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Research article

DNA methylation-modifiers reduced food intake in juvenile chickens (*Gallus gallus*) and Japanese quail (*Coturnix japonica*)

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ABSTRACT

S-Adenosylmethionine (SAM) is the major endogenous methyl donor for methyltransferase reactions, while 5-Azacytidine (AZA) is a synthetic drug inhibiting DNA methyltransferase activity. Both molecules can thus influence DNA methylation patterns in an organism and thereby affect gene expression and ultimately behavior in the long-term. Whether or not effects on behavior are exerted on a shorter time scale is unclear. The goal of this study was to explore the direct effects of SAM and AZA on appetite regulation, using broiler chicken and Japanese quail as the animal models. Fed or 180 min-fasted broilers (at day 4 post-hatch) or 360 min-fasted quail (at day 7 post-hatch) were intracerebroventricularly injected with SAM or AZA and food intake was measured for 360 min. For broilers, there was no effect of AZA, at any dose, on food intake in either fed or fasted chicks at any time point. In contrast, 1 and 10 μg doses of SAM reduced food intake in fed chicks at 60 min post-injection. In fasted chicks, although there were no differences for the first 30 min post-injection, SAM suppressed food intake during the second 30-min period. For quail, however, AZA (25 μg dose) decreased food intake at 60 and 150–360 min post-injection in fasted birds. A reduction in food intake was also observed at 120- and 360-min post-injection in fed quail in response to 5 and 25 μg doses of AZA, respectively. SAM had no effect when quail were fasted, whereas 1 μg dose of SAM suppressed food consumption in fed quail during the third 30-min period. Thus, when administered directly into the central nervous system, SAM may act as a transient appetite suppressant in both broilers and quail, whereas the direct inhibitory effect of AZA on food consumption depends on species and nutritional states.

1. Introduction

Appetite regulation is the result of a complex interplay between pathways that integrate at the level of the hypothalamus. Many of these pathways involve various hormones and neuropeptides originating from different organs and brain regions that communicate the energy and health status of the organism [2,22]. Novel regulators of appetite continue to be identified. To address the mounting obesity epidemic and understand the molecular basis for eating disorders, it is critical to have a complete understanding of the physiological pathways that regulate appetite.

Much of the knowledge on appetite regulation in avian species was derived from studies with chickens (*Gallus gallus*). Broilers have been bred for efficient meat production for over a century [12], with extensive studies focusing on improving feeding efficiency and welfare. Selection for growth-related traits led to correlated effects on feeding behavior and appetite regulation, with chickens consuming food beyond maintenance and growth energy requirements and being prone to metabolic disorders later in life. Broilers can thus serve as a model to understand compulsive eating behavior and obesity. On the other hand, Japanese quail (*Coturnix japonica*), although well-adapted to both laboratory conditions and handling procedures, is a less intensely-selected species, which may provide evolutionary perspective on physiological mechanisms that are conserved between birds and mammals.

Our group has identified a number of appetite regulators, mostly peptides, that when directly administered into the central nervous system of chicks, influence food consumption and hypothalamic physiology. For example, intracerebroventricular (ICV; into the left lateral ventricle) administration of neuropeptide Y potently stimulated feed intake [27], while melanocortins (such as α- and β-melanocyte-stimulating hormone) [7], corticotropin-releasing factor [24] and mesotocin [15] exerted anorexigenic effects, to name a few. In all of those studies,

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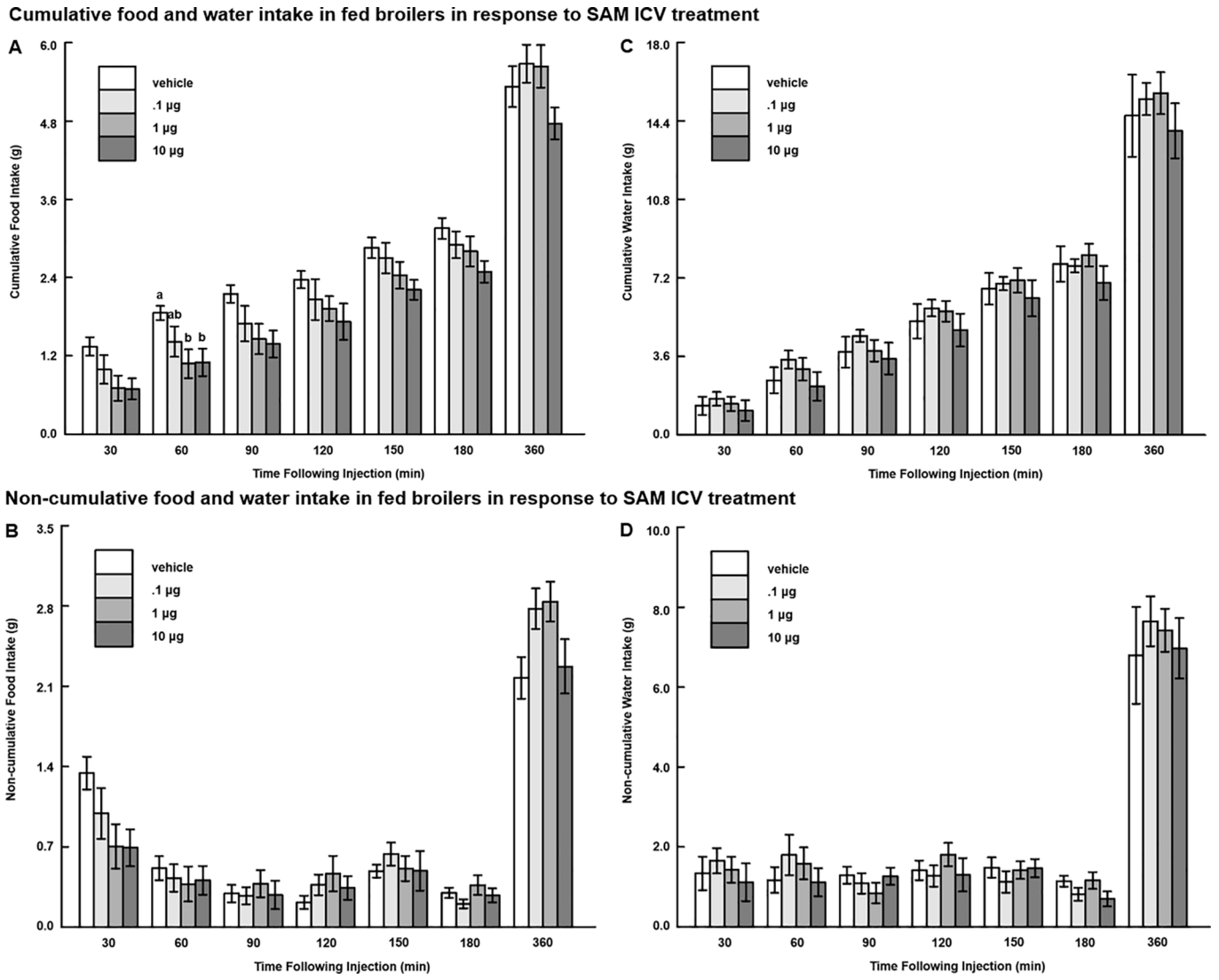

