

**Effects of Adolescent Caffeine Consumption on Addictive Behaviors in  
Adulthood**

By

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**ABSTRACT**

Caffeine is found in a variety of beverages such as soda, coffee, and tea, which are consumed by many people on a daily basis. Because of how commonly caffeine is consumed, it is important to discover the effects adolescent caffeine consumption may have on adult addiction behaviors. A rat model was used to explore the potential effects of adolescent caffeine consumption. Rats consumed caffeine during their adolescent period and underwent behavioral tests in adulthood to assess whether they displayed behaviors associated with enhanced vulnerability to drug addiction. Behavioral tests used to assess addictive behaviors in adulthood included drug self-administration (SA) and a Pavlovian Conditioned Approach (PCA) task. Our findings show that rats that consume caffeine during adolescence self-administer cocaine in adulthood at a higher rate than controls. Rats consuming caffeine during adolescence also have a higher breakpoint for cocaine self-administration in adulthood than controls, indicating that adolescent caffeine consumption increases motivation for drug taking. We also found that a greater percentage of rats consuming caffeine during early adolescence displayed “sign-tracking” behaviors during the PCA task that has been associated with altered dopamine signaling and correlated with increased drug self-administration. While the results were not as clear, a similar trend was also seen in rats that consumed caffeine during their late adolescent period. Overall, these findings illustrate that adolescent caffeine consumption enhances addictive behaviors in adulthood.

## INTRODUCTION

Recently, caffeine consumption among adolescents has greatly increased. The daily amount of caffeine consumed by 9- to 17- year olds has increased by more than 2-fold since 1980 (Frary *et al*, 2005). For this reason, it is important to be aware of the potential outcomes or effects that result from adolescent caffeine consumption.

The brain undergoes a variety of structural and functional changes during adolescence. Behaviors typical of adolescents, including increased risk-taking and impulsivity, may be attributable to brain changes during this period (Casey *et al*, 2008; Fareri *et al*, 2008; Steinberg, 2008). The prefrontal cortex (PFC), responsible for cognitive behaviors, is continuing to mature during adolescence (Wahlstrom *et al*, 2010). Additionally, the pruning of synaptic connections likely contributes to decreases in cortical gray matter and increases in white matter that are observed during adolescence (Bartzokis *et al*, 2008; Giedd *et al*, 1999; Giedd, 2004; Gogtay *et al*, 2004; Gogtay and Thompson, 2010; Jernigan *et al*, 1991; Luders *et al*, 2005; Paus *et al*, 2001; Pfefferbaum *et al*, 1994; Thompson *et al*, 2005; Sowell *et al*, 2003). The “architecture” of the dopamine system is well developed at birth, however changes in receptor expression and synaptic density are observed during adolescence. For example, dopamine hyperactivity is observed in adolescence relative to both adulthood and childhood. Increases in dopamine signaling and availability in adolescence compared to adulthood have been observed in rat and non-human primate models (Andersen *et al*, 1997; Goldman-Rakic and Brown, 1982; Irwin *et al*, 1994; Stamford, 1989). This observation may help to explain the dopamine-driven behaviors, such as increased impulsivity and risk-taking, which are observed in adolescents.

Alterations in the dopamine system are also observed as a result of caffeine consumption. Caffeine acts in the brain by antagonistically binding adenosine receptors that are co-expressed with dopamine receptors in the striatum (Fredholm *et al*, 1999). Antagonism of adenosine receptors by caffeine not only increases dopamine release, but also increases dopamine signaling in the striatum (Fredholm *et al*, 1999). Using *c-fos* mRNA expression as a marker of neuronal activity, prior studies have shown that caffeine increases *c-fos* expression in cells containing D<sub>1</sub> receptors and cells containing D<sub>2</sub>-type dopamine receptors (Johansson *et al*, 1994). Interestingly, this is similar to the effects of other psychostimulants, such as cocaine, that also increase *c-fos* expression in cells containing D<sub>1</sub>-type dopamine receptors. These findings suggest that caffeine may be inducing brain changes in ways similar to cocaine.

Prior research has shown that caffeine consumption during adolescence, but not adulthood, results in brain changes and differential expression of proteins involved in the dopamine system (O'Neill *et al* 2015). Adolescent caffeine consumption resulted in increased dopamine D<sub>2</sub> receptor and dopamine transporter expression, and decreased expression of adenosine A<sub>2A</sub> receptor in adulthood (O'Neill *et al* 2015). Adolescent caffeine consumption was also associated with increased cocaine-induced locomotor activity (O'Neill *et al* 2015). These results lead us to hypothesize that adolescent caffeine consumption acts as a vulnerability factor for drug addiction because of its effects on the mesolimbic dopamine system. The mesolimbic dopamine system is involved in reward and motivation, and plays a significant role in drug addiction.

A theory of drug addiction proposed by Robinson and Berridge (2008) is the incentive sensitization theory of addiction. This theory suggests that the learned stimulus-

response habit of drug taking alone is not sufficient to result in pathological drug addiction. The incentive sensitization theory proposes that in addition to the learned stimulus-response habit, brain cells and circuits that normally regulate the incentive salience applied towards stimuli become hypersensitive, and as a result, apply pathological levels of incentive salience to drugs and drug-associated cues (Robinson and Berridge 2008).

Dopamine is highly involved in motivational aspects of incentive salience and plays an important role in the incentive sensitization theory of addiction. This is because the dopamine system is involved in learning via classic conditioning that enables the development of salience to be attributed to a stimulus cue. In classic (Pavlovian) conditioning, the subject learns to associate a reward (unconditioned stimulus, US) with a reward-predictive cue (conditioned stimulus, CS) that is inherently neutral. After presentations of the CS and US pairing, the subject learns to associate the CS and the US and will display a conditioned response (CR) as a result of the presentation of the CS alone. It is common that when an animal or person has learned about a reward (US) via classic conditioning, they will exhibit a phasic increase in dopamine transmission in response to the predictive cue (CS) rather than in response to the reward itself. This phenomenon is often seen in drug addicts where pathological levels of incentive salience are directed toward drugs and drug-associated cues. Recent advances in the incentive sensitization theory suggest that incentive salience toward non-drug cues may be an important predictor of an individual's susceptibility to developing incentive sensitization towards drugs and drug-associated cues.

A Pavlovian Conditioned Approach (PCA) task can be a measure of incentive salience for drug and non-drug rewards. In a PCA task, behavioral responses to CS presentation have been distinguished into two different approach phenotypes – goal tracking and sign tracking (Flagel *et al* 2007). Goal trackers tend to approach the location of reward delivery, while sign trackers tend to approach the cue itself (Flagel *et al* 2007). The sign-tracking response behavior has been shown to be dependent on dopamine, while the goal-tracking response is not (Flagel *et al* 2011). The sign-tracking response indicates that a large amount of incentive salience has been applied towards the cues predictive of the reward.

The following studies aim to understand what effect adolescent caffeine consumption has on behaviors associated with drug addiction during adulthood. The two behavioral tests used to assess the rats' vulnerability towards addictive behaviors are drug self-administration and a PCA task. Each behavioral test was preceded by a unique caffeine consumption procedure, although both behavioral tests took place in adulthood. Adolescent caffeine consumption prior to self-administration followed the adolescent caffeine consumption protocol used in O'Neill *et al* 2015 where caffeine was consumed during a 28-day adolescent period. Adolescent caffeine consumption prior to the PCA task was divided into a 14-day early and 14-day late adolescent period. By dividing the adolescent period into early and late adolescence, we hoped to determine whether caffeine consumption in early or late adolescence leads to an especially distinct vulnerability to addictive behaviors. Based on prior research described above, it was expected that rats that consumed caffeine during adolescence would display enhanced cocaine self-administration compared to controls. In regards to the PCA task, it was

expected that rats that consumed caffeine during adolescence (both early and late) will display sign-tracking behaviors at a higher rate than controls.

## METHODS

### *Animals*

Male Sprague-Dawley rats (Charles River) were received on postnatal day 21 for both drug self-administration and PCA studies. Rats were housed doubly and were kept on a 12 – hour light/dark cycle. Rats were given food and water *ad libitum*. All behavioral tests occurred during the light period and were completed in accordance with guidelines established by the Institutional Animal Care and Use Committee at the University of Colorado Boulder.

