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Central Administration of Agouti-Related Peptide Increases Food Intake in Japanese Quail

Tyler Lindskoog, Mark Bohler, Elizabeth R. Gilbert, Mark A. Cline[1]

Department of Animal and Poultry Sciences
Virginia Polytechnic Institute and State University
Blacksburg, Virginia, United States

ABSTRACT

Agouti-related peptide is a 132-amino acid peptide associated with stimulating food intake in birds and mammals. The aim of the present study was to investigate the effect of AgRP in seven-day old Japanese quail. In Experiment 1, we tested 0.25, 0.5 and 1.0 nmol AgRP and found no effect on food or water intake over a three-hour period. In Experiment 2, we tested 0.25, 0.5 and 1.0 nmol AgRP and found no effect on food or water intake over 24 hours. In Experiment 3, we tested 0.0625 and 0.125 nmol AgRP and found no effect on food intake over a 24-hour duration, but found an increase in water intake 900 minutes following injection. In Experiment 4, we found an increase in food and water intake 900 minutes following injection in quail which received 1.5, but not 3.0, nmol AgRP. In Experiment 5, we found that AgRP had no effect on behaviors other than food intake. These results suggest that AgRP might have a stimulatory effect on food intake in Japanese quail.

INTRODUCTION

Agouti-related peptide (AgRP) is a peptide consisting of 132 amino acids that was first found in the hypothalamus of mice in 1997 (Ollmann, Wilson et al. 1997, Shutter, Graham et al. 1997). Since then, the orexigenic effect of AgRP has been studied in many species including the rat (Csiffáry, Görcs, et al. 1990, Cowley, Pronchuk et al. 1999, Hagan, Rushing et al. 2000, Hagan, Benoit et al. 2001), mouse (Rossi, Kim et al. 1998, Cowley, Pronchuk et al. 1999, Wang, Saint-Pierre et al. 2002), chicken (Tachibana, Sugahara et al. 2001), and ring-tail dove (Strader, Schiöth et al. 2003). AgRP is primarily found in the hypothalamus and is involved in energy homeostasis

[1] Corresponding author contact: macline2@vt.edu; phone: 00-1-540-231-4477

by acting on the melanocortin system. More specifically, AgRP acts as an antagonist for the melanocortin-3 receptor (MC3R) and melanocortin-4 receptor (MC4R) to stimulate food intake (Ollmann, Wilson et al. 1997, Rossi, Kim et al. 1998, Gropp, Shanabrough et al. 2005). Most of what is known regarding the central mechanisms mediating AgRP induced food intake was found in rodent models.

The arcuate nucleus (ARC) of the hypothalamus has been defined as a key mediator of food intake via signaling from two neuron populations: neuropeptide Y (NPY), which produce orexigenic factor NPY, and pro-opiomelanocortin (POMC) neurons that release anorexigenic factors (Funahashi, Hori et al. 2000). NPY neurons co-release γ -aminobutyric acid (GABA) (Horvath, Bechmann et al. 1997) and AgRP (Hahn, Breininger et al. 1998), while POMC neurons release POMC-derived peptides and co-express cocaine-amphetamine related transcript (CART) (Vrang, Larsen et al. 1999). Many neuropeptides can be cleaved from POMC, including α-melanocyte stimulating hormone (α-MSH) (Eipper and Mains 1980, Drouin, Chamberland et al. 1985) which is the endogenous ligand for MC3R (Roselli-Rehfuss, Mountjoy et al. 1993) and MC4R (Mountjoy, Mortrud et al. 1994). In mice, NPY and AgRP neurons are stimulated by increased levels of circulating ghrelin (Wang, Saint-Pierre et al. 2002) and are hyperpolarized by circulating leptin (Cowley, Smart et al. 2001). Upon release, AgRP antagonizes the MC3R (Cowley, Smith et al. 2003) on NPY neurons (Mounien, Bizet et al. 2005), preventing inhibition via α-MSH binding (Cowley, Smith et al. 2003), and POMC neurons, preventing POMC-derived factor release (Rau and Hentges 2017). AgRP is also known to mediate food intake by blocking α-MSH action in the paraventricular nucleus (PVN) (Cowley, Pronchuk et al. 1999).

