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Epigenetic modifiers identified as regulators of food intake in a unique hypophagic chicken model

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ABSTRACT

DNA methylation is an epigenetic modification that influences gene transcription; however, the effects of methylation-influencing chemicals on appetite are unknown. We evaluated the effects of single administration of a methyl donor, S-Adenosylmethionine (**SAM**), or methylation inhibitor, 5-Azacytidine (**AZA**), on immediate and later-age food intake in an anorexic chick model. The doses of intracerebroventricularly-injected SAM were 0 (vehicle), 0.1, 1, and 10 μg, and of AZA were 0 (vehicle), 1, 5, and 25 μg. When injected on day 5 posthatch, there was no effect of SAM on food intake in either fed or fasted chicks, whereas AZA increased food consumption in the fasted state but decreased it in fed chicks. We then performed a single injection (same doses) at hatch and measured food intake on day 5 in response to neuropeptide Y (**NPY**; 0.2 μg) injection. Irrespective of NPY, chicks injected with 1 μg of SAM ate more than others on day 5. In contrast, chicks injected with AZA (5 and 25 μg doses) consumed less on day 5. In conclusion, we identified DNA methylation-regulating chemicals as regulators of food intake. AZA but not SAM affected food intake in the short-term, feeding state dependently. Later, both chemicals injected on the day of hatch were associated with food intake changes at a later age, suggesting that feeding pathways might be altered through changes in methylation.

Implications

Appetite regulation is closely associated with food consumption and growth performance in poultry. However, the effects of epigenetic-modifying chemicals on appetite remain unknown. Here, we chose two epigenetic modifiers, S-Adenosylmethionine and 5-Azacytidine, to evaluate the effects of central injection on appetite regulation. 5-Azacytidine, but not S-Adenosylmethionine, was associated with immediate effects, which is a novel finding in chickens. Both S-Adenosylmethionine and 5-Azacytidine affected later-age appetite regulation possibly through epigenetic modifications. Identification of these novel regulators of appetite sheds light on our understanding of appetite regulation mechanisms and provides potential strategies in dealing with abnormal food consumption in chickens.

Introduction

Appetite regulation involves a complex interaction of pathways that are activated through the integration of signals that converge on the hypothalamus. The hypothalamus serves as the regulatory center for energy homeostasis, integrating cues from a variety of central and peripheral factors to balance energy intake and expenditure (Berthoud, 2002; Suzuki et al., 2010). Identifying novel regulators of feeding behavior and elucidating the associated molecular pathways will continue to facilitate the development of strategies to treat eating disorders.

An attractive model with which to understand appetite regulatory mechanisms is the Virginia lines of chickens. These lines, originating from a common founder population, have been continuously selected for low (**LWS**) or high (**HWS**) juvenile BW, and after, more than 60 consecutive generations of selection differ in BW at selection age (56 days) by more than 12-fold, which is accompanied by divergent appetite phenotypes (Lillie et al., 2018). The LWS are lean and hypophagic and display varying severities of anorexia, whereas all HWS are compulsive eaters that become obese (Jambui et al., 2017). This model is the only one of under- and over-eating behavior resulting from long-term divergent selection for BW originating from a common founder population (Rubin et al., 2010).

Intracerebroventricular (**ICV**; into left lateral ventricle) injection of anorexigenic peptides, including α-melanocyte-stimulating hormone (**α-MSH**) (Cline et al., 2008), corticotropin-releasing factor (**CRF**) (Cline et al., 2009), insulin (Smith et al., 2011) and ghrelin

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(orexigenic in mammals) (Xu et al., 2011), inhibited food intake in both lines, with greater sensitivity to satiety-inducing effects in LWS than HWS. Central administration of human recombinant leptin reduced food intake in LWS but had no effect on HWS (Kuo et al., 2005), while neuropeptide Y (**NPY**) (Newmyer et al., 2013) and obestatin (Xu et al., 2011) increased food intake in HWS but not LWS. These results collectively suggest that long-term selection for BW altered the hypothalamic satiety mechanisms in HWS and LWS lines. Also observed in these studies were hypothalamic changes in c-Fos (Cline et al., 2008; Newmyer et al., 2013), a marker for neuronal activity, and appetite-related peptide gene expression (Yi et al., 2017), which implied molecular changes as a result of direct activation or inhibition of central feeding pathways. Moreover, the resistance of the hypophagic LWS to NPY is early-life stress (cold ambient temperature and food deprivation)-exposure-dependent (Yi et al., 2016; Wang et al., 2017) and the mechanism involves stress-induced anorexigenic tone originating from CRF-ergic neurons in the paraventricular nucleus of the hypothalamus. Because the effect on feeding behavior was observed at a later age than when the stressor was applied, we hypothesized that the mechanism was epigenetic and occurred exclusively in the LWS because the changes were not observed in HWS (Yi et al., 2016; Wang et al., 2017). Indeed, this was confirmed when we identified a 5′-cytosine-phosphate-guanine-3′ (**CpG**) in the promoter region of the CRF gene that was hypomethylated in response to stress in the LWS (Xiao et al., 2020). This reduced methylation disrupted the binding of a transcriptional repressor, methyl domain-binding protein 2, which was associated with increased CRF mRNA in the paraventricular nucleus of the hypothalamus (Xiao et al., 2020). Thus, changes in appetite regulation can be modulated by epigenetic modifications that occur in response to environmental influences.

Epigenetics, including DNA methylation, histone acetylation and regulation of microRNAs, etc., involves changes in gene expression that do not alter the DNA sequence (Gibney and Nolan, 2010). DNA methylation is a covalent modification requiring DNA methyltransferases (**DNMTs**) that catalyze the addition of a methyl group to cytosines that exist in the context of CpG dinucleotides in the DNA sequence, and is generally considered to be inhibitory with respect to effects on transcriptional regulation. S-Adenosylmethionine (**SAM**) is the major endogenous methyl donor (Loenen, 2006) and is an essential component of one carbon metabolism, which involves several dietary precursors, including folate, choline, and methionine (McKay and Mathers, 2011; Feil and Fraga, 2012). Dietary deficiencies in methyl donors led to widespread DNA demethylation or hypomethylation (Laird and Jaenisch, 1994; Detich et al., 2003; Anderson et al., 2012). However, the effects of central administration of SAM on DNMT activity and DNA methylation are unknown. At the opposite end of the spectrum, there exist several inhibitors of DNA methylation, such as 5-Azacytidine (**AZA**), which was first identified as a chemotherapeutic drug (Christman, 2002). However, at doses that do not induce cell death, once incorporated in DNA, AZA binds to DNMT1 and inhibits its activity, causing a rapid decline in DNA methylation and subsequent increases in gene transcription (Creusot et al., 1982; Christman et al., 1983).