Fig. 1. Food and water intake in SAM-injected fed broiler chickens. Cumulative (A) and non-cumulative (B) food intake at times post-injection of 4-day-old fed broiler chicks (*Gallus gallus*) that were intracerebroventricularly (ICV) injected with 0 (vehicle), 0.1, 1, or 10 µg of SAM (n = 9–10 per group). Cumulative (C) and non-cumulative (D) water intake at times post-injection of the same chicks (n = 10 per group). Values represent means ± standard errors. Unique letters denote a significant difference ($P < 0.05$) among groups within a time point, Tukey's test. Note that in A, 1 and 10 µg of SAM injection tended to decrease food intake in broilers at 30 min post-injection relative to vehicle injection ($P = 0.07$).

changes in feeding were accompanied by distinct changes in neuronal activation and gene expression of appetite-associated factors in hypothalamic nuclei, such as the arcuate nucleus and paraventricular nucleus. We have also identified novel regulators of appetite, that previously had no appetite-related roles ascribed to their function in any species, including visfatin [6] and xenopsin [14]. While most factors that exert effects on appetite are proteinaceous, it is clear that other bioactive molecules have the potential to affect appetite as well.

S-Adenosylmethionine (SAM), the principal methyl donor in the body [13], is an essential component of the methionine cycle [10,16]. It can directly influence DNA methylation and thereby affect gene expression, and there are many rodent studies focusing on the effects of methyl donors on DNA methylation as a result of dietary deficiency [18,19] or supplementation [25,26]. Researchers evaluated the influence of methyl analogues on feeding in Leghorn chicks. Both L- and D-methionine could stimulate food intake, whereas SAM appeared to suppress feeding at a high dose (100 µg), although no significance was detected [3]. These results were controversial, since methionine could be converted into SAM and thereby provide a methyl group to other substrates, leaving the role of central-injected SAM on appetite regulation remaining unclear.

At the other end of the spectrum, there are molecules that can inhibit or prevent DNA methylation. 5-Azacytidine (AZA) was first synthesized almost 60 years ago [17], and was demonstrated to be a chemotherapeutic agent [4]. With its ability to incorporate into DNA at high concentrations, AZA inhibited DNA synthesis in tumor cells [23]. However, at low doses, AZA irreversibly binds to DNMT and inhibits its activity, leading to reductions in maintenance DNA methylation [5,8].

The objective of this study was thus to evaluate the effects of two molecules known for their role in DNA methylation, SAM and AZA, on appetite regulation in broiler chicks and Japanese quail.

2. Materials and methods

2.1. Animals

Day-of-hatch Cobb-500 broiler chicks were obtained from a local hatchery. Chicks were group-caged the same day and then individually caged on day 2 post-hatch in a room at a constant temperature of 30 ± 2 °C and 50 ± 5% relative humidity, with 24-h of light. Chicks were

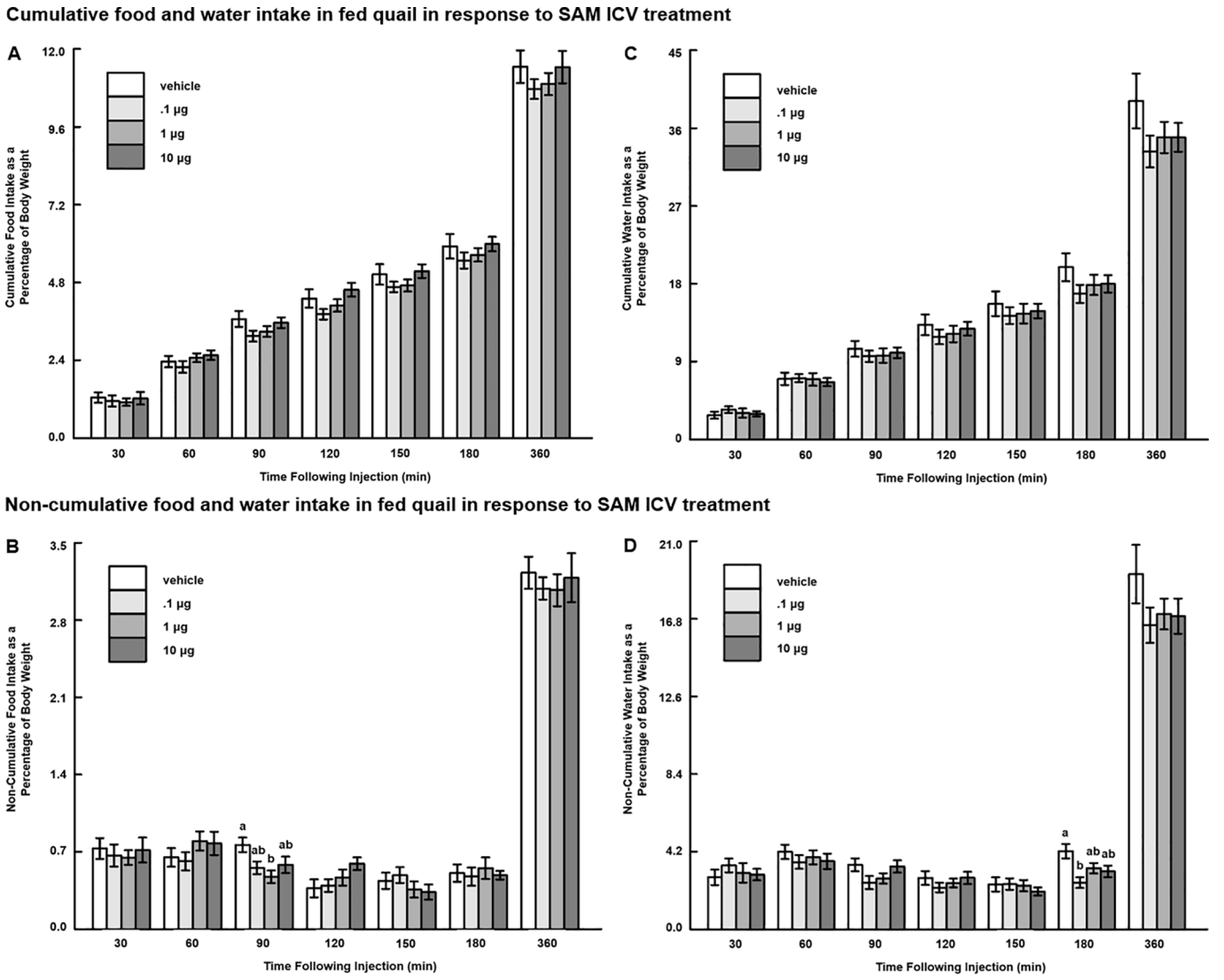

Fig. 2. Food and water intake in SAM-injected fed quail. Cumulative (A) and non-cumulative (B) food intake as a percentage of body weight at times post-injection of 7-day-old fed quail (*Coturnix japonica*) that were intracerebroventricularly (ICV) injected with 0 (vehicle), 0.1, 1, or 10 µg of SAM (n = 10–12 per group). Cumulative (C) and non-cumulative (D) water intake as a percentage of body weight at times post-injection of the same chicks (n = 11–12 per group). Values represent means ± standard errors. Unique letters denote a significant difference ($P < 0.05$) among groups within a time point, Tukey's test.

briefly handled for 5 s, once daily, to adapt to handling and minimize stress, and had ad libitum access to food (energy: 3000 kcal metabolizable energy/kg and 21.5% crude protein) and water.