### *Caffeine consumption protocol for cocaine self-administration*

Upon arrival, rats were double housed. Caffeine consumption began 7 days after arrival. Rats in the caffeine-consuming group were given a single water bottle containing 0.3g/L caffeine. They had 24-hour access to the caffeinated water for 28 consecutive days from postnatal day (PND) 28 – 55 (Figure 1a). Caffeine bottles were replaced with water bottles at the end of the 28 days and rats were given one week of water consumption before self-administration began. A group of age-matched adolescent rats consumed only water during PND28 – PND55 as a control group. Fluid consumption and body weight was monitored for rats drinking caffeine and control rats throughout the 28-day consumption period.

### *Cocaine self-administration (SA) procedure*

Self-administration procedures were performed in operant conditioning chambers (MedAssociates, St Albans, VT) equipped with two response levers and an infusion pump system. Surgery and self-administration procedures were similar to those described in Kavanagh *et al*, 2015. Rats (PND 58 – 60) were implanted with jugular catheters under

halothane anesthesia (1–2.5%). Rats were allowed 3–4 days recovery in their home cage before experimental procedures began. During this time, catheters were flushed daily with 0.1 ml heparinized saline and animals were administered (S)-(+)-ketoprofen (5 mg/kg, s.c.), a non-steroidal anti-inflammatory analgesic (Carabaza et al. 1996). After recovery, animals were allowed to self-administer intravenous cocaine (0.5 mg/kg/100  $\mu$ l injection) on a fixed ratio 1 (FR1) reinforcement schedule in daily 2 h sessions for 5 days per week for 2 weeks (10 total sessions). The animals were then allowed to self-administer the same dose of intravenous cocaine on a fixed ratio 5 (FR5) reinforcement schedule in daily 2 h sessions for 3 days. Finally, the animals were allowed to self-administer cocaine in the same manner on a progressive ratio (PR) reinforcement schedule in sessions capped at 5 hours for 3 days. The PR reinforcement schedule required that throughout the session the rats increase their lever press responses in order to receive an infusion of cocaine. The progression of the response/infusion ratio was determined according to the following formula:  $[5e^{(\text{injection number} \times 0.2)}]-5$  (e.g. 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50 etc.) (Kavanagh *et al*, 2015). In all schedule conditions, cocaine injections were delivered over 5 s concurrent with the illumination of a cue light above the active lever and were followed by a 15 s time-out period when the house light remained off and responding produced no consequence. Inactive lever responses produced no consequence throughout testing.

An additional cohort of rats was used to compare acquisition of sucrose self-administration. Rats were initially trained to lever-press for 45 mg sucrose pellets (Bio-Serv, Flemington, NJ) in standard operant test chambers on an FR1:TO20 s schedule of reinforcement under food-restricted conditions. Self-administration sessions were

terminated after the acquisition of 50 sucrose pellets or at 1 h. Rats underwent 10 self-administration sessions and a rat was deemed to have acquired self-administration behaviors when they self-administered 50 sucrose pellets at an asymptotic level of responding.

*Caffeine consumption protocol for PCA task*

For the PCA task, a different caffeine consumption model was used. Several modifications were made in this model. First, we compared the effects of caffeine consumption that was restricted to the early adolescent period with the late adolescent period. The early adolescent group consumed caffeine from PND30 – PND43, and the late adolescent group consumed caffeine from PND45 – PND59 (Figure 1b). Each age group had the opportunity to consume caffeine for a 2-week period. Second, the rats only had access to caffeine during their 12-hour active (dark) cycle. During the other 12 hours (the light, inactive cycle), rats had access to one standard water bottle. Third, throughout the consumption procedure two bottles (a standard water bottle or a bottle containing a 0.3g/L caffeine and 0.3% sucrose solution) were available to eliminate forced consumption of caffeine and allow the animals to choose which solution was consumed. A cohort of age-matched control rats had the choice between a standard water bottle or a bottle containing 0.3% sucrose with no caffeine during the 12-hour active cycle. Rats had access to the caffeine or sucrose bottle 5 days per week during the 2-week consumption period (10 total days of consumption). Consumption from each bottle was monitored daily, and the body weight of the rats was monitored twice a week. After the 2 weeks of caffeine consumption, rats only had access to a standard water bottle for the duration of

testing. Both the early adolescent and late adolescent age groups had 2 rest weeks immediately after their caffeine consumption, and behavioral testing began in adulthood.

*Pavlovian conditioned approach (PCA) task*

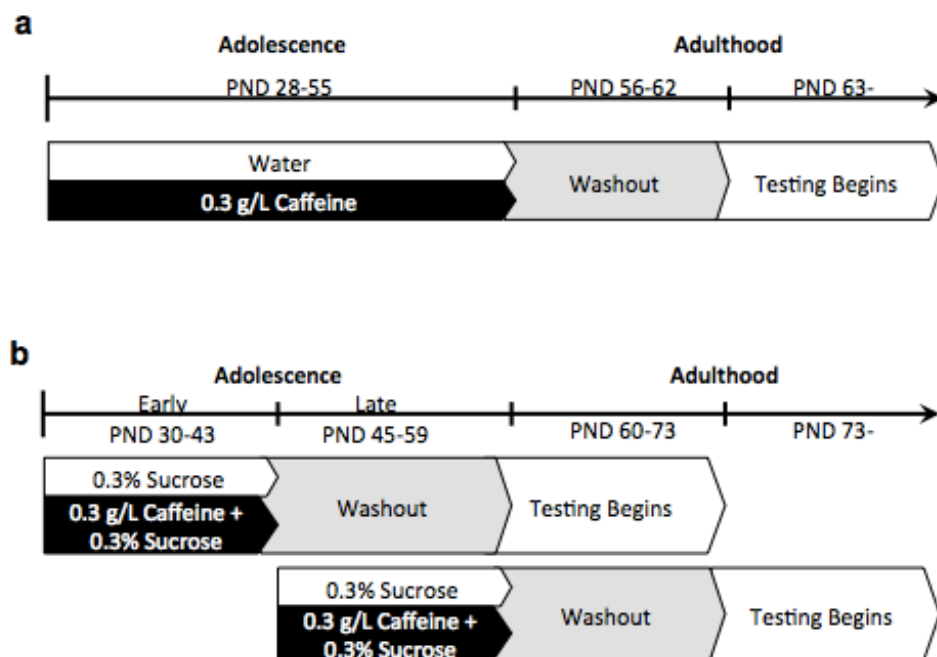
This task was performed in operant conditioning chambers (MedAssociates, St Albans, VT) equipped with two response levers and a magazine that dispensed banana-flavored sucrose pellets. Approximately one week prior to beginning the task, rats were given sucrose pellets once a day in their home cages for a few days. For 2 days the rats were placed in the chambers for pre-training sessions in which 50 pellets were randomly delivered on a variable interval 30-sec schedule. No cues were present and no operant response was required for pellet delivery. After training, rats began the PCA task, completing one session per day, 5 days per week for 2 weeks (a total of 10 sessions). One session consisted of 25 trials. A single trial included the presentation of the lever and a stimulus light (a compound conditioned stimulus, CS) for 8 seconds. After this time the light turned off, lever retracted, and a sucrose pellet (unconditioned stimulus, US) was delivered into the magazine. No operant response at the lever or the magazine was required for the delivery of the sucrose pellet. During the 8-second latency period when the lever and stimulus light became available, the time the rat spent interacting with the lever, and/or the magazine was recorded. The amount of time that passed prior to the rat approaching the lever or magazine (latency) was also recorded. The time between trials was randomized and could range from 30-90 seconds, but was on average 60 seconds. The following events were automatically recorded using Med Associates software: (1) duration of lever-CS responding, (2) the latency to the first lever C-S contact, (3) duration

of magazine responding during lever-CS presentation, and (4) the latency to the first magazine entry during lever-CS presentation.

