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The buzz on caffeine in invertebrates: effects on behavior and molecular mechanisms

Julie A. Mustard

School of Life Sciences, Arizona State University, Tempe AZ 85287 USA

Abstract

A number of recent studies from as diverse fields as plant-pollinator interactions, analyses of caffeine as an environmental pollutant, and the ability of caffeine to provide protection against neurodegenerative diseases have generated interest in understanding the actions of caffeine in invertebrates. This review summarizes what is currently known about the effects of caffeine on behavior and its molecular mechanisms in invertebrates. Caffeine appears to have similar effects on locomotion and sleep in both invertebrates and mammals. Furthermore, as in mammals, caffeine appears to have complex effects on learning and memory. However, the underlying mechanisms for these effects may differ between invertebrates and vertebrates. While caffeine's ability to cause release of intracellular calcium stores via ryanodine receptors and its actions as a phosphodiesterase inhibitor have been clearly established in invertebrates, its ability to interact with invertebrate adenosine receptors remains an important open question. Initial studies in insects and mollusks suggest an interaction between caffeine and the dopamine signaling pathway; more work needs to be done to understand the mechanisms by which caffeine influences signaling via biogenic amines. As of yet, little is known about whether other actions of caffeine in vertebrates, such as its effects on GABA_A and glycine receptors, are conserved. Furthermore, the pharmacokinetics of caffeine remains to be elucidated. Overall behavioral responses to caffeine appear to be conserved amongst organisms; however, we are just beginning to understand the mechanisms underlying its effects across animal phyla.

Keywords

Drosophila; honey bee; insect; methylxanthine; adenosine receptor; ryanodine receptor

Caffeine may be the most widely consumed neuroactive compound on the planet, and it might not just be humans using it for a buzz. Caffeine can be synthesized by a number of species of plants, including those used to make coffee, tea and maté [1]. Compounds such as caffeine and nicotine in plants have generally been regarded as defense mechanisms to reduce damage from herbivores. Thus, it is somewhat surprising to discover that caffeine also appears in the nectar from a number of species of plants [2–4], and recent work suggests plants may be using caffeine to manipulate the behavior of their invertebrate pollinators [5, 4]. These results suggest complex interactions between the caffeine produced by plants and the invertebrates that interact with them that may affect pollination levels and influence the coevolution of plants, pollinators and herbivores.

Given its popularity, it is not surprising that caffeine occurs as a significant pollutant in wastewater. Even after sewage treatment, caffeine may be released into aquatic environments and present in the sludge, which may then contaminate soils via its use as a

fertilizer [6, 7]. Although these concentrations are generally low, in the nanomolar range for treated effluent, little is known about the chronic effects of caffeine on aquatic invertebrates. Recent studies using environmentally relevant concentrations have shown chronic caffeine exposure may be deleterious. Caffeine treatment leads to upregulation of Hsp-70 in the mussel *Mytilus californianus* [8] and to destabilization of lysosomal membranes in the clam *Ruditapes philippinarum* and the crab *Carcinus maenas* [9, 10], two indicators of cellular stress. Increasing our knowledge regarding how caffeine affects aquatic organisms, especially chronic exposure, is important for assessing the risks associated with caffeine contamination of the environment.

In addition, a number of studies show that caffeine consumption provides protection against neurodegenerative diseases, such as Parkinson's and Alzheimer's, and dementia [11–14]. However, little is known about the molecular mechanisms by which caffeine is providing protection. Invertebrate models have provided invaluable insight on the mechanisms through which drugs such as ethanol and cocaine affect the nervous system (for recent reviews see [15, 16]). For example, forward genetic approaches in the nematode, *Caenorhabditis elegans*, and the fruit fly, *Drosophila melanogaster*, have identified new targets for the actions of ethanol that were then verified as involved in responses to ethanol in mammals [17–19]. In addition, reverse genetic approaches using data from human or mouse genome wide studies have shown that genes of interest can be studied in the simpler systems provided by invertebrates [20, 21]. A greater understanding of the mechanisms by which caffeine acts in invertebrates would allow the extensive genetic, behavioral and neurophysiological tools available in invertebrates to be used to examine the relationship between caffeine and neurodegeneration.