Japanese quail were bred and hatched in our vivarium. After removal from the hatcher, chicks were group-caged in a brooder for 4 days, then individually caged in a separate room (35 ± 1 °C and 50 ± 5% relative humidity), with a 14-h light/10-hour dark cycle. The individual cages allowed visual and auditory contact between chicks. Quail were provided ad libitum access to a mash starter diet (energy: 2900 kcal metabolizable energy/kg and 24% crude protein) and water. Quail handling was different from broilers and the details are given below.

Each quail was acclimated twice daily once individually caged. The acclimation procedure consisted of the chick being removed from its cage, being briefly transferred to two different Plexiglas boxes, having its head inserted into a restraining device for 5 s, transferred into another Plexiglas box, and then placed into the restraining device once again. Upon completion of this procedure, the chick was returned to its home cage. The restraining device was a block of hardened clay, which had been molded around the head of an 8-day-old quail chick cadaver and was designed such that the entire area of the frontal bone was not obstructed and was designed with two air vents positioned to end at the nostrils. This device allowed for a free-hand ICV injection. The acclimation procedure was conducted leading up to the day of data collection.

All broiler experiments were conducted at 4 days post-hatch, whereas all quail experiments were done at 7 days post-hatch. These ages were selected for consistency with our previous food intake studies. An older age of quail is used because of its slower rate of development relative to the broiler chicken. Experimental procedures were performed according to the National Research Council Publication, Guide for Care and Use of Laboratory Animals and were approved by the Virginia Tech Institutional Animal Care and Use Committee.

2.2. Intracerebroventricular injection procedure

On the day of the experiment, birds were ICV-injected using a method that does not appear to induce physiological stress, adapted from [9,11,21]. The head of the bird was briefly inserted into a restraining device that left the cranium exposed to allow for free-hand injection. Anatomical landmarks were determined visually by using the restraining device and plastic tubing sheath. The quail restraining device coordinated the injection point at 2 mm anterior to the coronal

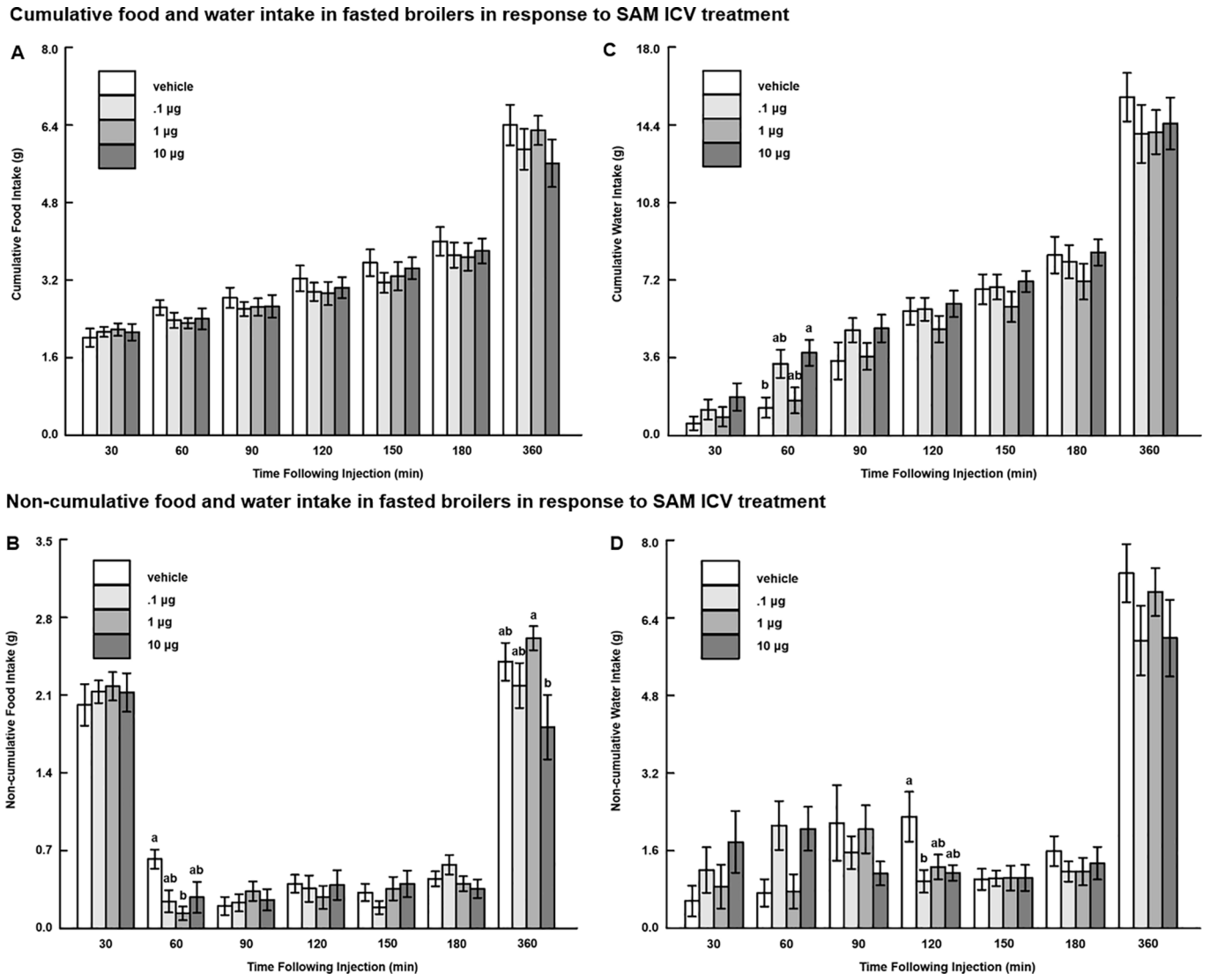

Fig. 3. Food and water intake in SAM-injected fasted broiler chickens. Cumulative (A) and non-cumulative (B) food intake at times post-injection of 4-day-old fasted broiler chicks (*Gallus gallus*) that were intracerebroventricularly (ICV) injected with 0 (vehicle), 0.1, 1, or 10 µg of SAM (n = 7–9 per group). Cumulative (C) and non-cumulative (D) water intake at times post-injection of the same chicks (n = 10 per group). Values represent means ± standard errors. Unique letters denote a significant difference ($P < 0.05$) among groups within a time point, Tukey's test.

suture and 0.75 mm lateral from the sagittal suture. The plastic tubing sheath over the needle controlled the injection depth at 1.5 mm. Injection coordinates for broiler chickens, however, were 3 mm anterior to the coronal suture, 1 mm lateral from the sagittal suture, and 2 mm deep targeting the left lateral ventricle. The needle remained at injection depth in the un-anesthetized bird for 5 s post-injection to reduce backflow. Chicks were assigned to treatments at random. SAM or AZA (Sigma, St. Louis, MO, USA) was dissolved in artificial cerebrospinal fluid [1] 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 eliminated from analysis. Sex was determined visually by dissection and gonadal inspection at the time of decapitation.