ICV injection of AgRP increases food intake in rats (Hagan, Rushing et al. 2000, Hagan, Benoit et al. 2001), layer type chickens (Tachibana, Sugahara et al. 2001), and ring doves (Strader, Schiöth et al. 2003). While NPY is a potent initiator of food intake, AgRP causes a prolonged increase in food intake in mice (1 week) (Krashes, Shah et al. 2013) and rats (24 hours) (Hagan, Rushing et al. 2000). To our knowledge, no one has explored the effects of AgRP on food intake in Japanese quail. That AgRP differentially affected layer and broiler type chickens (Tachibana, Sugahara et al. 2001) may be explained by genetic differences between strains. The chicken has undergone the most intense artificial selection of any bird, which has likely influenced the neurobiology and physiology mediating feeding behavior. More specifically, the broiler chicken is selected for growth and meat production purposes and is known to eat without cessation, likely because it lacks a satiety mechanism (Furuse 2002). It is hypothesized that broiler chicks produce less glucagon like peptide 1 (GLP-1) and α-MSH than layers which may explain the differences in food intake (Furuse 2002). Because of this intense selection the chicken may not be an ideal model for general avian physiology. The Japanese quail (*Coturnix japonica*) has undergone less intense selection than the chicken, but still adapts well to caged environments and handling procedures. The objective of this research was thus to explore the orexigenic effect of AgRP in Japanese quail.

MATERIALS AND METHODS

Animals

Japanese quail were bred and hatched in our vivarium. Upon removal from the hatcher, chicks were group-caged in a brooder for four days, then individually in galvanized wire cages (8 cm wide, 7 cm deep and 8 cm high) in a room at a constant temperature of 35 ± 1 °C, 50 ± 5% relative humidity, with a 14-hour light/10-hour dark period (lights on at 00:00 hours). At all times, unless otherwise noted, chicks had ad libitum access to tap water and a mash starter diet formulated to meet or exceed the recommended nutrient specifications for the starter phase of growth (2900 kcal ME/kg and 24% CP) (Poos 1994). The individual cages allowed visual and auditory contact with other chicks.

After the chicks were individually caged, they were handled twice daily to reduce the effects of stress on the day of data collection. The handling procedure consisted of the chick being removed from its cage and placed in a small Plexiglas box where it remained momentarily before being transferred to another Plexiglas box where it remained momentarily. The chick was removed from the second Plexiglas box and its head inserted into a restraining device and held in this position for five seconds. The chick was then placed back into the Plexiglas box momentarily before having its head repositioned in the restraining device for another five seconds. Following removal from the restraining device for the second time the chick was placed back into its home cage. This procedure was conducted twice daily from the day they were caged (day 3 post hatch) to the day before the experiment (day 6 post hatch). The restraining device was designed with two air vents positioned to end at the nostrils. All 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.

ICV injection procedure

On the day of the experiment, quail were injected using an adapted method that does not appear to induce physiological stress (Lear, Liu et al. 2017, Yuan, Gilbert et al. 2017). The head of the quail was briefly inserted into the restraining device. Injection coordinates were 2 mm anterior to the coronal suture, 0.75 mm lateral from the sagittal suture, and 1.5 mm deep, targeting the left lateral ventricle. Anatomical landmarks were determined both visually and by palpation. Injection depth was controlled by placing a plastic tubing sheath over the base of the needle. The needle remained at injection depth in the un-anesthetized quail for five seconds post-injection to reduce backflow. Quail were assigned to treatments at random. Human AgRP (83-132) (Phoenix Pharmaceuticals, Burlingame, CA, USA) was dissolved in 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 quail was decapitated, and its head sectioned along the frontal plane to determine the site of injection. Any quail without dye present in the lateral ventricle system was eliminated from analysis. The sex of the quail was determined visually by dissection at the time of decapitation.

Experiment 1: Food and water intake following 0, 0.25, 0.5, or 1.0 nmol doses, 3-hours

Quail were randomly assigned to receive either 0 (vehicle only), 0.25, 0.5, or 1.0 nmol AgRP by ICV injection. After injection, quail 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 minutes for 180 minutes post-injection. Water weight (g) was converted to volume (ml; 1 g = 1 ml). The data were analyzed using analysis of variance (ANOVA) within each time point via the GLM procedure in SAS 9.3 (SAS institute, Cary, NC, USA) with the statistical model including the main effect of dose. When dose effects were significant, Tukey's method of multiple comparisons was used to separate the means within each time point. Statistical significance was set at $P < 0.05$ for all experiments. Food and water intake results are shown on a cumulative and non-cumulative basis.