### *Data analysis*

The effects of adolescent caffeine consumption on adult cocaine self-administration were analyzed using a two-way mixed-design ANOVA with consumption group (between) and session (within) as factors to assess differences in the number of infusions per self-administration session. Data for the PCA task was analyzed separately for rats consuming caffeine in early adolescence or late adolescence. Average lever and magazine responding, latency, and percent of approaches (% of trials/session where either the lever or the magazine was approached first) were analyzed using a two-way mixed-design ANOVA with consumption group (between) and session (within) as independent variables. Goal tracker (GT) and sign tracker (ST) designations were made in two ways. The first method for designating goal or sign tracker status was made by comparing total lever and magazine responding, latencies, and approaches across all ten sessions. For responding and approaches, if the lever responding or approaches were greater than magazine responding or approaches the rat was designated a sign tracker. If the opposite was true, the rat was designated a goal tracker. For latency, if the lever latency was shorter than the magazine latency (indicating the rat approached the lever before approaching the magazine), the rat was designated a sign tracker. If the opposite was true the rat was designated a goal tracker. The percent of goal trackers and sign trackers within each consumption group was calculated for each of the above variables. The second method of analysis used to designate goal or sign tracker status was creating a response bias score. This score was calculated by subtracting total magazine duration

from total lever duration, and dividing this difference by the sum of the total magazine duration plus total lever duration:  $(\text{total lever duration} - \text{total magazine duration}) / (\text{total lever duration} + \text{total magazine duration})$ . This resulted in a response bias score for each rat for each session that ranged from -1 (complete goal tracker) to +1 (complete sign tracker). Response bias score was analyzed using a two-way mixed-design ANOVA with consumption group (between) and session (within) as factors. For both models of caffeine consumption, caffeine consumption per day was measured as the mg of caffeine consumed per kg of body weight.

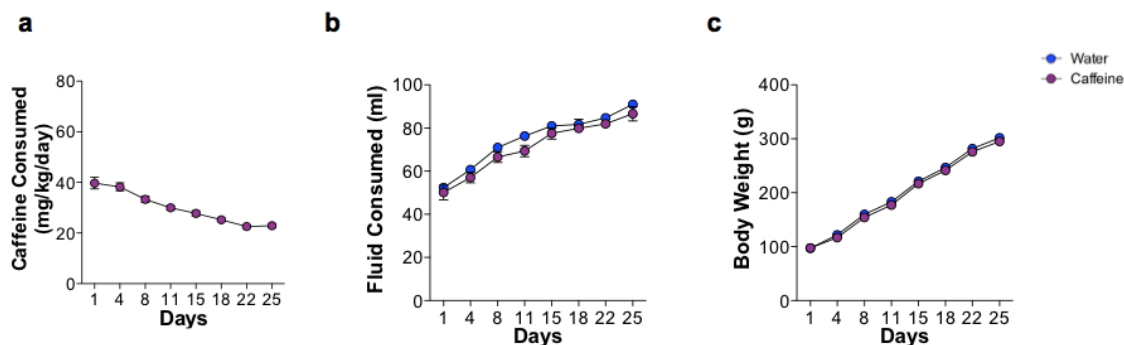


**Figure 1.** Caffeine Consumption Timelines. (a) Timeline for caffeine consumption model used for rats that would proceed to self-administration studies. (b) Timeline for caffeine consumption model used for rats that would proceed to PCA task.

## RESULTS

### *Caffeine consumption preceding self-administration*

The dose of caffeine consumed (mg/kg) decreased slightly across the adolescent caffeine consumption period and averaged  $31.43 \pm 3.88$  (Figure 2a). Total fluid consumption was comparable for caffeine-consuming rats and water-consuming controls, and increased throughout the adolescent consumption period at a similar pace for both groups (Figure 2b). Body weight was also similar for caffeine-consuming rats and water controls, and both groups gained weight at a similar pace throughout the adolescent consumption period (Figure 2c). These findings were comparable to previously published results using this procedure (O'Neill *et al*, 2015).



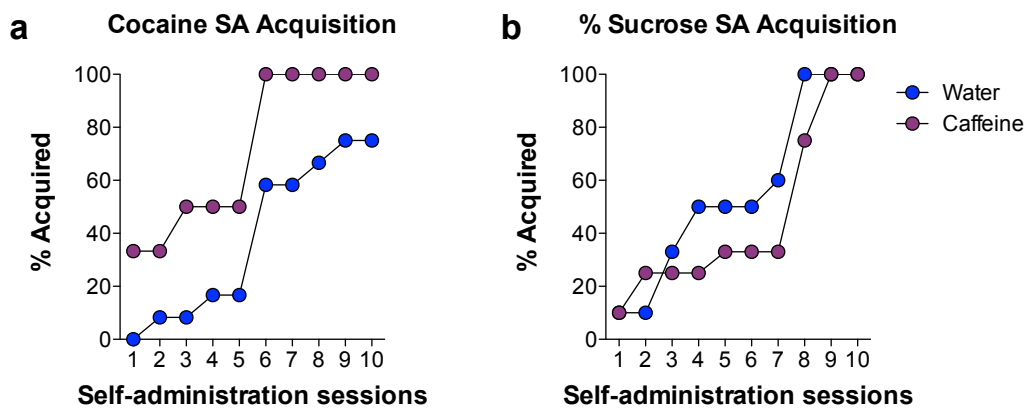
**Figure 2.** Caffeine Consumption During Adolescence Prior to SA. (a) The caffeine dose decreased across the consumption period. The average caffeine dose was  $31.43 \pm 3.88$  mg/kg. (b) The total fluid consumed by the water controls and the caffeine-consuming rats was comparable and increased across the consumption period. (c) The body weight of water controls and caffeine-consuming rats was similar and increased across the adolescent consumption period.

*Adolescent caffeine consumption increases the acquisition of cocaine self-administration and enhances cocaine self-administration behaviors*

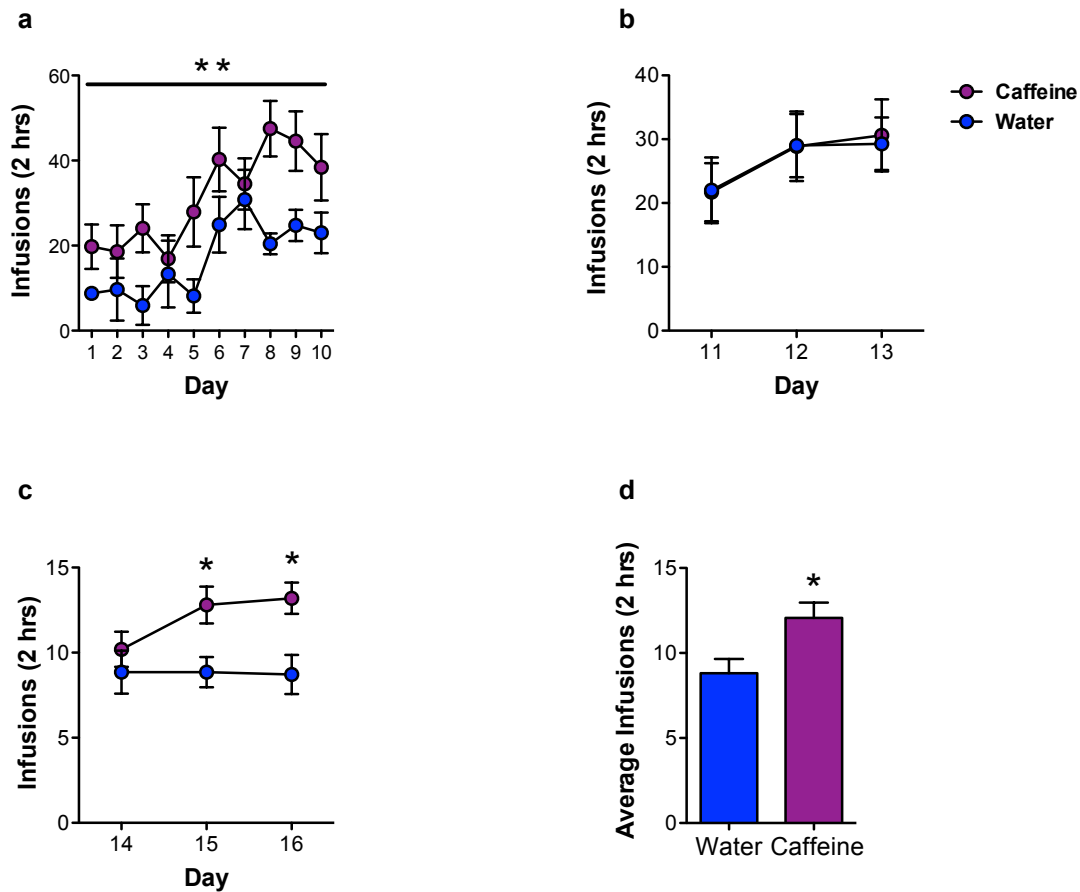
To evaluate the overall effects of caffeine consumption on the acquisition of cocaine and sucrose self-administration, we calculated the percentage of rats that reached a criterion (15 cocaine infusions or 50 sucrose pellets by the end of the self-administration session). Using this measure, we observed that rats that consumed caffeine during adolescence acquired cocaine-taking faster than control rats that drank water only during adolescence (Figure 3a). Rats that consumed caffeine during adolescence acquired sucrose taking at the same rate as their water-drinking counterparts (Figure 3b). These findings suggest that adolescent caffeine consumption may increase the sensitivity to cocaine reinforcement corroborating previous work (O'Neill *et al*, 2015).