2.3. Experiment 1: food and water intake in SAM-injected fed broilers and quail

Using a randomized complete design, chicks were assigned to receive 0 (vehicle only), 0.1, 1, or 10 µg dose of SAM, the doses of which were based on a former study [3], by ICV injection (in each group: n = 10 for broilers and n = 12 for quail, initial numbers). Body weights (BWs) for each SAM treatment in broilers were 83.5 g, 84.9 g, 85.8 g, and 83.9 g, respectively ($F(3, 33) = 0.13$; $P = 0.939$), and in quail were 16.1 g, 16.3 g, 16.1 g, and 14.7 g, respectively ($F(3, 39) = 3.48$; $P = 0.025$). After injection, chicks were returned to their individual home cages and given ad libitum access to both food and water. Food and water intake were monitored (0.01 g) every 30 min for the first 180 min post-injection and at 360 min post-injection. Water weight (g) was converted to volume (ml: 1 g = 1 ml). Food and water intake were calculated as a percentage of BW to better account for the variation in BWs of quail. Male (%) in each SAM treatment in broilers were 50, 56, 33, and 22, respectively, and in quail were 60, 27, 50, and 58, respectively.

2.4. Experiment 2: food and water intake in SAM-injected fasted broilers and quail

Procedures were identical to Experiment 1, except that broilers and quail were fasted for 180 and 360 min, respectively, before SAM

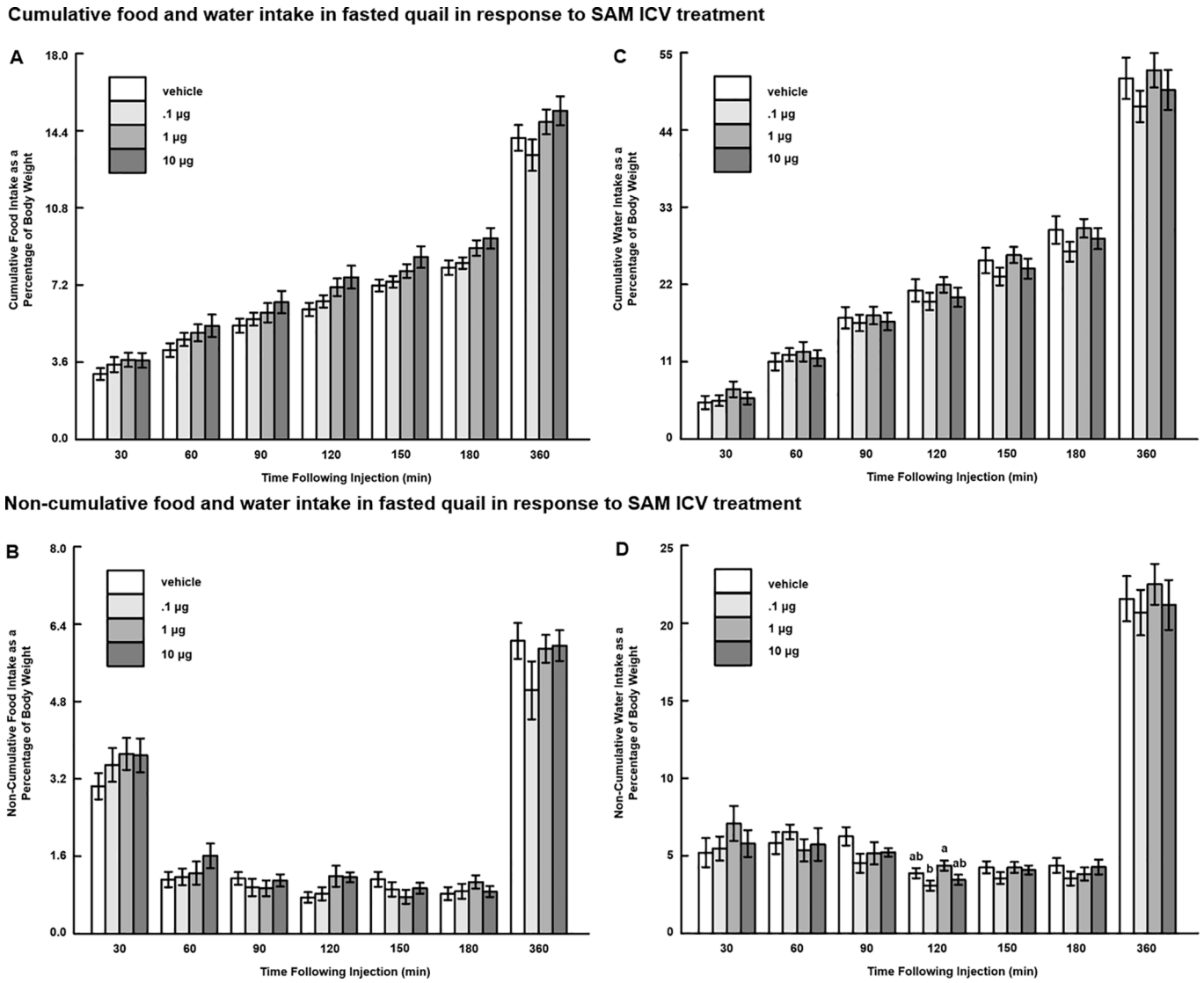

Fig. 4. Food and water intake in SAM-injected fasted quail. Cumulative (A) and non-cumulative (B) food intake as a percentage of body weight at times post-injection of 7-day-old fasted quail (*Coturnix japonica*) that were intracerebroventricularly (ICV) injected with 0 (vehicle), 0.1, 1, or 10 µg of SAM (n = 7–9 per group). Cumulative (C) and non-cumulative (D) water intake as a percentage of body weight at times post-injection of the same chicks (n = 11 per group). Values represent means ± standard errors. Unique letters denote a significant difference ($P < 0.05$) among groups within a time point, Tukey's test.

injection, while access to water remained during fasting. BWs for each SAM treatment in broilers were 78.3 g, 79.7 g, 81.9 g, and 78.2 g, respectively ($F(3, 30) = 0.42$; $P = 0.742$), and in quail were 13.2 g, 13.5 g, 13.0 g, and 14.2 g, respectively ($F(3, 36) = 1.26$; $P = 0.302$). Male (%) in each SAM treatment in broilers were 11, 43, 22, and 44, respectively, and in quail were 30, 50, 36, and 33, respectively.