Epigenetic-modifying chemicals thus have the potential to facilitate changes in behavior that persist over time through effects on gene regulation. Unclear, however, is whether such molecules can exert direct effects on behavior that are independent of their effects on DNA methylation. To our knowledge, there are few reports of effects of methyl donors or methylation inhibitors on feeding behavior. Because we observed changes in appetite regulation in the LWS but not in HWS as a result of early posthatch environmental stressors, we hypothesize that direct administration of an epigenetic modifier has the potential to have a similar influence as stress on feeding behavior at a later age. Thus, the objective of the present study was to measure the immediate (inject on day 5 posthatch) and long-term (inject on day of hatch and measure effects 5 days later) effects of central administration of SAM and AZA on food and water intake in LWS chicks.

Material and methods

Animals

The lines of chicks are from a long-term divergent selection program for either low or high BW at 56 days of age (Siegel, 1962). The founding population consisted of crosses of seven inbred lines of White Plymouth Rocks, and the selection program is reviewed in a previous study (Márquez et al., 2010). All eggs were from age contemporary LWS parents from the 60th generation of selection and were incubated in the same incubator and hatcher. After hatch, chicks were group-caged for 1 day and then transferred to individual cages in a room at a constant temperature of 32 ± 1 °C and 50 ± 5% relative humidity with 24 h of light. Chicks had free access to a mash diet (3 000 kcal ME/kg and 22% CP, Table 1) (McConn et al., 2018) and tap water. The individual cages allowed visual and auditory contact among chicks, which were handled twice daily to adapt to handling.

Intracerebroventricular injection procedure

On the day of hatch and/or day 5 posthatch, chicks were ICV-injected using a method that does not appear to induce physiological stress (Furuse et al., 1999; Saito et al., 2005), adapted from previous research (Davis et al., 1979). The head of the chick was briefly inserted into a restraining device that left the cranium exposed to allow for free-hand injection. Injection coordinates were based on the Kuenzel and Masson chicken stereotaxic atlas (Kuenzel and Masson, 1988). Anatomical landmarks were determined visually by using a restraining device and plastic tubing sheath. The restraining device is a piece of clay with scales that was molded

Table 1
Ingredient and chemical composition of the chicken experimental diet.

Ingredient (% as fed)[1]	Experimental diet
Corn grain	60.37
Soybean meal	33.99
Dicalcium phosphate	1.5
Limestone	1.21
Soybean oil	1.13
Vitamin/mineral premix[2]	1
Methionine 99%	0.26
L-Lysine HCl 78%	0.19
Sodium bicarbonate	0.15
L-Threonine	0.09
Coban 90[3]	0.05
Choline-Cl 60%	0.05
Phytase-Ronozyme10000[4]	0.01
Energy; kcal ME/kg	3 000
CP (%)[5]	22
Crude fat (%)[5]	3.0

Abbreviations: HCl = hydrochloride; Cl = chloride; ME = metabolizable energy.
[1] Diets were formulated to meet or exceed minimum recommended specifications for Cobb-500 broilers during the starter phase (Cobb-Vantress).
[2] Guaranteed analyses (per kg of premix): Manganese, 25.6 g; selenium, 120 mg; zinc, 30 g; Vitamin A, 4 409 171.076 IU; Vitamin D3, 1 410 934.744 ICU; Vitamin E,13 227.513 IU; d-biotin, 88.183 mg.
[3] Coban 90 (Elanco Animal Health, Greenfield, IN, USA) contains 90 g of Monensin sodium per pound of premix and is included in the diet as a coccidiostat.
[4] DSM Nutritional Products, Ltd. (Arcadia, WI, USA).
[5] Analyzed at Experiment Station Chemical Laboratories at University of Missouri.

with the use of a chick cadaver. The restraining device leaves the cranium exposed and coordinates the injection point at 3 mm anterior to the coronal suture and 1 mm lateral from the sagittal suture. The plastic tubing sheath over the needle was used to control injection depth (2 mm). The needle remained at injection depth in the un-anesthetized chick for 5 s postinjection to reduce backflow. SAM (A7007, Sigma, St. Louis, MO, USA), AZA (A2385, Sigma) or chicken NPY (AnaSpec, San Jose, CA, USA) was dissolved in chicken artificial cerebrospinal fluid (Anderson and Heisley, 1972) as a vehicle for a total injection volume of 5 μl with 0.1% Evans Blue dye to facilitate injection site localization. After data collection, the chick was decapitated and its head sectioned along the frontal plane to determine the presence of dye in the lateral ventricle. Any chick without dye present in the lateral ventricle system was deleted from statistical analysis. Sex was determined visually by dissection and gonadal inspection at the time of decapitation.

Experiment 1: Food and water intake in S-Adenosylmethionine-injected fed low weight-selected chicks

On day 5 posthatch, chicks were randomly assigned to receive either 0 (vehicle), 0.1, 1, or 10 μg of SAM, the doses of which were based on a former study (Bungo and Shiraishi, 2010), by ICV injection (n = 10 in each group, starting number). BWs for each treatment were 20.3 g, 19.5 g, 19.9 g, and 18.1 g, respectively (RMSE = 2.58; F (3, 32) = 1.20; P = 0.325). After injection, chicks were returned to their individual home cages and given *ad libitum* access to both food and water. Food and water were placed in two separate white plastic cups right outside the cage, with the amount in each being half a cup-full, providing easy access for birds while preventing spill to the largest extent. At the time of injection and every 30 min thereafter, both cups were removed for 10 s, which provides sufficient time to measure their weights without interfering with feeding or drinking. Food and water intake were monitored (0.01 g) every 30 min for 180 min postinjection and at 360 min postinjection. Male (%) in each SAM treatment in LWS chickens were 56, 67, 33, and 44, respectively.

Experiment 2: Food and water intake in S-Adenosylmethionine-injected fasted low weight-selected chicks

Procedures were identical to Experiment 1, except that chicks were fasted for 180 min before SAM injection. BWs for each treatment were 17.9 g, 18.7 g, 20.3 g, and 18.0 g, respectively (RMSE = 2.53; F (3, 34) = 1.77; P = 0.172). During the fasting period, only food was withheld, while birds still had *ad libitum* access to water. Male (%) in each SAM treatment were 20, 56, 33, and 30, respectively.