To further clarify these effects, additional analysis of cocaine self-administration behavior was conducted for each schedule of reinforcement. On the FR1 reinforcement schedule, rats that consumed caffeine during adolescence received significantly more infusions than controls (Figure 4a;  $F_{1,220} = 28.13$ ,  $p < 0.0001$ ). No significant differences in the total number of infusions per session were observed between rats that consumed caffeine as adolescents compared with controls for the FR5 reinforcement schedule (Figure 4b). On the PR schedule, however, rats that consumed caffeine as adolescents received significantly more infusions per session than control rats (Figure 4c;  $F_{1,45} = 13.65$ ,  $p = 0.0006$ ). This effect was most robust on the final 2 out of the 3 total sessions of the PR reinforcement schedule of cocaine self-administration. When averaging the total number of infusions for all 3 PR cocaine self-administration sessions, rats that consumed

caffeine during adolescence received significantly more infusions than control rats (Figure 4d;  $t_{15} = 2.541$ ,  $p = 0.0226$ ).



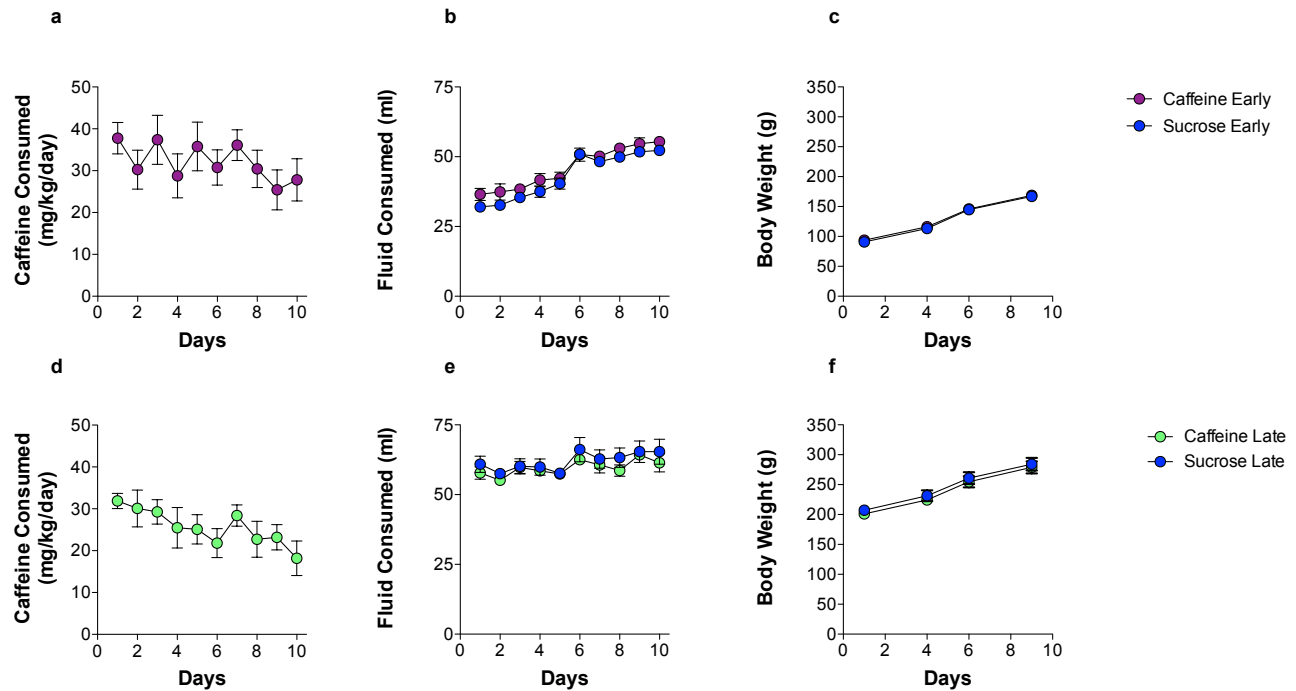
**Figure 3.** Comparison of Self-Administration Acquisition Behaviors. (a) Rats that consumed caffeine during adolescence acquired cocaine-taking behaviors quicker than control rats. (b) Rats that consumed caffeine during adolescence acquired sucrose-taking behaviors at a rate similar to control rats.



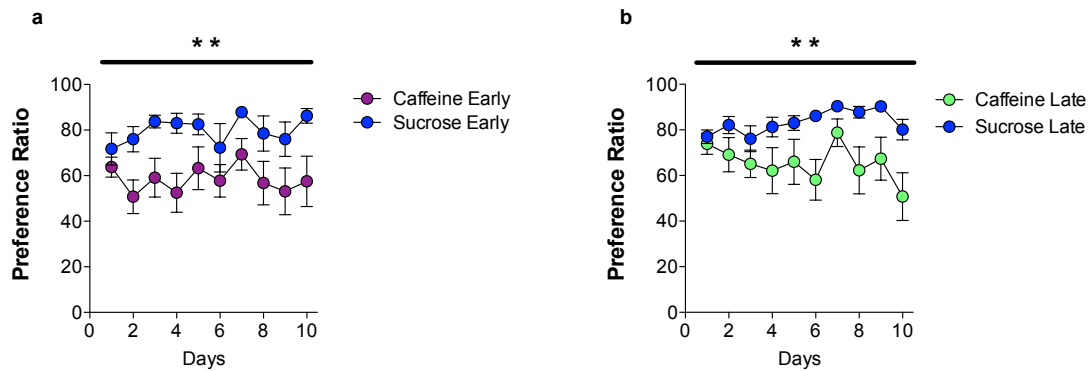
**Figure 4.** Cocaine Self-Administration Behaviors. (a) On the FR1 reinforcement schedule rats that consumed caffeine during adolescence received significantly more cocaine infusions than water controls ( $F_{1,220} = 28.13$ ,  $p < 0.0001$ ). (b) There was no significant difference in the number of cocaine infusions received by caffeine-consuming or control rats on the FR5 reinforcement schedule. (c) For the second 2 days of the PR reinforcement schedule, rats that consumed caffeine during adolescence received significantly more cocaine infusions after 2 hours than controls ( $F_{1,45} = 13.65$ ,  $p = 0.0006$ ). (d) On average, rats that consumed caffeine during adolescence received significantly more infusions after 2 hours of the PR session than water controls ( $t_{15} = 2.541$ ,  $p = 0.0226$ ).

### *Caffeine consumption for PCA task*

The caffeine dose for rats consuming caffeine during the early adolescent period remained relatively constant throughout the consumption period (Figure 5a). The average caffeine dose for rats that consumed caffeine during early adolescence was 32.05 mg/kg. Total fluid consumption for caffeine rats during early adolescence was consistent with the sucrose-consuming control cohort, and increased at the same rate for both groups throughout the early adolescent consumption period (Figure 5b). Body weight for rats consuming caffeine during early adolescence was also comparable to sucrose-consuming controls, and increased at similar rates for both groups throughout the early adolescent consumption period (Figure 5c). The caffeine dose for rats that consumed caffeine during the late adolescent period decreased slightly throughout the 2-week consumption period (Figure 5d). The average caffeine dose for rats that consumed caffeine during late adolescence was 25.62 mg/kg. During the late adolescent consumption period, caffeine-consuming rats' total fluid consumption was comparable to that of the sucrose-consuming controls and remained relatively constant throughout the 2-week consumption period (Figure 5e). Body weight for caffeine-consuming rats and sucrose-consuming controls during the late adolescent period was comparable and increased at a similar rate throughout the consumption period (Figure 5f). The control rats showed a significantly higher preference for their 0.3% sucrose bottle than their standard water bottle compared to the preference that the caffeine rats showed for their 0.3 g/L caffeine + 0.3% sucrose bottle to their standard water bottle for both the early adolescent ( $F_{1,126} = 7.37$ ,  $p = 0.0168$ ) and late adolescent ( $F_{1,126} = 126$ ,  $p = 0.0125$ ) consumption periods (Figure 6).