2.5. Experiment 3: food and water intake in AZA-injected fed broilers and quail

Procedures were identical to Experiment 1, except that chicks were treated with 0 (vehicle only), 1, 5, 25 µg dose of AZA, the doses of which were based on a former study [20]. BWs for each AZA treatment in broilers were 81.1 g, 83.9 g, 84.1 g, and 82.4 g, respectively ($F(3, 34) = 0.17$; $P = 0.919$), and in quail were 13.3 g, 14.7 g, 14.0 g, and 13.4 g, respectively ($F(3, 36) = 1.25$; $P = 0.306$). Male (%) in each AZA treatment in broilers were 50, 67, 50, and 60, respectively, and in quail were 40, 27, 60, and 67, respectively.

2.6. Experiment 4: food and water intake in AZA-injected fasted broilers and quail

Procedures were identical to Experiment 3, except that broilers and quail had ad libitum access to water but no access to food for 180 and 360 min, respectively, prior to AZA injection. BWs for each AZA treatment in broilers were 75.3 g, 79.6 g, 80.4 g, and 76.3 g, respectively ($F(3, 31) = 0.97$; $P = 0.420$), and in quail were 14.1 g, 14.3 g, 13.7 g, and 13.9 g, respectively ($F(3, 38) = 0.27$; $P = 0.844$). Male (%) in each AZA treatment in broilers were 56, 63, 44, and 78, respectively, and in quail were 20, 50, 40, and 30, respectively.

2.7. Statistical analyses

Results are expressed as means ± standard errors. Data were analyzed using analysis of variance (ANOVA) with SAS 9.4 (SAS institute, Cary, NC, USA) using the GLM procedure within each time point, with the statistical model including the main effect of dose (SAM or AZA). When dose effects were significant, Tukey's method of multiple comparison was used to separate the means within each time point.

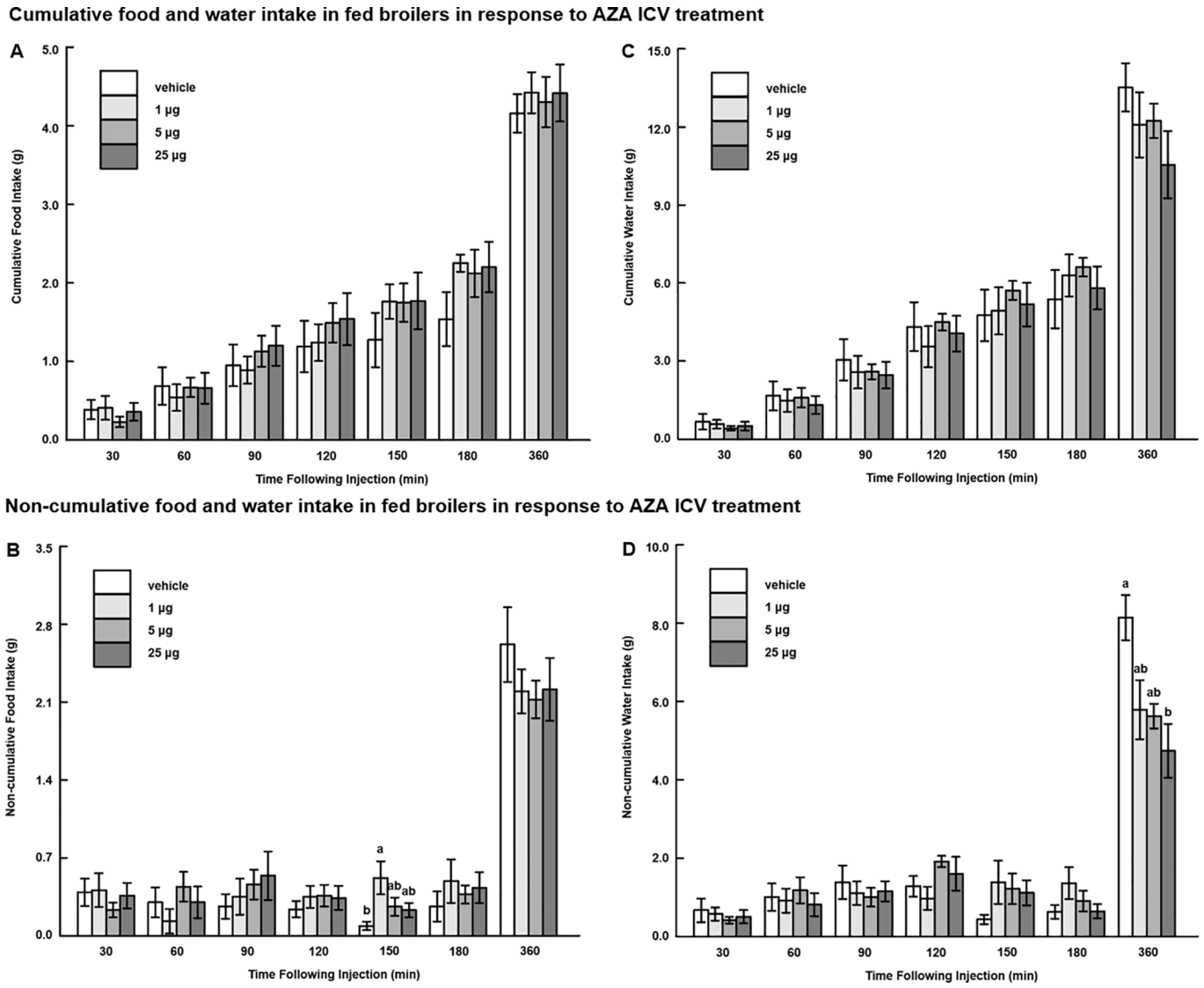

Fig. 5. Food and water intake in AZA-injected fed broiler chickens. Cumulative (A) and non-cumulative (B) food intake at times post-injection of 4-day-old fed broiler chicks (*Gallus gallus*) that were intracerebroventricularly (ICV) injected with 0 (vehicle), 1, 5, or 25 µg of AZA (n = 8–10 per group). Cumulative (C) and non-cumulative (D) water intake at times post-injection of the same chicks (n = 8–10 per group). Values represent means ± standard errors. Unique letters denote a significant difference ($P < 0.05$) among groups within a time point, Tukey's test.

Statistical significance was set at $P < 0.05$ for all experiments.