Experiment 3: Food intake in neuropeptide Y-injected low weight-selected chicks after S-Adenosylmethionine injection

Before being group-caged on day of hatch, chicks were randomly assigned to receive either 0 (vehicle), 0.1, 1, or 10 μg of SAM by ICV injection (n = 20 in each group, starting number, and two trials were conducted). After SAM injection, chicks were group-caged and then individually caged as described in 2.1, where they had free access to food and water prior to NPY injection. On day 5 posthatch, chicks were randomly assigned to receive either 0 (vehicle) or 0.2 μg of NPY (equal numbers of both treatments within each SAM-treated group) by ICV injection (n = 10 in each sub-group). BWs for each SAM treatment with 0 μg of NPY were 20.6 g, 20.5 g, 21.2 g, and 23.3 g, respectively (RMSE = 7.17; F (3, 137) = 1.19; P = 0.317). BWs for each SAM treatment with 0.2 μg of NPY were 21.2 g, 21.4 g, 21.4 g, and 20.8 g, respectively (RMSE = 2.89; F (3, 135) = 0.39; P = 0.762). After injection, chicks were returned to their individual home cages and given *ad libitum* access to both food and water. Food intake was monitored (0.01 g) at 60 min postinjection. Male (%) in each SAM treatment with 0 μg of NPY were 33, 48, 56, and 27, respectively. Male (%) in each SAM treatment with 0.2 μg of NPY were 67, 33, 30, and 56, respectively.

Experiment 4: Food and water intake in 5-Azacytidine-injected fed low weight-selected chicks

Procedures were identical to Experiment 1, except that chicks were treated with either 0 (vehicle), 1, 5, or 25 μg of AZA by ICV injection (n = 14 in each group, starting number, and two trials were conducted). The doses were based on a previous study (Qiang et al., 2015). BWs for each treatment were 25.3 g, 24.3 g, 25.1 g, and 24.4 g, respectively (RMSE = 3.04; F (3, 85) = 0.59; P = 0.626). Male (%) in each AZA treatment were 71, 36, 48, and 57, respectively.

Experiment 5: Food and water intake in 5-Azacytidine-injected fasted low weight-selected chicks

Procedures were identical to Experiment 4, except that chicks were fasted for 180 min before AZA injection (n = 16 in each group, starting number, and two trials were conducted). BWs for each treatment were 25.5 g, 25.6 g, 27.0 g, and 24.7 g, respectively (RMSE = 3.46; F (3, 106) = 2.09; P = 0.106). Chicks had *ad libitum* access to water but not food, when fasted. Male (%) in each AZA treatment were 50, 59, 52, and 41, respectively.

Experiment 6: Food intake in neuropeptide Y-injected low weight-selected chicks after 5-Azacytidine injection

Procedures were identical to Experiment 3, except that chicks were first treated with either 0 (vehicle), 1, 5, or 25 μg of AZA on day of hatch (n = 24 in each group, starting number, and two trials were conducted). BWs for each AZA treatment with 0 μg of NPY were 21.4 g, 18.9 g, 19.5 g, and 18.6 g, respectively (RMSE = 2.89; F (3, 129) = 6.50; P = 0.0004). BWs for each AZA treatment with 0.2 μg of NPY were 20.9 g, 18.9 g, 19.6 g, and 20.0 g, respectively (RMSE = 3.40; F (3, 119) = 2.26; P = 0.085). After AZA injection, as described in Experiment 3 for SAM, chicks were given *ad libitum* access to food and water prior to NPY injection. Male (%) in each AZA treatment with 0 μg of NPY were 50, 44, 67, and 33, respectively. Male (%) in each AZA treatment with 0.2 μg of NPY were 33, 56, 48, and 41, respectively.

Statistical analyses

Food and water intake data for each experiment were expressed as a percentage of BW on a cumulative basis before statistical analysis. For all experiments, data were analyzed by ANOVA with SAS 9.4 (SAS Institute, Cary, NC, USA) using the GLM procedure. The interaction between dose effect and sex (or trial) was evaluated first. When the interaction was not significant, the effect of sex was not considered in the following analyses, while the trials were pooled. Otherwise, the main effect of sex (or trial) was evaluated. For experiments 1, 2, 4, and 5, further analyses were performed within each time point, with the statistical model including the main effect of dose (SAM or AZA). When the main effect was significant, Tukey's method of multiple comparisons was then used to separate the means. For experiments 3 and 6, the interaction between SAM (or AZA) and NPY was further evaluated. When the interaction was not significant, the main effect of dose (NPY, and SAM or AZA) was then evaluated. Statistical significance was set at $P < 0.05$ for all experiments.

Results

Effects of sex and trial

There was no interaction between dose and sex (or trial) and no main effect of sex (or trial) for all experiments ($P > 0.05$). Thus, sex was not considered in further analyses and only the main effect of dose was evaluated, with different trials being pooled.

Experiments 1 and 2: Food and water intake in S-Adenosylmethionine-injected low weight-selected chicks

SAM had no effect on food or water intake at any dose or any time point in fed (Fig. 1A and B, respectively) or fasted (Fig. 2A and B, respectively) chicks on day 5 posthatch.