Experiment 2: Food and water intake following 0, 0.25, 0.5, or 1.0 nmol doses, 24-hours

Procedures were identical to Experiment 1, except that food and water was monitored every 30 minutes for 180 minutes and then at 6, 9, 12, 15 and 24 hours post-injection.

Experiment 3: Food and water intake following 0, 0.0625 or 0.125 nmol doses, 24-hours

Procedures were identical to Experiment 2, except that the doses used were 0, 0.0625 and 0.125 nmol AgRP.

Experiment 4: Food and water intake following 0, 1.5 or 3.0 nmol doses, 24-hours

Procedures were identical to Experiment 2, except that the doses used were 0, 1.5 and 3.0 nmol AgRP.

Experiment 5: Behavioral analysis

The behavior test was conducted in the light cycle. The quail were tested in the same room where birds were housed to avoid the stress from a novel environment and the technician was not visible to the bird during the recording. In addition to the injection acclimation procedure, to acclimate quail to the behavior recording arenas, quail were placed in mock acrylic recording arenas for one hour each day. The quail were kept in the individual cages with auditory but not visual contact with each other (to reduce isolation stress during the observational period) and were randomly assigned to receive either vehicle or 1 nmol by ICV injection. Injections were performed, and the quail were immediately placed in a 290 mm × 290 mm acrylic recording arena with food and water containers in diagonal corners. Quail were simultaneously and automatically recorded from three angles for 30 minutes post-injection on DVD and the data were analyzed in five-minute intervals using ANY-maze behavioral analysis software (Stoelting, Wood Dale, IL). At 30 minutes post-injection, food intake was measured. Locomotion (m traveled), the amount of time spent standing, sitting, preening, or in deep rest, as well as the number of defecations, jumps, steps, feed pecks, exploratory pecks, and escape attempts were quantified. Food pecks were defined as pecks within the food container, whereas any other pecks were counted as exploratory. Deep rest was defined as having the eyes closed for greater than three seconds (and its timing starting three seconds after eye closure, ending when eyes reopened). Preening was defined as trimming or dressing down with the beak. Due to non-heterogeneous variance, behavioral data were analyzed

by the Mann–Whitney U test. Pecking efficiency at 30 minutes post-injection was calculated by dividing the amount of food consumed by the number of food pecks for each quail. Pecking efficiency and food intake data were analyzed by ANOVA.

RESULTS

Experiment 1: Food and water intake following 0, 0.25, 0.5, or 1.0 nmol doses, 3-hours

Quail which received an ICV injection of AgRP consumed the same amount of food as vehicle-only quail on a cumulative (Figure 1a) and non-cumulative (Figure 1b) basis. Similarly, quail which received an ICV injection of AgRP consumed the same amount of water as vehicle-only quail on a cumulative (Figure 2a) and non-cumulative (Figure 2b) basis.

Experiment 2: Food and water intake following 0, 0.25, 0.5, or 1.0 nmol doses, 24-hours

Quail which received an ICV injection of AgRP consumed the same amount of food as vehicle-only quail on a cumulative (Figure 3a) and non-cumulative (Figure 3b) basis. Similarly, quail which received an ICV injection of AgRP consumed the same amount of water as vehicle-only quail on a cumulative (Figure 4a) and non-cumulative (Figure 4b) basis.

Experiment 3: Food and water intake following 0, 0.0625 or 0.125 nmol doses, 24-hours

Quail which received an ICV injection of AgRP consumed the same amount of food on a cumulative (Figure 5a) and non-cumulative (Figure 5b) basis. However, quail which received AgRP increased water intake (Figure 6). While there was no effect of AgRP on cumulative water intake (Figure 6a), on a non-cumulative basis (Figure 6b) those which received 0.0625 and 0.125 nmol AgRP increased water intake 900 minutes following injection.