3. Results

3.1. Food and water intake in SAM-injected fed broilers and quail

At 60 min post-injection, food intake significantly decreased in broilers injected with 1 and 10 µg doses of SAM (Fig. 1A). There were no differences detected at any other time points or on a non-cumulative basis (Fig. 1A and 1B). Water intake was not affected by SAM injection in fed broilers (Fig. 1C and 1D).

SAM did not affect food intake at any time points on a cumulative basis in quail, but the 1 µg dose of SAM decreased food intake during the third 30-minute period post-injection on a non-cumulative basis (Fig. 2A and 2B). Water intake was not affected on a cumulative basis (Fig. 2C), but was suppressed by 0.1 µg of SAM at 180 min post-injection on a non-cumulative basis (Fig. 2D).

3.2. Food and water intake in SAM-injected fasted broilers and quail

For fasted broiler chickens, there were no effects of SAM on food intake (Fig. 3A). On a non-cumulative basis, however, 1 µg of SAM suppressed food intake at 60 min post-injection (Fig. 3B). We also detected a difference in food intake at 360 min post-injection between the 1 and 10 µg SAM-injected chicks. For water intake, there was an increase in response to the 10 µg dose of SAM at 60 min post-injection, compared to the vehicle group (Fig. 3C). At 120 min post-injection, 0.1 µg of SAM decreased water intake on a non-cumulative basis (Fig. 3D).

SAM had no effects on quail food intake (Fig. 4A and 4B) or cumulative water intake (Fig. 4C). However, a difference in water intake was detected between the 0.1 and 1 µg doses of SAM at 120 min on a non-cumulative basis (Fig. 4D).

3.3. Food and water intake in AZA-injected fed broilers and quail

Neither food nor water intake were changed in fed broilers in response to AZA injection on a cumulative basis (Fig. 5A and 5C). On a non-cumulative basis, however, the 1 µg dose of AZA increased food

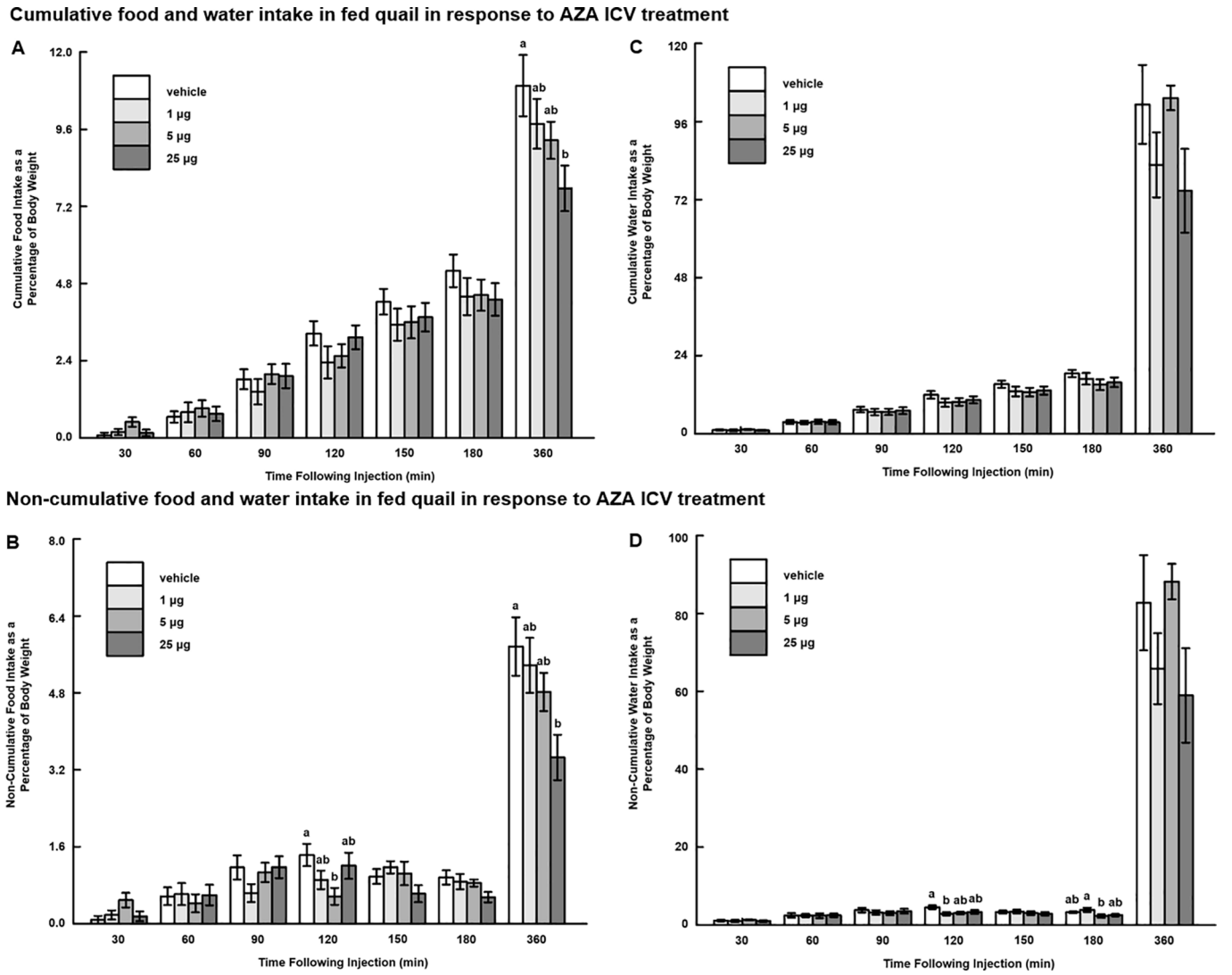

Fig. 6. Food and water intake in AZA-injected fed quail. Cumulative (A) and non-cumulative (B) food intake as a percentage of body weight at times post-injection of 7-day-old fed quail (*Coturnix japonica*) that were intracerebroventricularly (ICV) injected with 0 (vehicle), 1, 5, or 25 µg of AZA (n = 9–11 per group). Cumulative (C) and non-cumulative (D) water intake as a percentage of body weight at times post-injection of the same chicks (n = 9–11 per group). Values represent means ± standard errors. Unique letters denote a significant difference ($P < 0.05$) among groups within a time point, Tukey's test.

intake at 150 min post-injection (Fig. 5B), while the 25 µg dose of AZA decreased water intake at 360 min post-injection (Fig. 5D).

In fed quail, the 25 µg dose of AZA decreased food intake at 360 min post-injection (Fig. 6A). On a non-cumulative basis, this effect was also observed, and quail injected with 5 µg of AZA ate less than vehicle-injected birds at 120 min post-injection (Fig. 6B). Although water intake was not affected by AZA on a cumulative basis (Fig. 6C), on a non-cumulative basis, 1 µg of AZA caused quail to drink less compared with the vehicle-injected group at 120 min post-injection (Fig. 6D). Later at 180 min, more water was ingested in the 1 µg than the 5 µg dose group.