Thus, improving our understanding of the actions of caffeine is of growing interest from both an ecological and health perspective. The goal of this review is to provide an overview of what is known about the effects of caffeine on invertebrates and to highlight current questions.

Effects of caffeine on invertebrate behavior

Locomotion and sleep

In mammals, where its actions have been extensively studied in humans and rodent models, caffeine consumption is associated with increases in activity and alertness. Similarly, caffeine has been shown to increase locomotor activity in a variety of insects including hornets, *Vespa orientalis* [22], honey bees, *Apis mellifera* [22], the green scale insect, *Coccus viridis* [23] and flour beetles, *Tribolium castaneum* and *Tribolium confusum* [24, 25]. However, caffeine was also shown to inhibit swimming behavior in the jellyfish, *Aurelia aurita* [26], although the concentrations used in this case were very high.

The most detailed studies of caffeine on locomotion come from the analysis of its effects in the fruit fly where caffeine acts to increase activity, disrupt sleep patterns and increase the amount of time flies spend awake [27–31]. Sleep behavior in flies has many of the characteristics of sleep in mammals including circadian and homeostatic regulation, and rebound effects after sleep deprivation [28, 30]. Both chronic and acute exposure to caffeine lead to a dose-dependent decrease in amount of time flies spent asleep during the night [27, 31]. Sleeping flies that have consumed caffeine are more likely to be woken by mechanical stimulation suggesting caffeine consumption leads to a higher level of arousal [31]. Furthermore, chronic consumption of caffeine led to a lengthening of the circadian period [31]. These results are consistent with the effects of caffeine consumption in humans, which can reduce the amount of time spent sleeping and affect how much time is spent in different sleep stages [32]. Indeed, the parallels between the effects of caffeine on sleep in mammals

and the “sleep-like” behavior in flies was used to argue that a similar mechanism underlies sleep in flies and humans, and adds support to the idea that sleep is an important behavior conserved across animal phyla.

Learning and memory

In humans, whether or not caffeine acts as an actual cognitive enhancer beyond simply increasing alertness is unclear [33, 34]. However, there is clear evidence that caffeine affects learning and memory in invertebrates. When *Drosophila* are exposed to caffeine in their food for 20 hrs or more before conditioning, there is a significant reduction in their ability to associate a visual cue with an aversive stimulus [35, 36]. Similarly, honey bees fed an acute dose of caffeine, either in the reward solution during conditioning or prior to conditioning, showed a reduced level of acquisition during appetitive olfactory conditioning. However, the caffeine did not affect levels of recall during a 24 hr memory test, suggesting that the honey bees had actually learned the association between odor and reward, even though they did not respond during the conditioning phase [37]. In contrast, a number of studies also suggest that caffeine increases learning in invertebrates. Honey bees fed caffeine in the reward solution performed better during massed olfactory training than those fed sucrose alone [4]. In addition, honey bees given caffeine performed better at visual learning using a delayed-match-to-sample learning paradigm [38]. In the common snail, *Helix lucorum*, injection of caffeine, either before or after a conditioning trial, increased the number of positive responses (closing of the pneumostoma) to the conditioned stimulus (tapping on the shell) during training such that the rate of acquisition was increased in snails given caffeine [39].