Experiment 4: Food and water intake following 0, 1.5 or 3.0 nmol doses, 24-hours

Quail which received an ICV injection of AgRP increased food (Figure 7) and water (Figure 8) intake. Quail that received an injection of 1.5 nmol AgRP consumed more food 900 minutes following injection on a cumulative (Figure 7a) and non-cumulative (Figure 7b) basis. Chicks that received 3.0 nmol AgRP did not consume more than vehicle-only quail. Quail that received an injection of 1.5 nmol AgRP also consumed more water 900 minutes following injection on a cumulative (Figure 8a) and non-cumulative (Figure 8b) basis. The increase in food and water intake following injection of 1.5 nmol AgRP disappeared by 1440 minutes post-injection.

Experiment 5: Behavioral analysis.

Quail which received 1 nmol AgRP did not exhibit any differences in count-based behaviors (Table 1) or timed behaviors (Table 2).

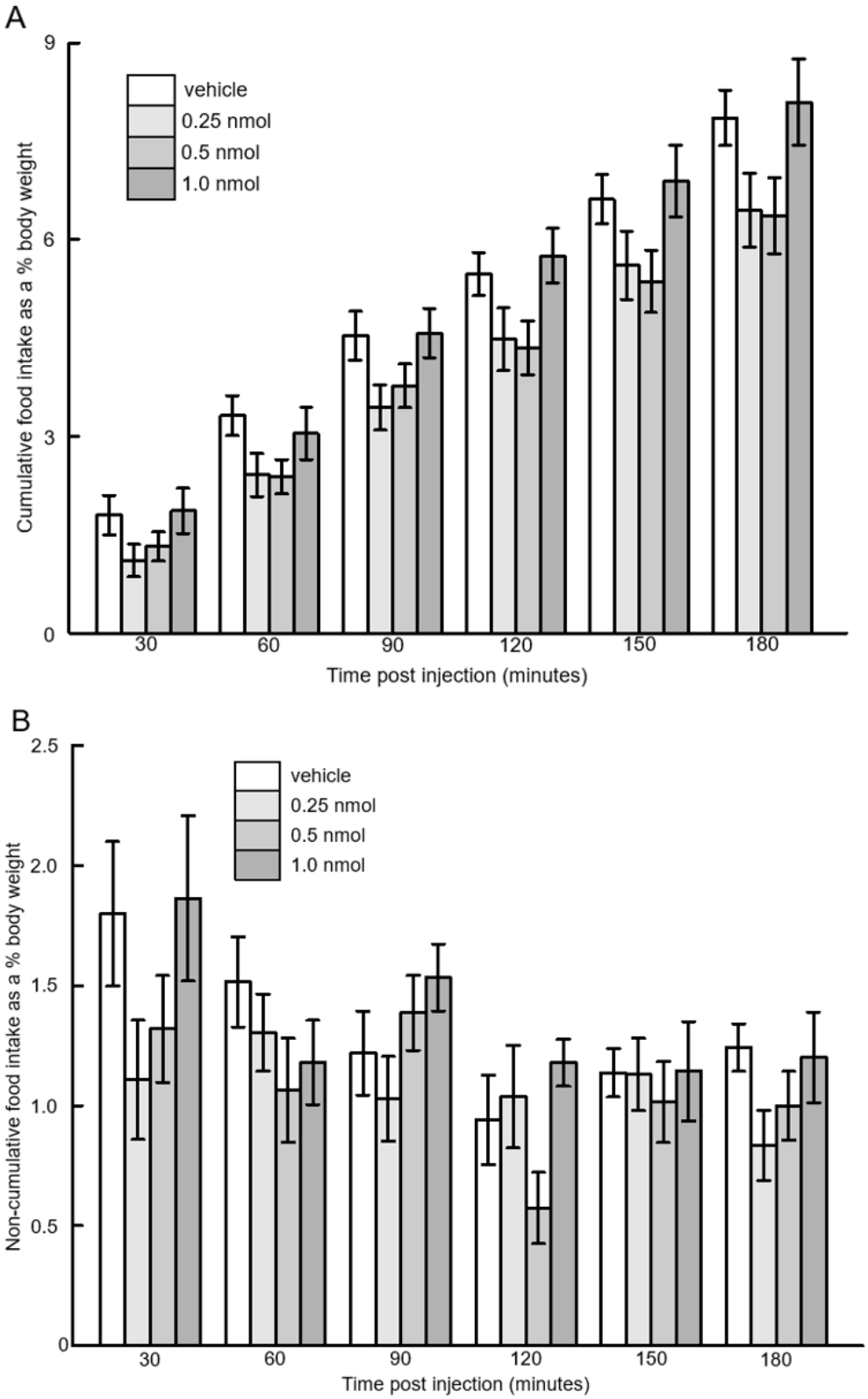

Fig 1. Experiment 1. Cumulative (a) and non-cumulative (b) food intake as a percentage of body weight following intracerebroventricular injection of AgRP in 7-day post-hatch Japanese quail. Values are means ± standard errors. For this experiment, there were 10-12 quail per treatment for analysis.