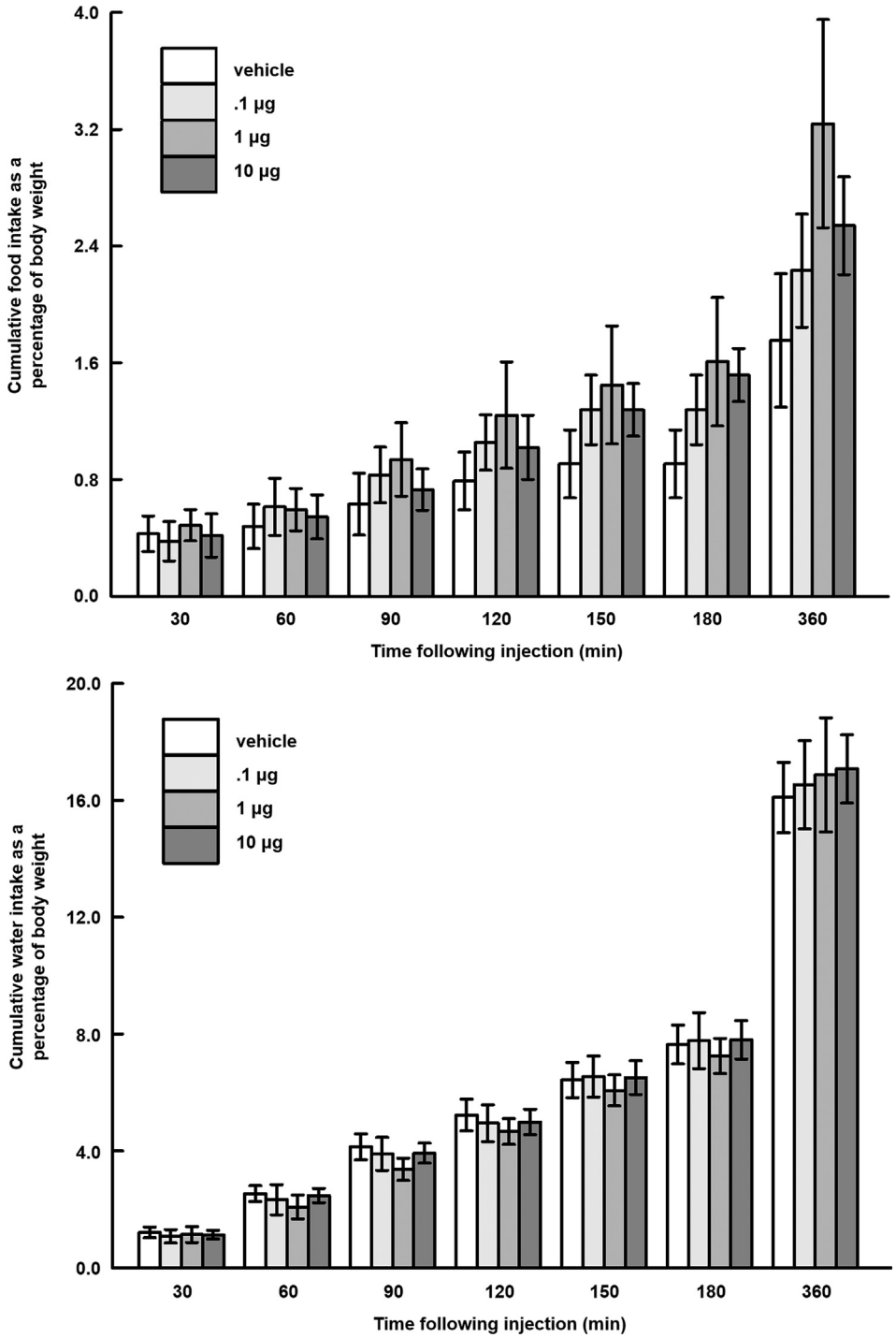

Fig. 1. Food and water intake in fed LWS chicks injected with SAM on day 5 posthatch. Cumulative food (A, $n = 9$ per group) and water (B, $n = 8–10$ per group) intake of 5-day-old fed low weight-selected (LWS) chicks that were intracerebroventricularly injected with 0 (vehicle), 0.1, 1, or 10 µg of S-Adenosylmethionine (SAM) on day 5 posthatch. Values represent means ± SE.