What might account for these different effects of caffeine on learning? One possible explanation is differences in the concentration of caffeine used. The experiments that resulted in a negative effect of caffeine on learning [35, 37, 36] used relatively high concentrations (10 – 50 mM) of caffeine, whereas the studies that showed positive effects on learning [39, 4] used lower concentrations (0.1 to 100 μ M). In the visual learning experiments done in honey bees [38], a dose of 100 mM caffeine was given via the use of dimethylformamide to carry the compound across the cuticle, but the proportion of caffeine that actually crossed in to the hemolymph is unknown making it difficult to compare these results. Another interesting factor is that, at least in honey bees, the studies that revealed a positive effect of caffeine were more difficult tasks. Difficult tasks are those that generally require more training trials to achieve the same level of response. For example, consumption of 100 μ M caffeine in the reward during spaced training had no effect on learning whereas caffeine at this concentration enhanced learning during massed training [37, 4]. Similarly, caffeine had no effect on learning during a Y-maze visual learning task, but the same concentration was shown to increase learning during the more difficult delayed-match-to-sample task [38]. This might suggest that the effects of caffeine on learning are somewhat subtle, and that more difficult learning tasks may be required to reveal caffeine’s influence.

There is also evidence that caffeine enhances recall of learned associations. Honey bees fed caffeine in the reward solution during massed olfactory training performed better on 24 hr recall tests than those fed sucrose alone [4]. Furthermore, honey bees given caffeine and conditioned to a visual task using a Y-maze had better long term memory recall [38]. Work from Perisse et al. suggests that calcium signaling might play an important role in caffeine’s effect on long term memory [40]. In the honey bee, a single pairing of an odor with a sucrose reward does not produce a robust long term memory. However, bees injected with caffeine before conditioning showed significantly higher levels of recall for a long term memory task than control bees. Interestingly, this effect was mimicked by the uncaging of calcium just prior to conditioning, suggesting that caffeine’s effects on intracellular calcium may play a significant role in enhancing memory [40].

What are the molecular mechanisms through which caffeine acts?

In *Drosophila*, increases in the concentration of cAMP were observed in the brain in response to caffeine consumption [36] or when caffeine was directly applied to the brain [31]. In addition, direct application of caffeine to the brains of honey bees results in an increase in intracellular calcium levels [40, 41]. However, exactly how caffeine causes increases in cAMP and calcium in invertebrates is still unknown. In vertebrate systems, caffeine has been shown to act through several mechanisms including increasing cAMP through the inhibition of phosphodiesterases, increasing intracellular calcium levels via release of intracellular stores through ryanodine receptors and as an antagonist at adenosine receptors (Figure 1). Because caffeine acts as an antagonist at A₁ and A_{2A} receptors at much lower concentrations than its other targets, it is expected that interactions with adenosine receptors is the main mechanism for caffeine's action in mammals [42]. The mechanisms of caffeine's action in invertebrates are much less clear.

Binding of caffeine to ryanodine receptors

Ryanodine receptors are ligand gated calcium channels that play important regulatory roles in many processes by releasing calcium from intracellular stores. Binding of caffeine to the receptor increases the affinity of the receptor for Ca²⁺, leading to activation of the channel at lower Ca²⁺ levels [43]. Whereas mammals have three genes encoding ryanodine receptors with distinct expression patterns and functional characteristics, invertebrates appear to only have one ryanodine receptor gene [44, 45]. Studies in nematodes [46], insects [47–49], mollusks [50], sea urchins [45] and crustaceans [51, 52] have shown that caffeine also interacts with invertebrate ryanodine receptors to release intracellular calcium stores. The concentrations of caffeine used in these studies are in the millimolar range (0.5 – 30 mM), similar to the concentrations of caffeine needed for calcium release via ryanodine receptors in mammals [42].