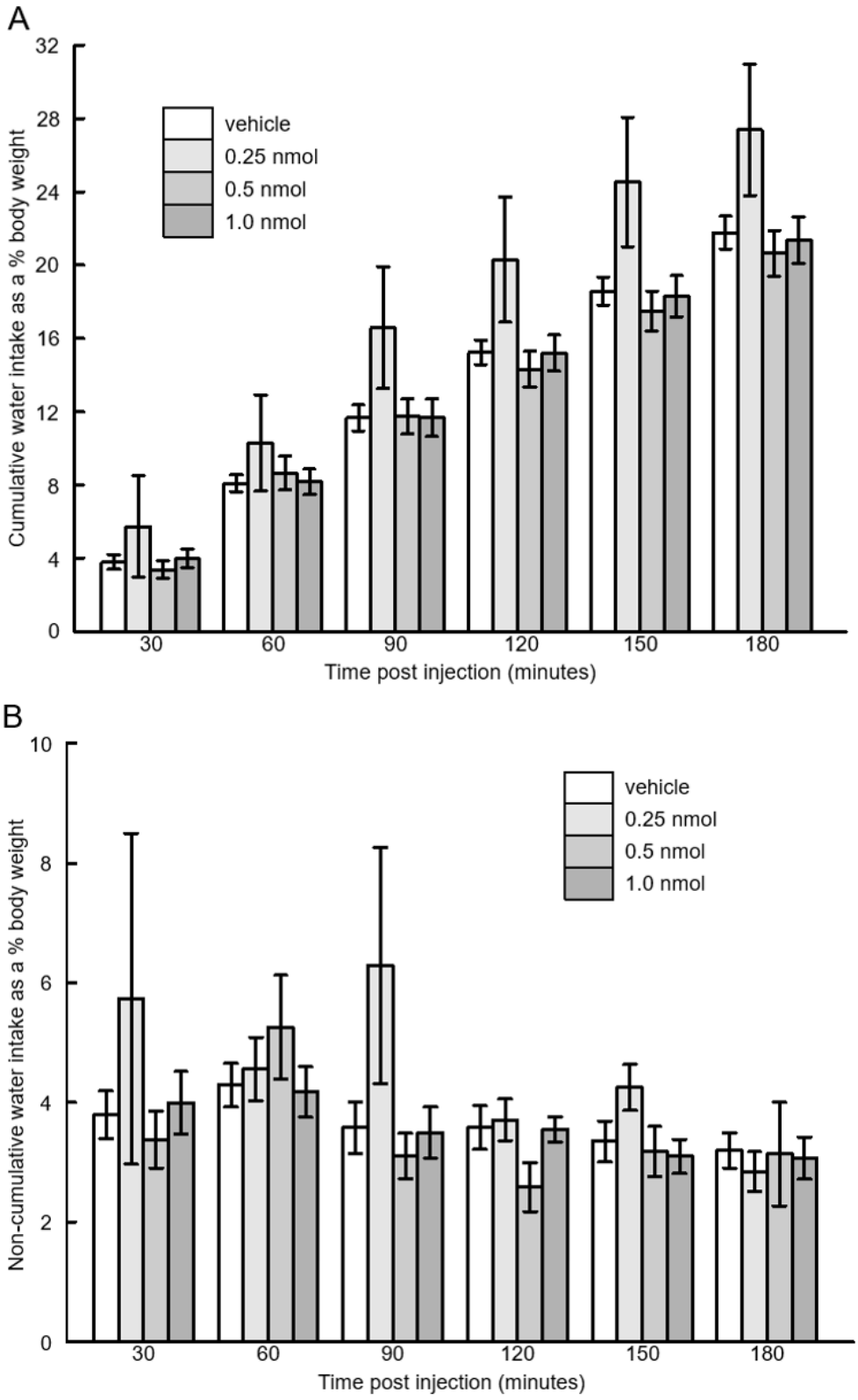

Fig 2. Experiment 1. Cumulative (a) and non-cumulative (b) water intake as a percentage of body weight following intracerebroventricular injection of AgRP in 7-day post-hatch Japanese quail. Values are means ± standard errors. For this experiment, there were 10-12 quail per treatment for analysis.