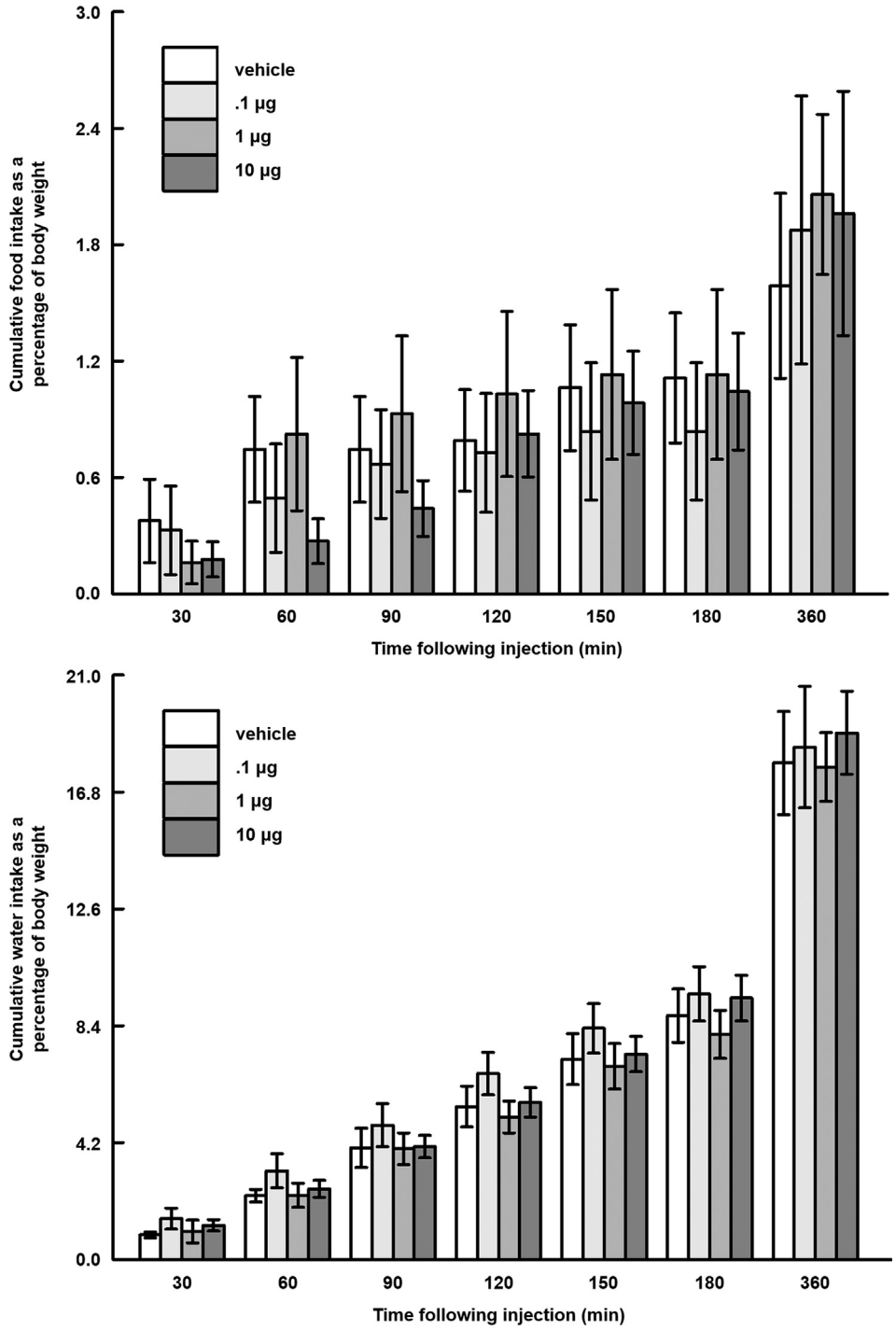

Fig. 2. Food and water intake in fasted LWS chicks injected with SAM on day 5 posthatch. Cumulative food (A, n = 9–10 per group) and water (B, n = 9–10 per group) intake of 5-day-old fasted low weight-selected (LWS) chicks that were intracerebroventricularly injected with 0 (vehicle), 0.1, 1, or 10 µg of S-Adenosylmethionine (SAM) on day 5 posthatch. Values represent means ± SE.

Experiment 3: S-Adenosylmethionine injection on day of hatch and neuropeptide Y injection on day 5: Food intake in low weight-selected chicks

There was no significant interaction between SAM and NPY injections, F (3, 272) = 0.60, P = 0.617 (Fig. 3). The main effects of SAM (F (3, 272) = 15.11, P < 0.0001) and NPY (F (1, 272) = 30.47, P < 0.0001) on food intake were both significant, with greater food consumption in chicks that received the 1 µg SAM dose compared to other groups, while NPY-injected chicks ate more than vehicle-injected counterparts (Fig. 3).

Experiment 4: Food and water intake in 5-Azacytidine-injected fed low weight-selected chicks

At 30 min postinjection, food intake was not different among groups. At 60 min and continuing through 180 min, food intake was reduced (as compared to vehicle-injected) in chicks that

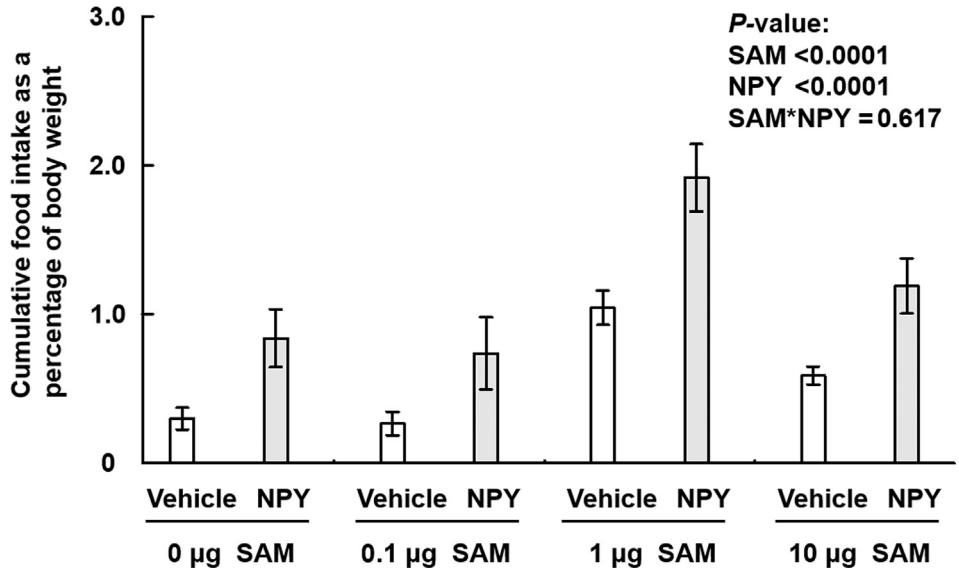

Fig. 3. Food intake in LWS chicks injected with SAM on day of hatch and NPY on day 5 posthatch. Food intake at 60 min postinjection of vehicle or neuropeptide Y (NPY) in 5-day-old fed low weight-selected (LWS) chicks that received a single intracerebroventricular injection of vehicle, 0.1, 1, or 10 µg of S-Adenosylmethionine (SAM) on the day of hatch (n = 31–38 per group). Values represent means ± SE.

received the 25 µg dose of AZA (Fig. 4A). At 90 through 180 min postinjection, food intake was also reduced in 1 µg-injected chicks. At 360 min, there were no differences among groups. The only differences observed for water intake were decreased in the 1 µg-injected chicks (relative to vehicle) at 60 and 90 min postinjection (Fig. 4B).

Experiment 5: Food and water intake in 5-Azacytidine-injected fasted low weight-selected chicks

When chicks were fasted prior to injection, the 25 µg dose of AZA was associated with increased food intake at 30 through 360 min postinjection (Fig. 5A). The only other difference was an increase in food intake for the 5 µg-injected group at 360 min postinjection. There were no effects of AZA on water intake in fasted chicks at any of the time points (Fig. 5B).

Experiment 6: 5-Azacytidine injection on the day of hatch and neuropeptide Y injection on day 5: Food intake in low weight-selected chicks

No significant interaction between AZA and NPY injection was observed, F (3, 248) = 1.87, P = 0.135 (Fig. 6). However, the main effect of AZA (F (3, 248) = 5.32, P = 0.0014) on food intake was significant such that chicks injected with 5 and 25 µg doses of AZA ate less than the vehicle- and AZA (1 µg)-injected birds (Fig. 6). The main effect of NPY (F (1, 248) = 9.72, P = 0.0020) on food intake was also significant, with greater food intake in chicks injected with NPY compared to those injected with vehicle (Fig. 6).