3.4. Food and water intake in AZA-injected fasted broilers and quail

Food and water intake were not unaffected by AZA in fasted broiler chickens (Fig. 7).

In fasted quail, however, the 25 µg dose of AZA decreased food and water intake from 60 to 360 min post-injection, although a significant difference in food intake was in comparison to the 5 µg rather than vehicle group at 90- and 120-minute post-injection (Fig. 8A and 8C). On a non-cumulative basis, quail injected with 25 µg of AZA ate less than those injected with 5 µg of AZA at 60- and 360-min post-injection and those in the other two groups at 360 min post-injection (Fig. 8B). Vehicle- and 1 µg AZA-injected quail drank more than 5 and 25 µg AZA-injected quail at 120- and 360-min post-injection, respectively (Fig. 8D). Greater water intake was observed in the 1 µg AZA group compared to the 25 µg dose of AZA at 60 min post-injection (Fig. 8D).

4. Discussion

The purpose of this study was to evaluate the effects of central administration of two methyl-modifying compounds, SAM and AZA, on food intake in two avian models. To our knowledge, the role of such molecules in appetite regulation is relatively unstudied. We observed a short-term appetite suppressive effect of SAM. At 30 min post-injection, both the middle and high doses of SAM tended to suppress appetite in broilers ($P = 0.07$), while the significant decrease occurred at 60 min post-injection. In layer-type Leghorn chicks, a similar tendency for inhibition of feeding was observed, but only in the 100 µg SAM-treated group and not at lower doses (1 and 10 µg) [3], indicating that broilers might have higher sensitivity to central administration of SAM than Leghorn chicks. This difference may be caused by different genetic backgrounds of meat- and egg-type chicks, which have been selected for

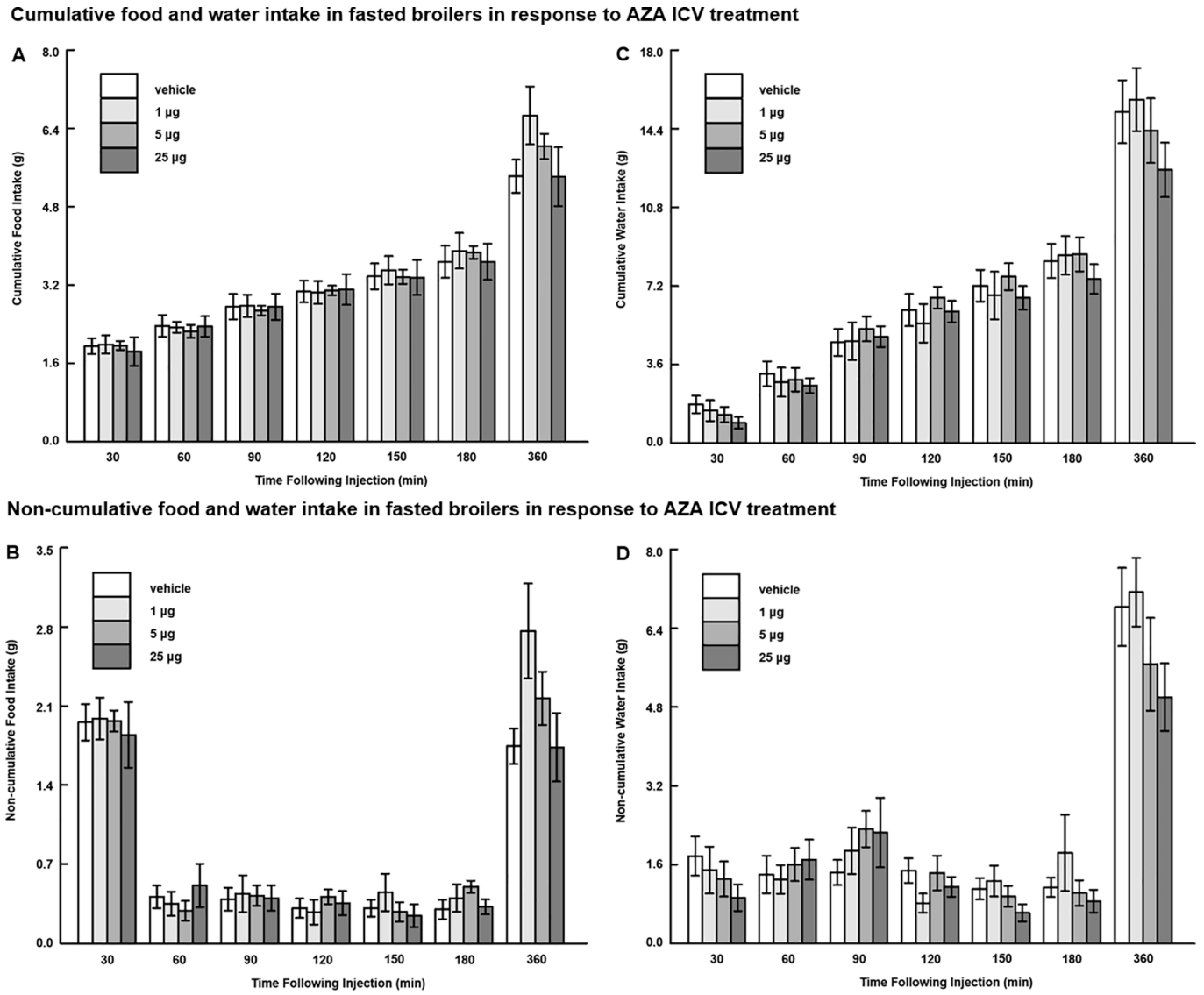

Fig. 7. Food and water intake in AZA-injected fasted broiler chickens. Cumulative (A) and non-cumulative (B) food intake at times post-injection of 4-day-old fasted broiler chicks (*Gallus gallus*) that were intracerebroventricularly (ICV) injected with 0 (vehicle), 1, 5, or 25 μg of AZA (n = 8–9 per group). Cumulative (C) and non-cumulative (D) water intake at times post-injection of the same chicks (n = 9–10 per group). Values represent means ± standard errors.

unique production purposes. In quail, we did not observe any effect when birds were fasted, but decreased food consumption was transiently observed in fed quail at 90 min post-injection. These results are consistent with broiler chicks, although quail appeared to be less sensitive than broilers, which might be attributed to species differences and artificial selection pressure. Typically, our feeding studies in chicks terminate at 3 h post-injection, because by this time there is a return to homeostatic feeding. However, because the effect of SAM on food intake is not well studied and it is known to play a role in epigenetic regulation due to its role as a methyl donor, we also measured food intake at 6 h post-injection, in order to determine whether there might be a longer-term effect on appetite regulation. However, no differences were detected at 360 min post-injection.