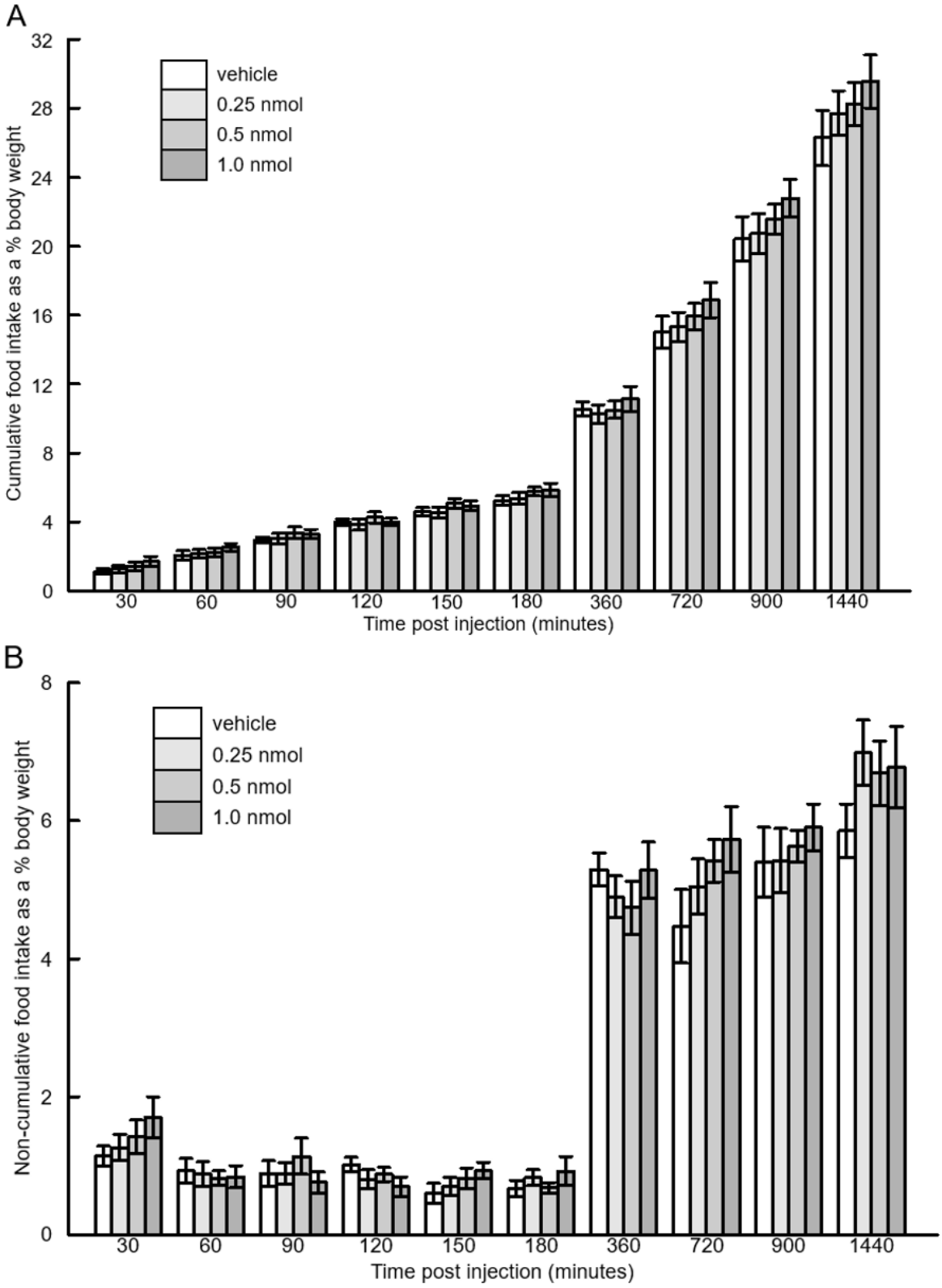

Fig 3. Experiment 2. Cumulative (a) and non-cumulative (b) food intake as a percentage of body weight following intracerebroventricular injection of AgRP in 7-day post-hatch Japanese quail. Values are means ± standard errors. For this experiment, there were 10-12 quail per treatment for analysis.