Discussion

Although it is well established that certain chemicals and nutrients can alter DNA methylation and thereby alter gene expression and ultimately behavior, the effects of direct administration of these chemicals on feeding behavior are unknown. It is also unclear whether such molecules can exert effects on feeding behavior that are independent of epigenetic-modifying effects. In the present study, we measured the effects of two such chemicals, SAM and AZA, on food and water intake using an avian model that is predisposed to anorexia. Evaluated were both direct and longer-term effects on feeding behavior. SAM and AZA were administered directly into the brain and in addition to quantifying food consumption, we evaluated the responsiveness to injection of a potent orexigenic factor to assess whether they had affected appetite regulation. Interestingly, SAM and AZA exerted different effects on food intake, both immediate and persistent, that were dependent of the feeding states of the chick. For water intake, we only observed transient effect in AZA-injected fed LWS chicks, while there were no influences found in other cases. Thus, AZA appeared to have limited effects on water intake, likely prandial. This is possibly because the main signaling pathways mediating water intake were not affected by either SAM or AZA. Typically, in studies like these, the effects of certain molecule on food and water intake follow a similar trend (increasing, decreasing, or no effects). A previous study found that a high dose of SAM (100 µg) only showed a tendency on decreasing food intake (Bungo and Shiraishi, 2010). Our doses were much lower than this. Thus, it is not surprising that we did not observe the effects on water intake. Since both SAM and AZA are epigenetic modifiers, their effects might take longer to appear. However, the investigation of effects on water consumption is beyond the scope of the present study. Our discussion will thus focus primarily on food intake effects and mechanisms.

SAM is the primary endogenous methyl donor and is critical for normal cellular methyl metabolism. Studies with various animal models have shown that dietary supplementation with methyl donors and participants in methyl metabolism can alter hypothalamic epigenetic regulation and thus affect gene expression and behavior (Schroeder et al., 2017). Rats that consumed a high-fat diet for 5 weeks displayed changes in hypothalamic NPY and pro-opiomelanocortin (**POMC**) promoter methylation that were inversely correlated with mRNA abundance (Cifani et al., 2015). High-fat diets fed during pregnancy led to elevated levels of POMC DNA methylation in rat pups and an obese phenotype at a later age (Marco et al., 2014). However, to our knowledge, information on hypothalamic DNA methylation in the context of appetite regulation is lacking in birds. Additionally, of relevance to putative effects on appetite regulation, SAM is required for the synthesis of monoamine neurotransmitters such as norepinephrine, dopamine, and serotonin, and other cellular pathways, as reviewed in a previous

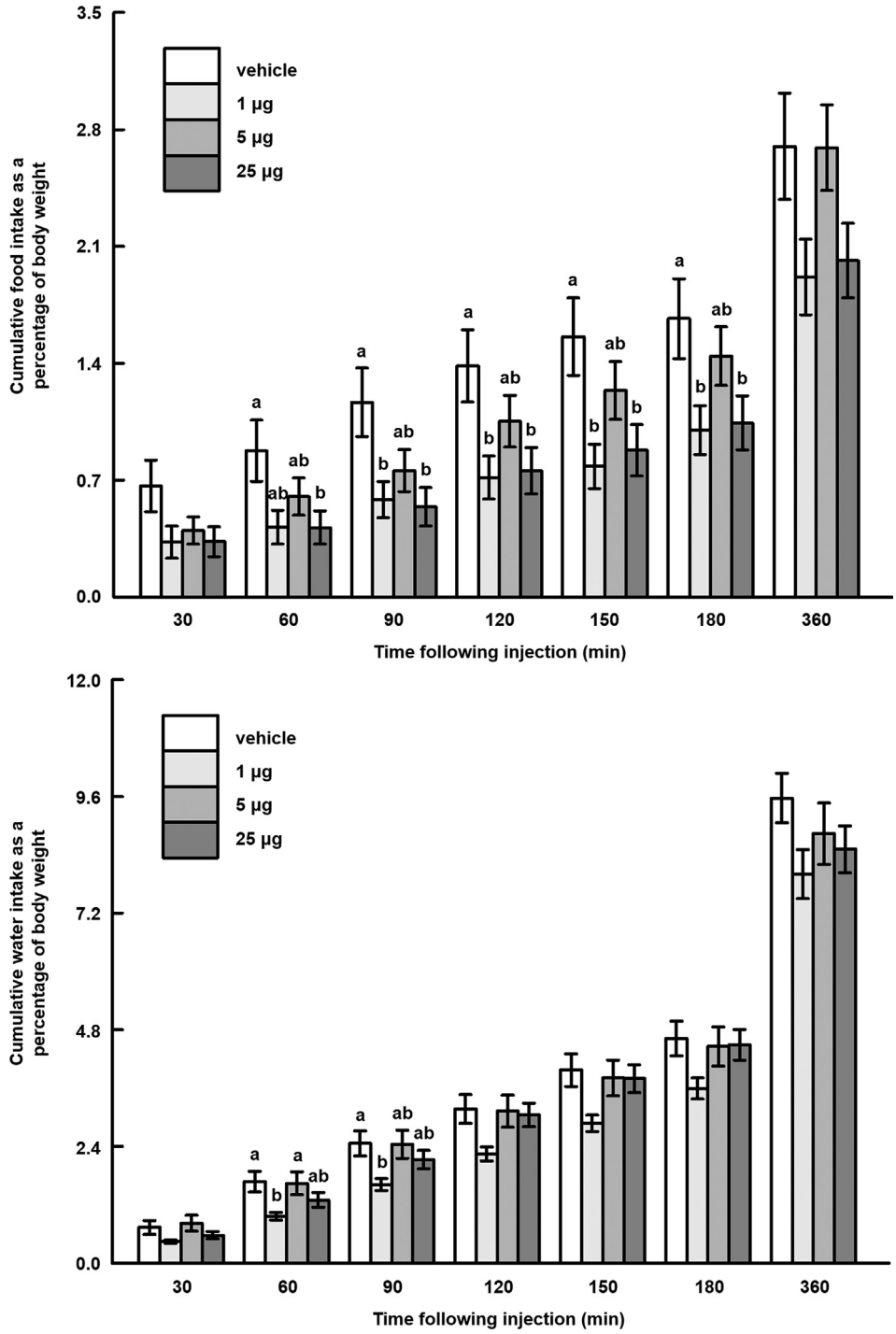

Fig. 4. Food and water intake in fed LWS chicks injected with AZA on day 5 posthatch. Cumulative food (A, n = 21–23 per group) and water (B, n = 25–28 per group) intake of 5-day-old fed low weight-selected (LWS) chicks that were intracerebroventricularly injected with 0 (vehicle), 1, 5, or 25 µg of 5-Azacytidine (AZA) on day 5 posthatch. Values represent means ± SE. Unique letters denote a significant difference (P < 0.05) among groups within a time point, Tukey's test.

study (Mischoulon and Fava, 2002). Thus, it is conceivable that the provision of SAM to the central nervous system might influence feeding behavior acutely.