**Figure 5.** Caffeine Consumption During Adolescence Prior to PCA. (a) Average caffeine dose across the early adolescent consumption period was 32.05 mg/kg. (b – c) Total fluid consumption and body weight were comparable for both caffeine-consuming rats and sucrose-consuming controls, and increased at similar rates throughout the early adolescent consumption period. (d) The average caffeine dose for the late adolescent consumption period was 25.62 mg/kg. (e) Total fluid consumption was similar for both caffeine-consuming rats and sucrose-consuming controls, and remained relatively constant throughout the late adolescent consumption period. (f) Body weight was comparable for caffeine-consuming rats and sucrose-consuming controls, and increased throughout the late adolescent consumption period.



**Figure 6.** Preference for Caffeine or Sucrose Bottle Compared to Standard Water Bottle. During the early and late adolescent period rats either had the choice between a 0.3g/L caffeine + 0.3% sucrose bottle and a standard water bottle (caffeine-consuming), or a 0.3% sucrose bottle and a standard water bottle (controls). (a) During the early adolescent consumption period, control rats had a significantly higher preference for their sucrose bottle than caffeine-consuming rats had for their caffeine + sucrose bottle ( $F_{1,126} = 7.37$ ,  $p = 0.0168$ ). (b) During the late adolescent consumption period, control rats had a significantly higher preference for their sucrose bottle than caffeine-consuming rats had for their caffeine + sucrose bottle ( $F_{1,126} = 126$ ,  $p = 0.0125$ ).

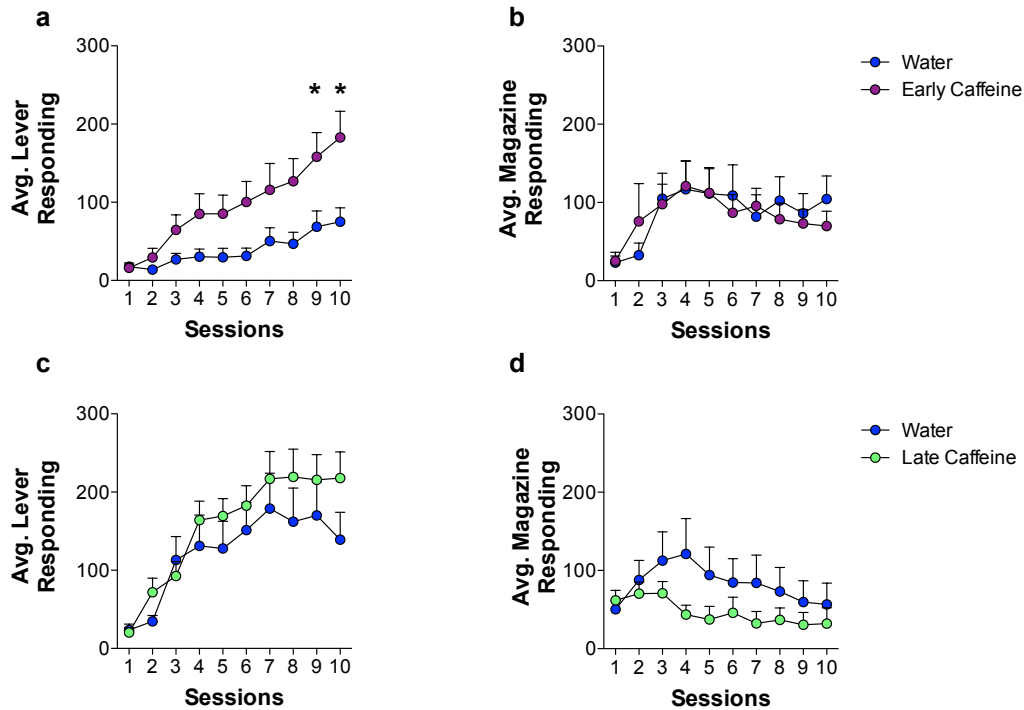
*Caffeine consumption during early adolescence enhances lever responding, lever latency, and lever approach behaviors consistent with the “sign-tracker” phenotype*

Early adolescent lever responding increased significantly across the sessions ( $F_{9,270} = 13.48, p < 0.0001$ ) for rats that consumed caffeine during the early adolescent period and controls. Caffeine consuming rats showed significantly greater lever responding on the final 2 PCA sessions (Figure 7a). Both caffeine-consuming and control rats of the early adolescent consumption group showed a significant increase in average magazine responding across sessions ( $F_{9,270} = 3.60, p = 0.0003$ ), however there was no difference in the average magazine responding between the early adolescent consumption groups (Figure 7b). Average lever latency for both rats that consumed caffeine during early adolescence and controls increased significantly across sessions ( $F_{9,270} = 25.81, p < 0.0001$ ) (Figure 8a). Rats that consumed caffeine during the early adolescent period had significantly shorter lever latencies compared with controls on the final 2 PCA sessions (Figure 8a). Average magazine latency for caffeine-consuming and control rats in the early adolescence consumption group remained relatively constant across sessions, and there were no significant difference between consumption groups (Figure 8b). The percentage of lever approaches increased across sessions for rats that consumed caffeine during early adolescence as well as controls ( $F_{9,270} = 25.47, p < 0.0001$ ). Caffeine-consuming rats had a significantly higher percentage of lever approaches compared to controls on the final PCA session (Figure 9a). The percent of magazine approaches increased significantly across sessions for both the caffeine-consuming and control rats of the early adolescence consumption group (Figure 9b;  $F_{9,270} = 4.88, p < 0.0001$ ). There was no difference in percent of magazine approaches between these consumption groups.

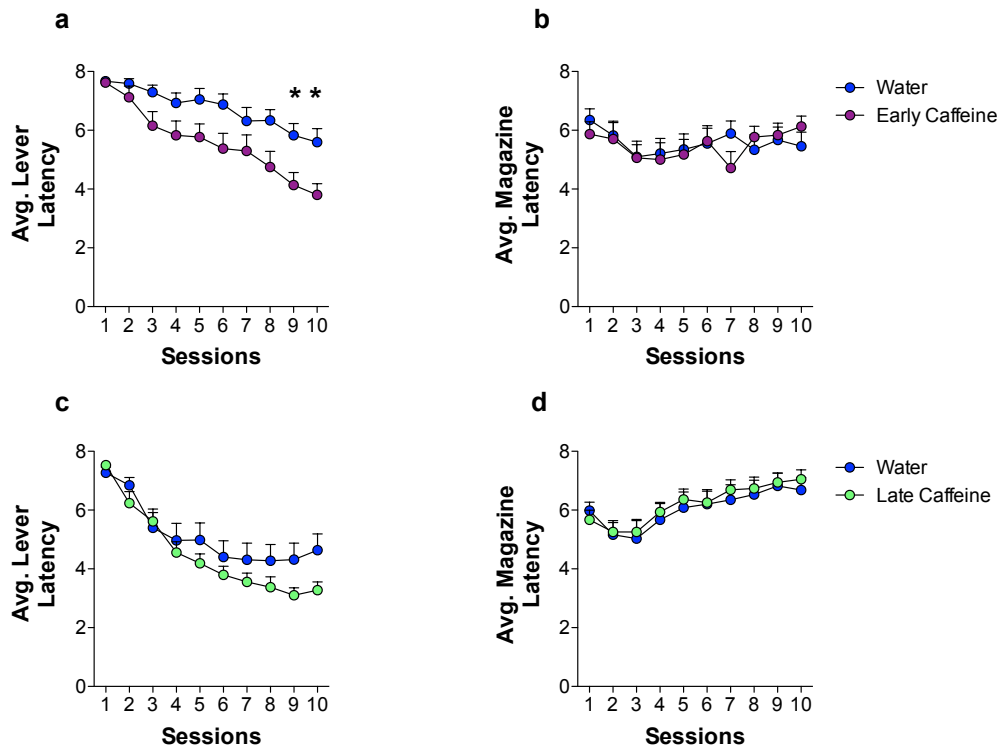
*Caffeine consumption during late adolescence results in a similar, though not as strong, trend towards behaviors consistent with the “sign-tracker” phenotype*