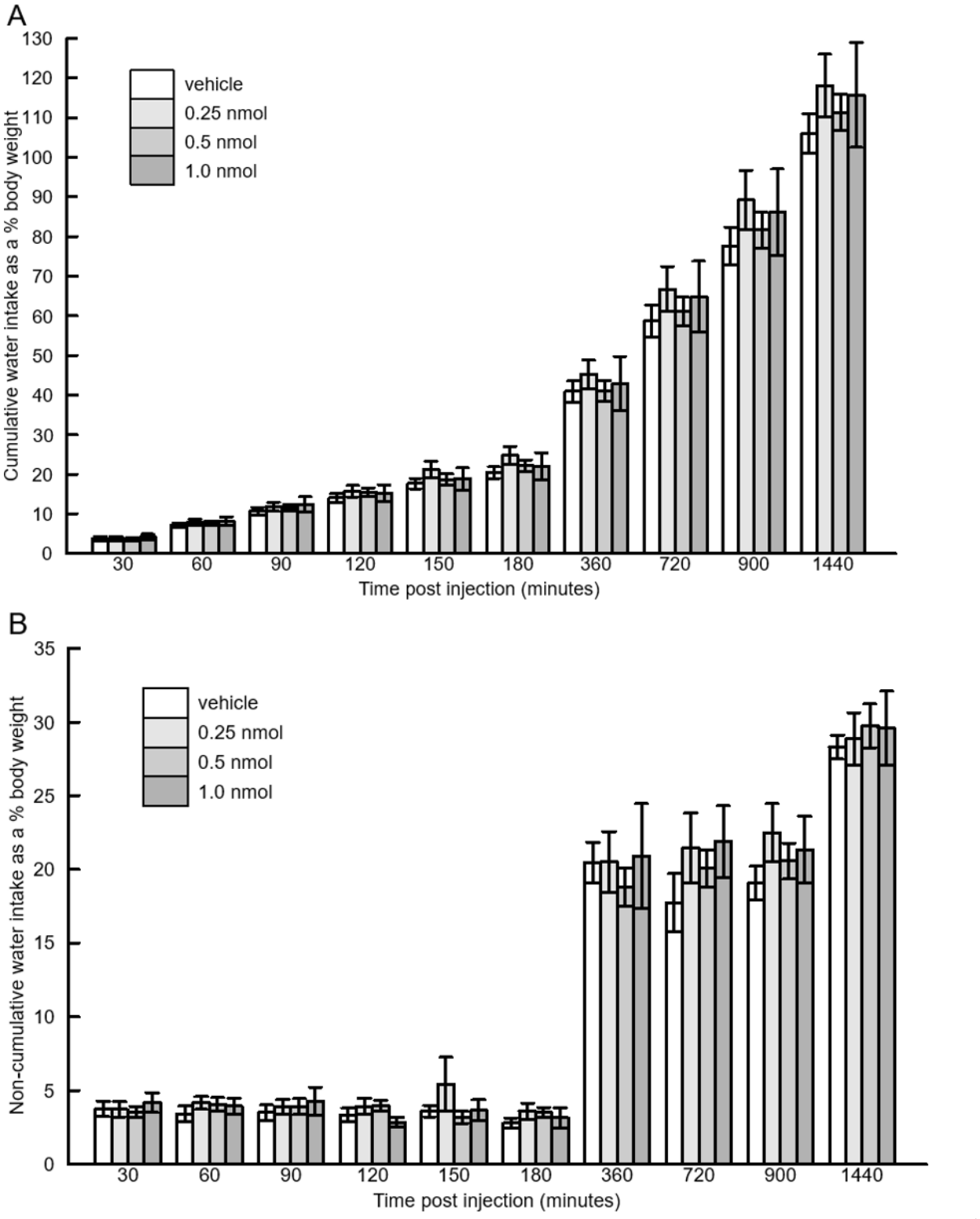

Fig 4. Experiment 2. Cumulative (a) and non-cumulative (b) water intake as a percentage of body weight following intracerebroventricular injection of AgRP in 7-day post-hatch Japanese quail. Values are means ± standard errors. For this experiment, there were 10-12 quail per treatment for analysis.