Fed broiler chickens treated with SAM ate less within one-hour post-injection. Although food intake was not affected during the first 30 min in fasted SAM-injected broilers, it decreased significantly during the second 30-minute period in the 1 μg SAM group. There was also a tendency in both 0.1 and 10 μg SAM groups ($P = 0.07$ and 0.09 respectively, not specified in Fig. 3B) for food intake to be reduced at 60 min post-injection. Taken together, both feeding and prior fasting resulted in suppression of feeding, indicating that there is an increase in anorexigenic tone.

While central injection of SAM caused a decreased in food intake in fasted broiler chicks, it increased water consumption in the same birds, although the effective doses were slightly different. This suggests that SAM might have opposite effects on appetite and thirst regulation when broilers have undergone fasting.

For AZA treatment in broiler chickens, we found no effects on cumulative food and water intake in either fed or fasted chicks, and only a few differences on a non-cumulative basis. These changes occurred at random time points and were not persistent, which might be caused by compensatory feeding of the birds to maintain the metabolic homeostasis or insensitivity of these broiler chicks due to selection. These results suggest that there are no direct effects of AZA on appetite regulation in broiler chicks.

Japanese quail responded to AZA more robustly, although the responses varied depending on nutritional states. The highest dose of AZA (25 μg) induced anorexia in both fed and fasted quail, but the effective duration was different. Fed quail ate less at 6 h post-injection, whereas for fasted quail this started at 1-h post-injection and lasted until the end

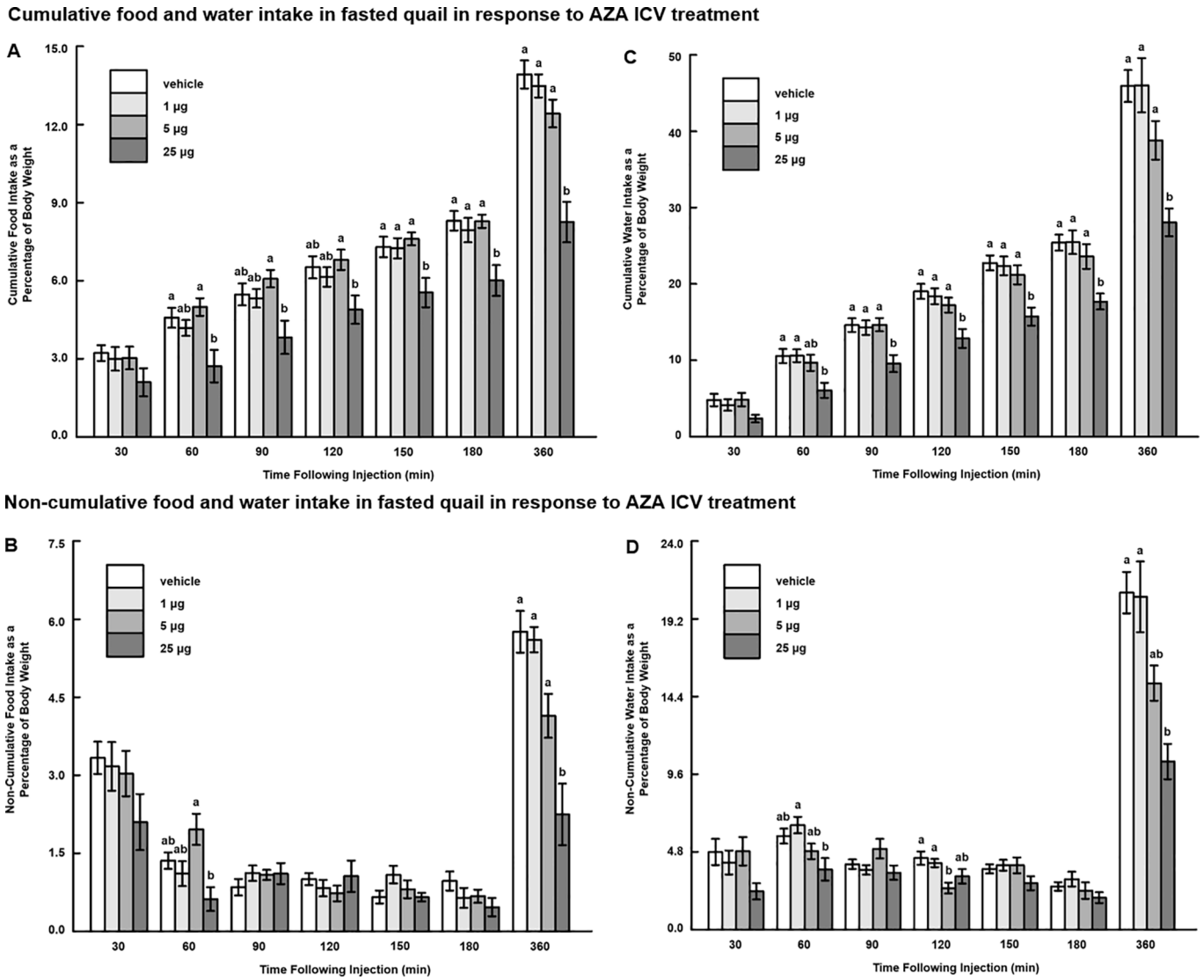

Fig. 8. Food and water intake in AZA-injected fasted quail. Cumulative (A) and non-cumulative (B) food intake as a percentage of body weight at times post-injection of 7-day-old fasted quail (*Coturnix japonica*) that were intracerebroventricularly (ICV) injected with 0 (vehicle), 1, 5, or 25 µg of AZA (n = 10 per group). Cumulative (C) and non-cumulative (D) water intake as a percentage of body weight at times post-injection of the same chicks (n = 10–11 per group). Values represent means ± standard errors. Unique letters denote a significant difference ($P < 0.05$) among groups within a time point, Tukey's test.

of the experiment, albeit the significance disappeared at 90 and 120 min. A similar study on low body weight selected (LWS) chickens (unpublished work from our group) suggested that the direct effects of AZA correlated with nutritional state, with reduced food intake in fed LWS but increased food intake in fasted LWS. The discrepancy of these two studies might be attributed to species differences and long-term artificial selection. It is worth noting that the anorexigenic effect of AZA was not observed immediately after injection yet became significant in fed and fasted quail at 6 h post-injection both on a cumulative and non-cumulative basis, suggesting that this effect might involve epigenetic changes modifying appetite-related genes' methylation and expression. Whether this persists to a later age is still unclear and warrants further investigation.

5. Conclusion

In conclusion, novel regulators of appetite are continuing to be identified. Here we evaluated two molecules related to methylation modification, in broiler chickens and Japanese quail. Central injection of methyl donor SAM suppressed food intake in both fed and fasted broilers and quail. In contrast, methylation inhibitor, AZA, did not affect food intake in either fed or fasted broiler chickens, whereas it decreased food intake in fed and fasted quail, although with differences in the effective duration. Thus, SAM induces transient short-term satiety in broilers and quail, while AZA-induced anorexia depends on species and nutritional states. Both of these molecules may thus elicit direct effects on regulating appetite. Further studies should involve exploration of the associated molecular mechanisms.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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