Similar to the early adolescent group, both caffeine-consuming and control rats from the late adolescence consumption group showed a significant increase in average lever responding across sessions (Figure 7c;  $F_{4,120} = 2.68$ ,  $p = 0.0348$ ). Average magazine responding for late adolescent caffeine-consuming and control rats showed a significant decrease across sessions (Figure 7d;  $F_{9,270} = 2.32$ ,  $p = 0.0159$ ). Though caffeine-consuming rats showed slightly higher lever responding and slightly lower magazine responding compared to controls, there were no significant differences between caffeine-consuming and control rats. For late adolescent rats, average lever latency significantly decreased for both caffeine-consuming and control groups across the sessions (Figure 8c;  $F_{9,270} = 37.46$ ,  $p < 0.0001$ ). There was no significant difference in average lever latency between late adolescent caffeine-consuming and control rats (Figure 8c). Average magazine latency for caffeine-consuming and control rats in the late adolescence consumption group showed a significant increase ( $F_{9,270} = 10.63$ ,  $p < 0.0001$ ) across sessions, but no significant differences between consumption groups (Figure 8d). Percent of lever approaches significantly increased across sessions for both caffeine and control rats ( $F_{9,252} = 41.68$ ,  $p < 0.0001$ ) of the late adolescence consumption group (Figure 9c). There was no significant difference in the percentage of lever approaches between rats that consumed caffeine during late adolescence and controls. The percent of magazine approaches was comparable for rats that consumed caffeine and controls of the late adolescent consumption group (Figure 9d). The percent of magazine approaches for both

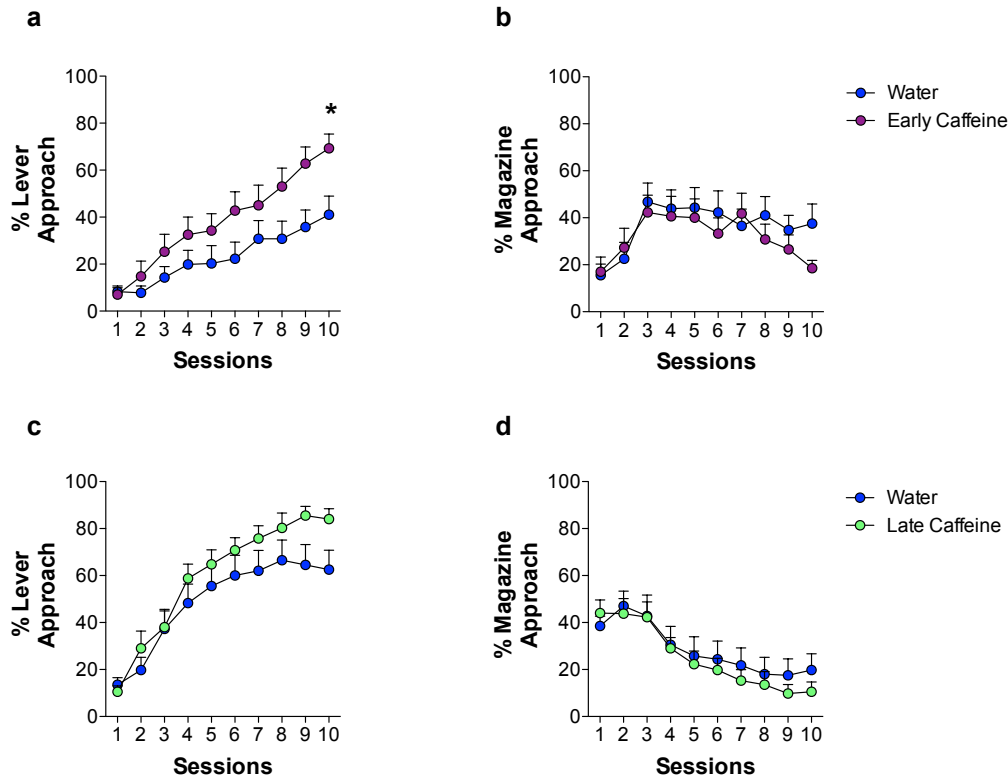
late adolescence consumption groups decreased significantly across sessions ( $F_{4,120} = 3.38, p = 0.0117$ ).



**Figure 7.** Average Lever and Magazine Responding for PCA Task. (a) Lever responding increased significantly across sessions for both caffeine-consuming and control rats of the early adolescence group ( $F_{9,270} = 13.48$ ,  $p < 0.0001$ ). Caffeine-consuming rats had significantly greater lever responding than controls ( $F_{1,270} = 7.08$ ,  $p = 0.0124$ ), however there was a significant interaction between session and consumption group ( $F_{9,270} = 2.81$ ,  $p = 0.0036$ ). Further analysis of the interaction revealed a significant difference between consumption group on sessions 9 ( $t_{30} = 3.134$ ,  $p < 0.05$ ) and 10 ( $t_{30} = 3.768$ ,  $p < 0.05$ ). (b) There was no significant difference in magazine responding between rats that consumed caffeine during early adolescence and control rats. Magazine responding significantly increased across sessions for both groups ( $F_{9,270} = 3.60$ ,  $p = 0.0003$ ). (c) There was no significant difference in lever responding between rats that consumed caffeine during late adolescence and controls. Both groups showed a significant increase in lever responding across sessions ( $F_{4,120} = 2.68$ ,  $p = 0.0348$ ). (d) Both rats that consumed caffeine during late adolescence and controls showed a significant decrease in magazine responding across sessions ( $F_{9,270} = 2.32$ ,  $p = 0.0159$ ). There was no significant difference in magazine responding between late adolescent caffeine-consuming and control rats.