Caffeine inhibits phosphodiesterases

Caffeine also works as an inhibitor of phosphodiesterases. Cyclic nucleotide phosphodiesterases (PDE) act to regulate cGMP and cAMP levels by hydrolyzing them to 5'-GMP or 5'-AMP, respectively. Both mammals and invertebrates have multiple genes for PDEs, and post-transcriptional mechanisms lead to a variety of protein isoforms. Perhaps the best known member of this protein family is the cAMP PDE II encoded by the *dunce* gene in *Drosophila* [53]. Given the well established role of the Dunce protein in learning [54], it might be expected that as an inhibitor of PDEs, caffeine would also affect learning. In fruit flies, treatment with the PDE inhibitor IBMX mimics the effects of caffeine on sleep, and blocking cAMP signaling prevents caffeine from having an effect [31]. Studies on purified enzymes and cell homogenates demonstrated that caffeine acts as a competitive inhibitor of PDEs from a number of insects [55–62]. Similar to the case with the ryanodine receptors, the concentration required for 50% inhibition of PDE activity by caffeine was in the millimolar range, from 0.5 to 10 mM. Although it is difficult to determine which specific form of PDE was being assayed in these studies, the overall trend suggests that concentrations above 0.5 mM would be required for significant inhibition of PDEs by caffeine.

Does caffeine act at invertebrate adenosine receptors?

Antagonism of adenosine receptors is believed to be the main molecular pathway for the effects of caffeine in mammals, as caffeine's affinity for adenosine receptors suggests it may affect adenosine receptors at biologically relevant concentrations. For comparison, the caffeine concentration to which human neural tissues are exposed after consumption of a cup of coffee is estimated to be in the range of 1 to 10 µM [42], a much lower level than the

caffeine concentrations associated with inhibition of phosphodiesterases or release of calcium via ryanodine receptors. Several adenosine receptor genes have been cloned from vertebrates with the receptors falling into four receptor families: A₁, A_{2A}, A_{2B}, and A₃. Caffeine binds with K_D values in the micromolar range to all four of the human adenosine receptors, although the A₁ and A_{2A} receptors are the most likely targets for the actions of caffeine [42]. The A₁ and A₃ receptors are coupled to G_i leading to a reduction in cAMP levels, whereas the A_{2A} and A_{2B} receptors interact with G_s leading to the activation of adenylyl cyclase. A number of other G proteins may interact with the adenosine receptors as well leading to tissue specific signaling cascades [63].

A number of pharmacological studies suggest that caffeine also interacts with adenosine receptors in invertebrates. Antagonists of mammalian A₁ and A₂ adenosine receptors mimic the behavioral effects of caffeine on sleep in *Drosophila* [27], whereas an A₁ receptor agonist acts in the opposite manner as caffeine by increasing the amount of time flies spend sleeping [28]. Whole cell recordings from honey bee Kenyon cells in the mushroom bodies also showed that the effects of caffeine were mimicked by a mammalian adenosine receptor antagonist [4]. In the mussel, *Mytilus californianus*, treatment with caffeine causes rounding up and a loss in the adhesion of the hemocytes that hold the mussel to the substratum; this effect of caffeine was reduced by the addition of adenosine or A₂ receptor agonists suggesting that the loss of adhesion was due to the interaction between caffeine and an adenosine receptor [64].

To date, adenosine receptors have only been cloned and functionally characterized from two invertebrates: the DmelAdoR receptor from *Drosophila* [65, 31] and the PminAdoR receptor from starfish [66]. Neither of these receptors have sequences that are closely conserved with the vertebrate adenosine receptor families (Figure 2), with the vertebrate A₂ receptor family being the most closely related to both the fly and starfish receptors. Interestingly, the fly and starfish receptors also share little sequence similarity between them (BLAST E value = 10⁻⁴⁴, with a max identity of 36%). When heterologously expressed in a Chinese hamster ovary cell line, stimulation of DmelAdoR with adenosine leads to increased levels of cAMP and Ca²⁺ [65]. However, the DmelAdoR receptor is endogenously expressed in a *Drosophila* neuroblast Bg2-c2 cell line, and treatment of these cells with adenosine leads to an increase in cAMP, but not calcium levels [67]. Thus, it is unclear whether or not the DmelAdoR receptor signals via Ca²⁺ under native conditions. Interestingly, in Bg2-c2 cells, caffeine does not antagonize the adenosine dependent increase in cAMP [67]; furthermore, caffeine still affects sleep behavior in DmAdoR knockout flies [31]. In contrast to the pharmacological studies, these results suggest that caffeine may not act through adenosine receptors in invertebrates. One possible explanation for the conflicting results between the pharmacological and DmelAdoR studies is that another adenosine, or other type of caffeine sensitive receptor, is present in invertebrates. Further support for there being another adenosine receptor type in invertebrates comes from studies on the actions of adenosine in pond snail [68] and blowfly [69] where the pharmacology of the adenosine receptors involved does not match the pharmacological profile for the DmelAdoR receptor [67].