In the present study, SAM injection on day 5 posthatch did not influence food or water intake of either fed or fasted LWS chicks. Our results were consistent with a study using 3-day-old layer-type chicks in the fed state (effects were not tested in the fasted state). ICV injection of SAM at doses not higher than 10 µg did not affect food intake at 30 min postinjection (Bungo and Shiraishi, 2010). It was stated that a 100 µg dose of SAM "tended" to decrease food intake, but the difference was not significant (Bungo and Shiraishi, 2010). This dose of SAM (100 µg, 0.2 µmol) was neither reported nor considered to induce toxicity, as a rat study found that a single ICV injection of SAM (1 µmol) did not cause brain damage (Charlton and Mack, 1994). Moreover, in an *in vitro* cell model, 1–5 mmol/L (2.5–12.5 µg) of SAM exposure to

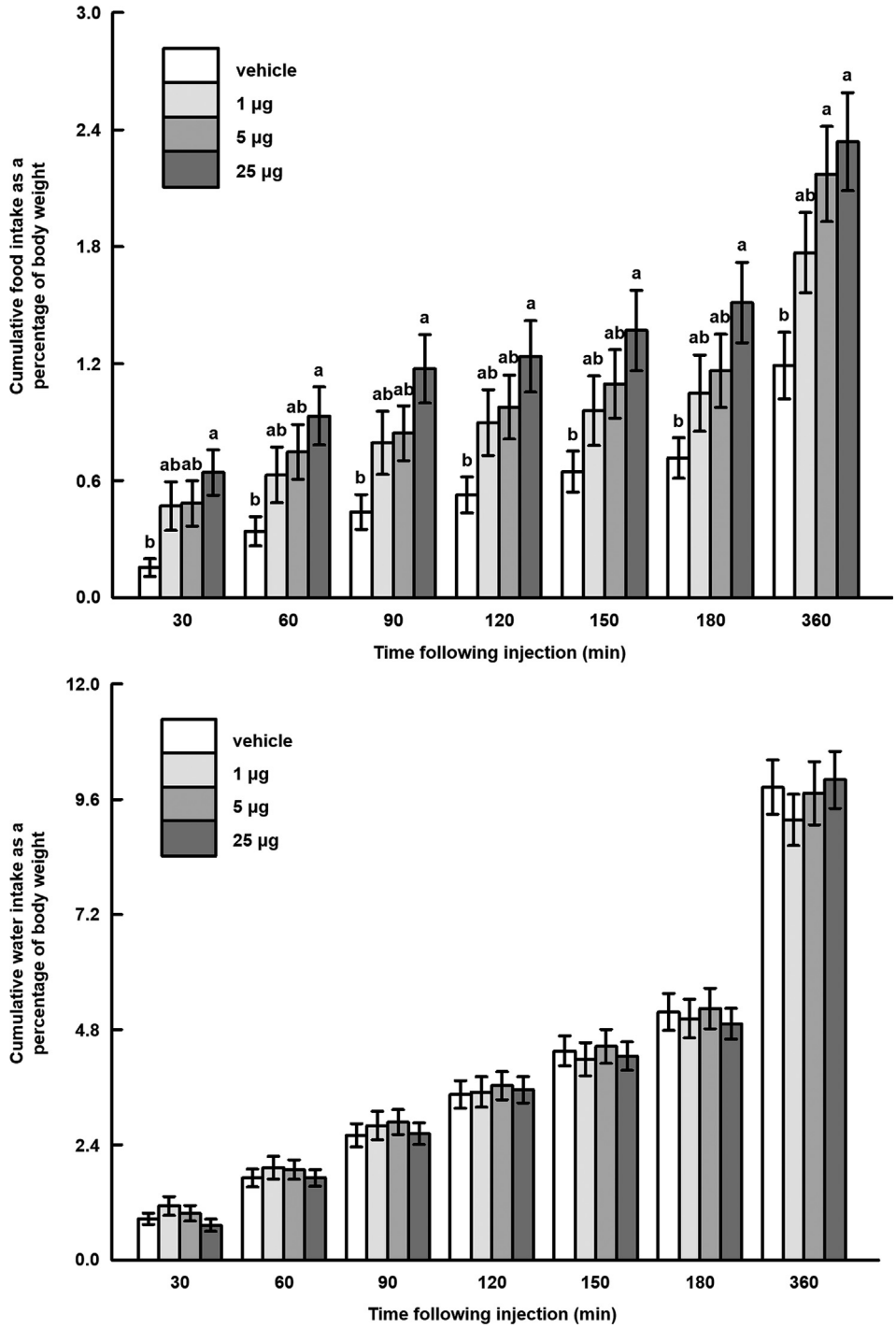

Fig. 5. Food and water intake in fasted LWS chicks injected with AZA on day 5 posthatch. Cumulative food (A, n = 25–31 per group) and water (B, n = 30–31 per group) intake of 5-day-old fasted low weight-selected (LWS) chicks that were intracerebroventricularly injected with 0 (vehicle), 1, 5, or 25 µg of 5-Azacytidine (AZA) on day 5 posthatch. Values represent means ± SE. Unique letters denote a significant difference (P < 0.05) among groups within a time point, Tukey's test.

cells did not affect the viability and elicit cytotoxicity (Lozano-Sepulveda et al., 2016). We thus based our initial dose tests on the doses used in the layer chick study but arrived at a lower dose range (0.1, 1, and 10 µg) as ones that would affect physiology without being toxic to the animal. The dose of 100 µg SAM will be utilized in future research but is beyond the scope of current study. We conclude from the data presented herein that SAM does not appear to have a direct short-term effect on feeding behavior in LWS chicks over the range used.