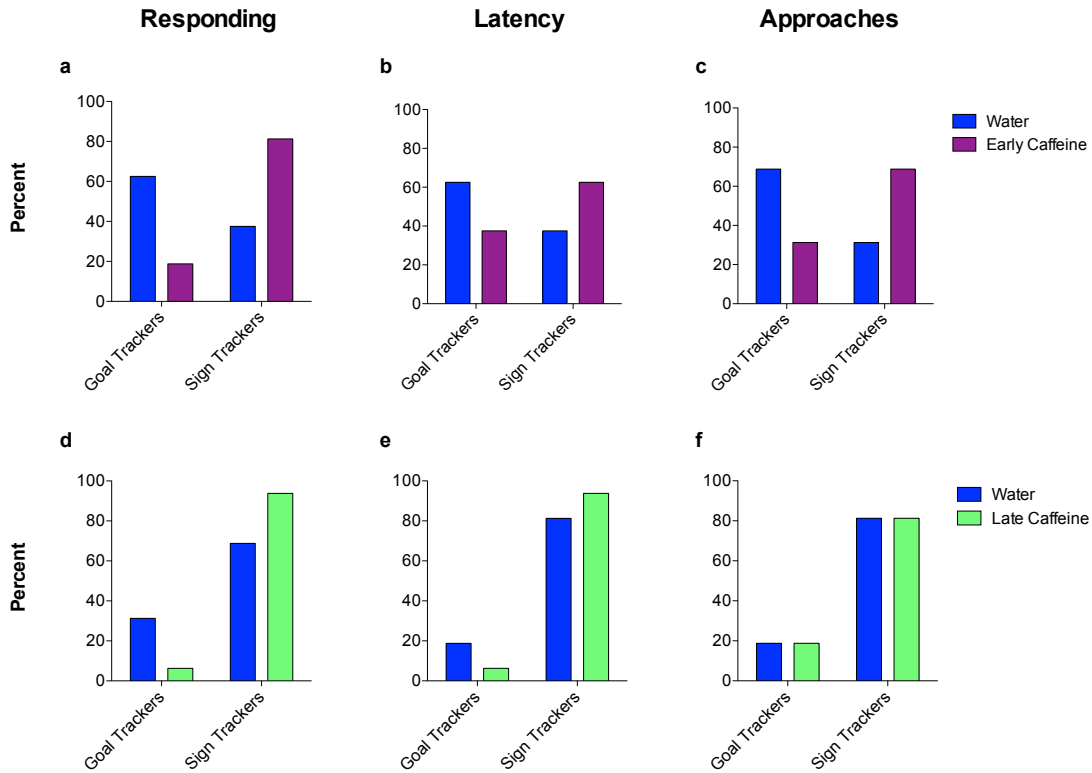
**Figure 8.** Average Lever and Magazine Latency for PCA Task. (a) Average lever latency significantly decreased across sessions for both caffeine-consuming and control rats of the early adolescence consumption group ( $F_{9,270} = 25.81$ ,  $p < 0.0001$ ). A significant difference was observed between caffeine-consuming and control rats ( $F_{1,270} = 6.99$ ,  $p = 0.0129$ ), however a significant effect of interaction was also observed ( $F_{9,270} = 2.19$ ,  $p = 0.0229$ ). Further analysis of the interaction revealed that caffeine-consuming rats had significantly shorter lever latencies than controls on the 9<sup>th</sup> ( $t_{30} = 3.003$ ,  $p < 0.05$ ) and 10<sup>th</sup> ( $t_{30} = 3.181$ ,  $p < 0.05$ ) sessions. (b) Average magazine latency remained fairly constant across sessions for rats that consumed caffeine during early adolescence and controls. There was no significant difference in average magazine latency between early adolescence caffeine-consuming rats and controls. (c) No significant difference in average lever latency exists between rats that consumed caffeine during late adolescence and controls. Lever latency decreased significantly across sessions for both groups ( $F_{9,270} = 37.46$ ,  $p < 0.0001$ ). (d) There was no significant difference in average magazine latency between late adolescent caffeine-consuming rats and controls. Average magazine latency showed a significant increase for both late consumption groups across sessions ( $F_{9,270} = 10.63$ ,  $p < 0.0001$ ).



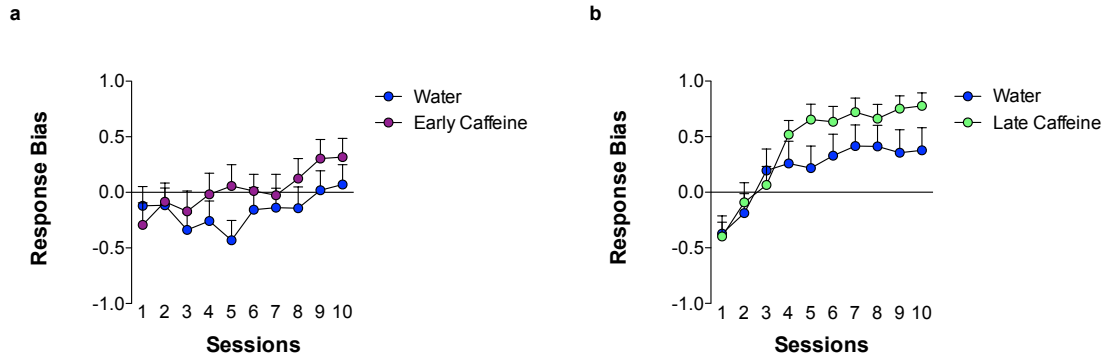
**Figure 9.** Percentage of Lever Approaches and Magazine Approaches for PCA Task. (a) The percentage of lever approaches increased for both caffeine-consuming and control rats of the early adolescence consumption group across sessions ( $F_{9,270} = 25.47$ ,  $p < 0.0001$ ). A significant effect between consumption groups was observed ( $F_{1,270} = 4.27$ ,  $p = 0.0474$ ), however a significant interaction was also observed ( $F_{9,270} = 2.11$ ,  $p = 0.0288$ ). Further analysis of this interaction revealed that on the 10<sup>th</sup> session, caffeine-consuming rats had a significantly greater percentage of lever approaches than controls ( $t_{30} = 2.942$ ,  $p < 0.05$ ). (b) Both early adolescence caffeine-consuming and control rats showed a significant increase in percent of magazine approaches across sessions ( $F_{9,270} = 4.88$ ,  $p < 0.0001$ ). There was no significant difference in percent of magazine approaches between early adolescent caffeine-consuming and control rats. (c) Rats that consumed caffeine during late adolescence and control rats showed a significant increase in percent of lever approaches across sessions ( $F_{9,252} = 41.68$ ,  $p < 0.0001$ ). There was no significant difference in percentage of lever approaches between caffeine-consuming and control rats. (d) Both rats that consumed caffeine during late adolescence and controls showed a significant decrease in percent of magazine approaches across sessions ( $F_{4,120} = 3.38$ ,  $p = 0.0117$ ). There was no significant difference in percent of magazine approaches between the late adolescence consumption groups.

### *Goal Tracker vs. Sign Tracker Comparisons*

For the early adolescence consumption group a higher percentage of caffeine-consuming animals were designated sign trackers compared with controls, and a lower percentage of caffeine-consuming animals were goal trackers compared with controls across all 3 variables analyzed: responding, latency, and approaches (Figure 10a-c). Additionally, a higher percentage of control rats were designated goal trackers than sign trackers for all 3 variables (Figure 10a-c). For the late adolescent group, across all 3 variables used to make a sign tracker or goal tracker designations, both caffeine and control rats had a higher percentage of sign tracker designations than goal tracker designations (Figure 10d-f). For the late adolescent group, based on responding and latency, a greater percentage of caffeine-consuming rats were sign trackers than control rats (Figure 10d-e). Based on approaches (Figure 10f), the percentage of rats that received sign tracker or goal tracker designations was equal between rats that consumed caffeine during late adolescence and controls. Response bias scores for both caffeine and control rats of the early adolescence consumption group increased significantly across sessions (Figure 11a;  $F_{9,270} = 2.19$ ,  $p = 0.0229$ ), trending slightly towards a sign tracker response bias score. However, there was no significant difference between caffeine-consuming and control rats. For the late adolescence consumption group, both caffeine and control rats' response bias score also showed a significant increase across sessions (Figure 11b;  $F_{9,270} = 18.63$ ,  $p < 0.0001$ ), trending towards a stronger sign tracker response bias score than seen in the early adolescence consumption group. However, similar to the early adolescence consumption group, there was no significant difference in response bias score between caffeine-consuming and control rats.



**Figure 10.** Percentage of Goal Trackers vs. Sign Trackers Based on Responding, Latency, and Approaches. (a-c) A higher percentage of rats that consumed caffeine during early adolescence were designated sign trackers compared to controls based on all three variables: responding, latency, and approaches. A lower percentage of rats that consumed caffeine during early adolescence were designated goal trackers than the controls. (d-e) A slightly greater percentage of rats that consumed caffeine during late adolescence were designated sign trackers than controls based on lever responding and lever latency. (f) An equal percentage of rats that consumed caffeine during late adolescence were designated sign trackers or goal trackers compared to controls.



**Figure 11.** Response Bias Scores. Response bias score was used to designate whether rats displayed sign-tracking ( $0 \leq +1$ ) tendencies or goal tracking ( $0 \geq -1$ ) tendencies. (a) Response bias scores for both rats that consumed caffeine during early adolescence and controls had a significant increase in response bias score across the sessions ( $F_{9,270} = 2.19$ ,  $p = 0.0229$ ), tending toward a slight sign-tracking score. There was no significant difference in response bias score between rats that consumed caffeine during early adolescence and controls. (b) Response bias scores for both rats that consumed caffeine during late adolescence and controls showed a significant increase in response bias score across the sessions ( $F_{9,270} = 18.63$ ,  $p < 0.0001$ ), tending toward a stronger sign-tracker score than seen in the early adolescence consumption group. There was no significant difference in response bias score between rats that consumed caffeine during late adolescence and control rats.