Interactions between caffeine and dopamine signaling

In mammals, caffeine has been shown to interact with the dopamine signaling pathway via three mechanisms: direct interactions between dopamine receptors and adenosine receptors, convergence of dopamine signaling pathways and adenosine signaling pathways on common second messengers (such as cAMP), and treatment with caffeine leading to dopamine release in the brain (for reviews see [70, 71]). Although little is known about the interaction between caffeine and dopamine signaling in invertebrates, several studies suggest that caffeine may act to influence dopamine signaling via one or more of these mechanisms. In

the central ganglion of the pond snail, *Planorbis corneus*, caffeine potentiated the inhibitory postsynaptic potentials produced by a dopaminergic neuron [72]. This effect was mimicked by treatment with dibutyryl cAMP, suggesting that this effect was due to caffeine's ability to raise cAMP levels, which could be due to its action as a phosphodiesterase inhibitor, its antagonism of adenosine receptors or via other mechanisms. Caffeine treatment of honey bees led to an increase in mRNA expression levels of a D2-like dopamine receptor [73] suggesting an interaction between caffeine and dopamine receptors. In addition, expression of a D1-like dopamine receptor is necessary for the reduction in sleep due to caffeine consumption in *Drosophila* [27]. Taken together, these results support the hypothesis that caffeine interacts with the dopamine signaling pathway in invertebrates, but more studies are necessary to determine the underlying mechanisms.

Other possible mechanisms

Several other actions for caffeine have been proposed including the blockade of GABA_A receptors [74–76], acting as a competitive inhibitor of glycine receptors [77, 76] and inhibition of acetylcholinesterase [78]. However, little information about the interactions between these targets and caffeine is known for invertebrates.

Caffeine pharmacokinetics

There is a dearth of information on the absorption and metabolism of caffeine in invertebrates. In mammals, caffeine is rapidly absorbed from the stomach and the gut, and it moves freely into fluids and tissues, including passage through the blood – brain barrier and the placenta [79]. Very little caffeine is excreted unchanged, instead it is metabolized to a number of different compounds, including theobromine, theophylline and paraxanthine [79]. The ratio of the compounds into which caffeine is converted varies greatly even amongst mammals [80]. This is of particular interest as several of these metabolites are also biologically active, for example theophylline is a more potent inhibitor of mammalian adenosine receptors than caffeine [42]. Thus, determining the metabolic pathways for caffeine is necessary for a true understanding of its actions in invertebrates. A few steps in this direction have been made. In mammals, caffeine metabolism is mainly dependent on the cytochrome P450 family member CYP1A2 [81]. Initial studies in *Drosophila* [82, 83] and honey bees [73] show that genes for members of the cytochrome P450 family (CYP proteins) are upregulated in the presence of caffeine. In general, studies in invertebrates have assumed that caffeine will also move throughout the tissues, including passage in to the brain. However, information regarding the absorption, tissue distribution and metabolites of caffeine in invertebrates represents a significant gap in our understanding.

