersion	6 min	Not reported	Mixed (males + females)	- males showed heightened anxiety-like behaviors in response to caffeine - females exhibited stronger alarm responses to conspecific alarm substance and aversion to predator sight	NTT	[19]
	0.3 mg/L to 600 mg/L (≈1.54–3089 µM)	Immersion	Acute	Not reported	Mixed (males + females)	-males exhibited more erratic and chaotic swimming patterns, reflecting stress-induced	NTT	[45]

						anxiety behaviors - females showed a longer latency to explore the upper zone of the tank, and freezing behavior, indicating heightened anxiety-like responses		
	100 mg/kg (systemic dose)	Dietary	30 min	166 (10-12 per group)	Not reported	- anxiety-like behaviors such as increased thigmotaxis (preference for the periphery of the tank) - freezing behavior - erratic swimming.	NTT	[53]
	25 mg/L and 60 mg/L (≈129–309 μM)	Immersion	10 min	Not reported	Mixed (males + females)	- increased bottom-dwelling - increased freezing behavior	NTT; SPT	[28]
	20 and 100 mg/L (≈103–	Immersion	10 min	Not reported	Not reported	- mild to pronounced increase in bottom-	NTT; SPT	[46]

	515 μM)					dwelling and freezing behavior, indicating heightened anxiety - reduced time spent near conspecifics, indicating decreased social interaction.		

Abbreviations: dpf: Days post-fertilization; LD: Light–dark (cycles); LDT: Light–dark test; NTT: Novel tank test; SPT: Social preference test; LDR: Larval diving response.

Table 2. Summary of caffeine-induced developmental effects in zebrafish embryos and larvae: Stages, doses, exposure durations, developmental defects, and references

Zebrafish stage	Caffeine dose	Exposure route	Exposure period	Sample size (*n*)	Sex	Developmental alterations	References
Embryos (2 hpf)	0.5 mM (≈500 µM)	Immersion	96 h	Not reported	Not reported	- increased malformations including edema, tail curvature - locomotor deficits	[25]
Embryos (2hpf)	31.25–2000 µM	Immersion	24-72 hpf	30 per concentration (developmental assays); 24 per group (behavioral assays)	Not reported	- minimal developmental defects, high survival rates, normal angiogenesis and no inflammatory response (31.25–125 µM) - pronounced developmental defects, low morphological scores (notochord, heart) (250–500 µM) - severe developmental defects, high	[27]

						mortality (1000–2000 μM)	
Embryos	250–350 ppm (≈1288–1802 μM)	Immersion	Not reported	Not reported	Not reported	- abnormal intersegmental vessels - dorsal longitudinal anastomotic vessels - subintestinal vein sprouting	[29]
Embryos	17.5, 35, 50, 100 and 150 mg/L (≈90–772 μM)	Immersion	Not reported	Not reported	Not reported	- reduced body length - decreased touch-induced movement - misaligned muscle fibers - defective motor axon projections	[67]
Larvae	10, 20, 50, and 100 μg/mL (≈52–515 μM)	Immersion	Not reported	Not reported	Not reported	- increased heart rate (125–140 bpm) - potential vascular developmental defects	[68]

Abbreviations: hpf: Hours post-fertilization; bpm: Beats per minute.

Table 3. Summary of research examining the impact of caffeine on oxidative stress and antioxidant responses in zebrafish

Zebrafish stage	Caffeine dose	Exposure route	Exposure time	Sample size (*n*)	Sex	Oxidative stress marker	Oxidative/Antioxidant effects	References
Embryos	100 µM	Immersion	24-96 hpf	Not reported	Not reported	HSP70 ↑ Cyclin G1 ↑ Bax/Bcl-2 ratio ↑	↑ Gene expression related to cell damage and apoptosis; mitochondrial dysfunction - oxidative stress during early development	[80]
Adult	0.16, 0.42, 1.09, 2.84, 7.40, 19.23, and 50 µg/L (≈0.0008–0.26 µM)	Immersion	28 days	15 per group	Not reported	SOD ↑ GRed ↑ GSH ↓ AChE ↓	↑ Antioxidant enzymes (SOD, GRed), ↓ glutathione, lipid peroxidation unchanged metabolic alterations (↑ lipid content); High doses (19.23 and	[81]

							50 µg/L) ↓ acetylcholinesterase, suggesting neurotoxicity	
Adult	100 mg/kg (systemic dose)	Dietary	Not reported	166 (10-12 per group)	Not reported	MDA ↑	↑ Lipid peroxidation in brain - antioxidant alpha-tocopherol prevented oxidative stress	[53]

↑ up-regulation/increase; ↓ down-regulation/decrease. Abbreviations: hpf: Hours post-fertilization; HSP70: Heat shock protein 70; SOD: Superoxide dismutase; GRed: Glutathione reductase; GSH: Reduced glutathione; AChE: Acetylcholinesterase; MDA: Malondialdehyde; LDH: Lactate dehydrogenase.

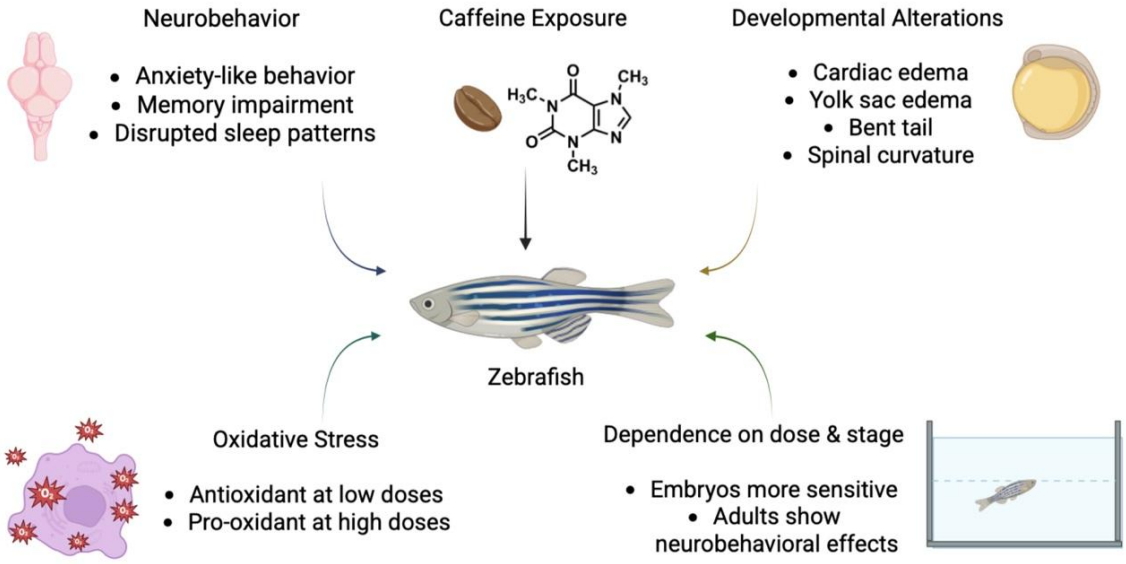

Figure 1. Conceptual schematic summarizing reported associations between caffeine exposure and dose- and stage-dependent outcomes in zebrafish. The diagram links caffeine exposure to neurobehavioral alterations and developmental alterations. Oxidative stress is highlighted as a contributing factor, depicted as antioxidant at low doses and pro-oxidant at high doses. The figure also emphasizes dependence on dose and developmental stage, indicating that embryos are more sensitive and that adults show neurobehavioral effects. *Created partially in BioRender.*