However, when SAM was injected at day of hatch, we observed differences in food intake among SAM-injected groups on day 5 posthatch. Regardless of NPY treatment, chicks that received the middle dose of SAM ate more than other chicks. The greater intake of 1 µg-injected chicks relative to other groups suggests that while SAM did not have an immediate effect on food intake, it elicited changes in appetite-related pathways that were manifested at a later age. Given that the LWS chickens are selected for a lower BW and show severe symptoms of anorexia, an anorexigenic tone

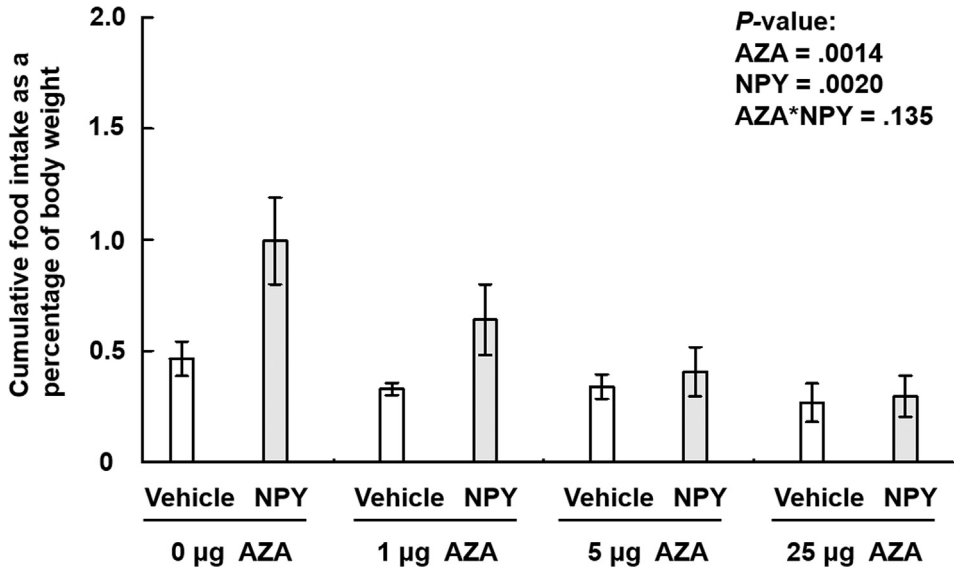

Fig. 6. Food intake in LWS chicks injected with AZA on day of hatch and NPY on day 5 posthatch. Food intake at 60 min postinjection of vehicle or neuropeptide Y (NPY) in 5-day-old fed low weight-selected (LWS) chicks that received a single intracerebroventricular injection of vehicle, 1, 5, or 25 µg of 5-Azacytidine (AZA) on the day of hatch (n = 20–42 per group). Values represent means ± SE.

may predominate within their hypothalamus (Yi et al., 2017) and central administration of a methyl donor may lead to changes in gene expression of some appetite-related factors that affect feeding-related circuits in the brain, such as the CRF pathway. Previously, we demonstrated that day of hatch stress-induced blunted responses to NPY on day 5 coincided with increased CRF gene expression in the hypothalamus (Yi et al., 2016; Wang et al., 2017) that was associated with hypomethylation at a site near the CRF promoter that disrupted binding of a transcriptional repressor (Xiao et al., 2020). It is thus tempting to speculate that because such changes were associated with DNA methylation, the provision of a methyl donor would potentially alter the expression of anorexigenic factors such as CRF, leading to a more sensitized response to orexigenic factors at a later age. However, since we did not observe an interaction between SAM and NPY injection in the present study, the CRF pathway might not be affected by SAM, leaving the possibility that other anorexigenic neuropeptides, like α-MSH, may be influenced. Thus, early-life SAM provision may have resulted in DNA methylation changes that were associated with reduced hypothalamic anorexigenic tone and greater food consumption at a later age.

In contrast to SAM, the methylation inhibitor AZA affected food intake within 1 h postinjection, with opposite effects on food intake observed under different feeding states. In fed chicks, AZA suppressed food intake, whereas when chicks had been fasted for 3 h, AZA had a stimulatory effect. This observation is intriguing because there are far fewer factors in nature that stimulate the appetite than those that induce satiety (Denbow and Cline, 2015). Important to note is that the orexigenic effect in fasted chicks appeared earlier and persisted for a longer duration than the anorexigenic effect in fed chicks. Typically, these types of feeding studies in the chick model are conducted within a period of 180 min during which there is cessation of the effect and restoration of homeostatic feeding (Cline et al., 2007; Bohler et al., 2019). It is uncommon to observe an effect (following a single ICV injection) at a later time. However, because of the potential for these molecules to elicit long-term effects due to their role in regulating epigenetic modifications (Zhou et al., 2009), we also included a 360-min time point for data collection. Chicks that were fasted prior to injection continued to have greater food intake at 360 min postinjection. That the high dose elicited an increase in food intake as early as 30 min that continued through 6 h suggests that the injection during the fasting state accentuated orexigenic signaling pathways while having the opposite effect during the fed state. It is possible that AZA's effect on DNMT activity, while global in terms of affecting overall DNA methylation (Wang et al., 2011), may have led to changes at methylation-susceptible loci, such as the CpG that we previously identified in the promoter of the CRF gene in LWS chicks (Xiao et al., 2020). Such effects may persist with time as we observed with stress-induced anorexia in the LWS chicks in our earlier studies (Yi et al., 2016; Wang et al., 2017). Future studies will evaluate whether AZA-related effects are associated with changes in CRF methylation.

Chicks that were injected with AZA, especially higher doses, at day of hatch displayed reduced food intake. It is enticing to conjecture that the opposite effects of day of hatch injection of SAM and AZA relate to methylation of appetite-associated factors, such as hypothalamic POMC or CRF. The administration of these chemicals has the potential to rewire the appetite circuitry by affecting the methylation of genes encoding key factors in feeding pathways. Elucidating such mechanisms was beyond the scope of the present study but is a target for future research.

Conclusions

In conclusion, we identified methylation-regulating chemicals as regulators of appetite in animals, using a unique avian model of hypophagia, the LWS line of chicks. We evaluated the effects of centrally injecting a methyl donor (SAM) and methylation inhibitor (AZA) on feeding behavior, immediately after injection, and days after injection, and observed multiple effects. While SAM did not directly affect feeding behavior after injection, it increased feeding 5 days later. Conversely, AZA had an inhibitory effect on food intake immediately after injection in fed chicks, but the opposite effect when they were fasted prior to injection. Consistent with the reduced food consumption in fed chicks, AZA affected food intake when chicks were injected with AZA at hatch, which is likely related to methylation of anorexigenic pathways. Thus, SAM and AZA exerted different effects on food intake, with some being immediate and feeding-dependent, while others persisted for days

after injection. To our knowledge, this is the first report, in any species, of the effects of AZA on feeding behavior.

Ethics approval

All animal protocols were approved by the Institutional Animal Care and Use Committee at Virginia Tech (#18-276).

Data and model availability statement

None of the data were deposited in an official repository. The data are available upon reasonable request to the corresponding author.

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M.A. Cline: Conceptualization, Methodology, Validation, Resources, Data Curation, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

Declaration of interest

The authors declare no conflict of interest.

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