Future considerations

Clearly, one of the most important outstanding questions on the actions of caffeine in invertebrates is the role of adenosine receptors. Does caffeine act as an antagonist at invertebrate adenosine receptors? While the overall functions of G protein coupled receptors for particular ligands are well conserved between orthologous mammalian and invertebrate receptors, their pharmacological profiles can differ significantly (for example, see [84]). Thus, it is not completely unexpected that caffeine may not affect the DmelAdoR receptor, which is the only invertebrate adenosine receptor to be pharmacologically characterized so far. Given that a number of studies in invertebrates suggest that caffeine is acting through an adenosine receptor, it is also possible that there is another receptor for adenosine in invertebrates. A number of genes for “orphan” G protein coupled receptors in the *Drosophila* genome still remain to be characterized, and one (or more) of them could be the adenosine receptor affected by caffeine. If, on the other hand, caffeine is not acting through an adenosine receptor in invertebrates, then the parallels in the effects of caffeine on

behavior in mammals and invertebrates suggests that caffeine may be acting on other pathways in mammals as well, and the assertion that its main function is as an adenosine receptor antagonist should be reexamined.

Secondly, the concentration of caffeine used in experiments is obviously important. In mammals, the behavioral responses to caffeine are biphasic with distinct effects at low versus high caffeine concentrations [33, 85–87]. This is most likely the case in invertebrates as well, for example, in the honey bee low doses of caffeine (as low as 0.1 μ M) increase memory retention, while higher doses (10 mM) reduce the levels of acquisition during appetitive olfactory conditioning [37, 4]. Since inhibition of phosphodiesterases and calcium release via ryanodine receptors require caffeine concentrations in the millimolar range, the ability of caffeine to affect behavior at lower concentrations suggests there is a role for adenosine receptors or other, as of yet unknown, targets for caffeine in invertebrates. One issue with many of the invertebrate studies is that the actual amount of caffeine to which an individual subject is exposed is unknown. For example, many studies use caffeine added to the food source, which the animals then consume freely. Because caffeine has an aversive taste [88, 89, 37, 90], it is possible that higher caffeine concentrations lead to decreased food consumption, leading to a lower dose of caffeine than expected. A better understanding of the effective dose would strengthen our understanding of the targets on which caffeine is acting to produce specific changes in behavior.

Conclusions

In general, caffeine appears to have similar effects on the behavior of invertebrates as those observed in humans and rodent models. However, whether or not caffeine is acting via the same molecular mechanisms is still an open question. Even within the extremely large number of species encompassed by the invertebrate category, almost all of the work was done using the fruit fly and honey bee model systems. Questions regarding the interactions between plants, their herbivores and their pollinators, and issues concerning the ecotoxicity of caffeine suggest that we need a broader understanding of the effects of caffeine across animal phyla.

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Figure 1.

The known actions of caffeine in vertebrates include: interaction with ryanodine receptors leading to intracellular calcium release; inhibition of phosphodiesterases leading to increased levels of cAMP or cGMP; inhibition of adenosine receptors; release of dopamine and the modulation of dopamine signaling via interaction between adenosine receptors and dopamine receptors; inhibition of the ligand-gated chloride channel GABA_A and glycine receptors; and inhibition of acetylcholinesterase. Only two of these mechanisms, interaction with ryanodine receptors and inhibition of phosphodiesterases, have been confirmed in invertebrates.

Figure 2.

Invertebrate adenosine receptors share only low levels of similarity with their vertebrate counterparts. The amino acid sequences of the two functionally characterized invertebrate adenosine receptors (fruit fly, DmelAdoR, NM_001276172; starfish, PminAdoR, AF521907) are compared to the sequences of adenosine receptors from human (HsapA1, X68485; HsapA2a, X68486; HsapA2b, X68487; HsapA3, L22607), mouse (MmusA1, NM_001008533; MmusA2a, NM_009630; MmusA2b, NM_007413; MmusA3, NM_009631), chicken (GgalA1, U28380; GgalA2B, AY169692; GgalA3, AF115332) and zebrafish (DrerA1, XM_001345932; DrerA2a, AY945800; DrerA2b, AY945802; DrerA3, XM_694994). The sequence of the human M1 muscarinic receptor (HsapMus1, X52068) was used to root the tree. The phylogenetic tree was generated using ClustalW2 [91].