## DISCUSSION

Our experiments resulted in findings indicating that caffeine consumption during adolescence impacts adult behaviors that are predictive of an enhanced vulnerability to addiction. We found that rats that consumed caffeine during adolescence acquired cocaine self-administration, but not sucrose self-administration, more quickly than controls. Rats that consumed caffeine during adolescence also self-administered greater amounts of cocaine than controls on the FR1 and PR reinforcement schedules. Rats that consumed caffeine during both early and late adolescence displayed more behaviors consistent with the sign-tracker phenotype than the goal-tracker phenotype. However, this effect was stronger and more distinct when compared to controls for rats that consumed caffeine during early adolescence than for rats that consumed caffeine in the late adolescence period.

Dopamine plays a large role in addictive behaviors. Previous work has shown that adolescent caffeine consumption influences the dopamine system (O'Neill *et al*, 2015) in a way that predicts that adolescent caffeine consumption may enhance vulnerability for addictive behaviors in adulthood. We explored whether adolescent caffeine consumption actually influences cocaine-taking behaviors in adulthood. Adolescent caffeine consumption enhanced cocaine self-administration acquisition in adulthood relative to controls, but this same effect was not seen for sucrose-self administration. These findings indicate that adolescent caffeine consumption may only alter addiction vulnerability and incentive salience for strictly drug rewards. Additionally, adolescent caffeine consumption significantly increased cocaine self-administration compared to controls on the FR1 and PR reinforcement schedule, but had no effect on cocaine self-administration

for the FR5 reinforcement schedule. These findings suggest that adolescent caffeine consumption may strictly affect the motivational aspect of drug taking. During the FR1 schedule, rats are experiencing cocaine for the first time and are learning about the self-administration process and its contingencies. For example, the rats are learning that a lever press results in an infusion of drug and that this is accompanied by the presence of a cue light. This is the period where incentive salience is being applied towards the drug-related cues like the cue light. Previous studies have shown that rats that consume caffeine during adolescence are more sensitive to cocaine (O'Neill *et al*, 2015). These findings, combined with our results that rats that consumed caffeine during adolescence received more infusions of cocaine than controls on the FR1 schedule, indicate that cocaine is more rewarding to rats that consumed caffeine during adolescence. Therefore, the caffeine-consuming rats that are more sensitive to cocaine attribute greater incentive salience towards the drug and drug-associated cues. During the FR5 schedule, the rats have already learned the self-administration task and are familiar with the cues. At this point, drug taking has become more of a habit, and we are observing the maintenance of the drug-taking behaviors that rats acquired during the FR1 sessions. Additionally, incentive sensitization to drug cues has already occurred; therefore, it is not surprising that we did not observe differences between rats that consumed caffeine during adolescence and controls on the FR5 reinforcement schedule. The effect seen during the PR reinforcement schedule especially corroborates this possibility, because the PR reinforcement schedule is meant to be a direct measure of how reinforcing a rat finds the drug, or the amount of incentive salience they have associated with the lever-pressing action that predicts the receipt of the drug reward. With the PR reinforcement schedule

we can observe the “breakpoint,” or the point at which the rat decides that receiving an infusion of drug requires too much work. Differences in breakpoint indicate differences in motivation. Our results showed that rats that consumed caffeine as adolescents had a significantly higher breakpoint for cocaine self-administration than controls, indicating that rats that consumed caffeine during adolescence had a higher motivation for cocaine taking.

Our findings from the PCA task demonstrate that both rats that consumed caffeine during early adolescence and those that consumed caffeine during late adolescence displayed more behaviors consistent with sign-tracking phenotypes than goal-tracking phenotypes. Interestingly, it has been shown that rats that consumed alcohol throughout the adolescent period also showed a stronger sign-tracker response in a PCA task in adulthood than control rats (Spoelder *et al*, 2015). These findings are similar to what we observed for adolescent caffeine consumption. This indicates that there are a variety of exogenous factors that, if present during the critical developmental period of adolescence, can impact the dopamine system and increase susceptibility for addictive behaviors in adulthood. Additionally, it has been shown that animals with sign-tracking phenotypes tend to apply greater incentive salience towards other drugs of abuse. Other work has shown that sign-trackers approach a classically conditioned opioid cue more readily and worked more avidly to receive the opioid reward than goal-trackers (Yager *et al*, 2015). These findings support the idea that sign-tracker and goal-tracker designations are involved in the motivational aspect of drug taking. Further, sign-tracker rats have also been shown to acquire cocaine self-administration more quickly than goal-tracker rats (Beckmann *et al*, 2011).

In our findings, we saw that the effect of increased sign-tracking behaviors relative to controls was more robust in rats that consumed caffeine during early adolescence than those that consumed caffeine during late adolescence. The difference in how strongly caffeine consumption enhances behaviors indicative of addiction vulnerability between early and late adolescent groups may imply that early adolescence is a more vulnerable period than late adolescence. Evidence suggesting that the brain may be more vulnerable and less developed in early adolescence compared to late adolescence has shown that sex hormones are involved in myelination and are important in organizing functional connections made during this period of development (Peper *et al*, 2011). Because the presence of sex hormones occurs during puberty, the pre-pubertal period of adolescence, which occurs earliest in adolescence, may be more vulnerable than late adolescence due to an insufficient quantity of sex hormones for myelination of important neural connections. Additionally, studies have shown that myelin in the frontal lobe continues to increase throughout adolescence (Giedd *et al*, 1999; Baird *et al*, 1999; Giedd, 2004). These findings indicate that earlier in the adolescent period there is less myelination of connections in the frontal lobe, which may contribute to early adolescence being a more vulnerable period of brain development than late adolescence. Alternately, the differences seen between rats that consumed caffeine during early adolescence and those that consumed caffeine during late adolescence may be attributable to procedural differences. For example, the average caffeine dose for early adolescent caffeine-consuming rats was 32.05 mg/kg, while the average caffeine dose for rats that consumed caffeine during late adolescence was only 25.62 mg/kg. The dose is likely higher for rats that consumed caffeine during early adolescence because they had smaller body weights

during consumption; therefore, the dose they received was greater per kg of body weight. The lower caffeine dose for rats that consumed caffeine during late adolescence could potentially explain why the effects seen with rats from this group were not as strong as the effects observed in rats that consumed caffeine during early adolescence. Additionally, because each caffeine consumption group (early and late) began the task 2 weeks after the end of their respective consumption periods, the rats that consumed caffeine during the early adolescent period performed the task earlier in adulthood than when the rats that consumed caffeine during late adolescence performed the PCA task. This may also account for the differences in effects seen between these groups. Another flaw of the caffeine consumption model used prior to the PCA task, and a flaw of the consumption model used prior to drug self-administration is that the rats stop drinking caffeine after adolescence. This is likely not representative of caffeine consumption in humans, who most likely continue drinking caffeine throughout adulthood. However, it is necessary to limit caffeine consumption to the adolescent period in order to determine that the differences in adult behaviors are the result of caffeine consumption specifically during the adolescent period.

The distinction between caffeine consumption during early vs. late adolescence should be further explored by using a caffeine consumption model that separates early and late phases of adolescence prior to testing cocaine self-administration behaviors. It would also be helpful to use this type of consumption model and then test for changes in levels of proteins that are part of the dopamine system, as was done in O'Neill *et al*, 2015. Furthermore, it would be useful to measure dopamine responses during the PCA task (as done in Spoelder *et al*, 2015) to confirm that the differences in behaviors are due

to caffeine-induced alterations in the dopamine system. Testing the effects of adolescent caffeine consumption on other behaviors that are predictive of addiction vulnerability could further support our results. For example, impulsivity has been associated with increased vulnerability for addictive behaviors. It may be valuable to test the effects of adolescent caffeine consumption on impulsivity using a delayed discounting behavioral task.

### **Conclusions**

Overall, adolescent caffeine consumption enhanced cocaine self-administration behaviors on the FR1 and PR reinforcement schedules. Rats that consumed caffeine during adolescence also tended to display more behaviors typical of sign-trackers. This effect was more robust for rats that consumed caffeine during early adolescence compared to rats that consumed caffeine during late adolescence. Taken together, these findings indicate that adolescent caffeine consumption enhances incentive sensitization towards drug and non-drug predictive cues. The increased incentive salience that rats that consume caffeine during adolescence apply towards these cues likely reflects a greater vulnerability toward addictive behaviors in adulthood.

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