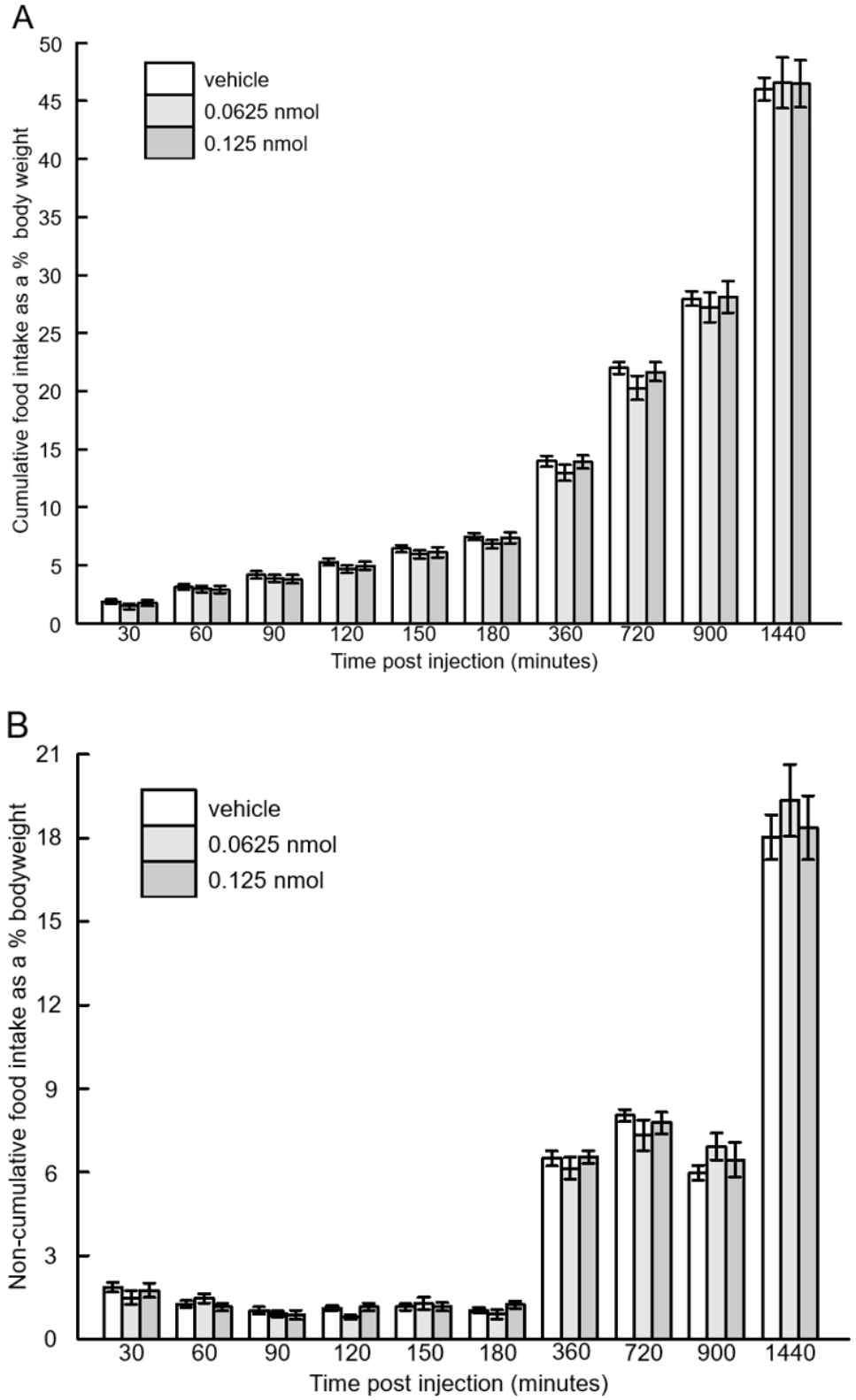

Fig 5. Experiment 3. Cumulative (a) and non-cumulative (b) food intake as a percentage of body weight following intracerebroventricular injection of AgRP in 7-day post-hatch Japanese quail. Values are means ± standard errors. For this experiment, there were 10-12 quail per treatment for analysis.

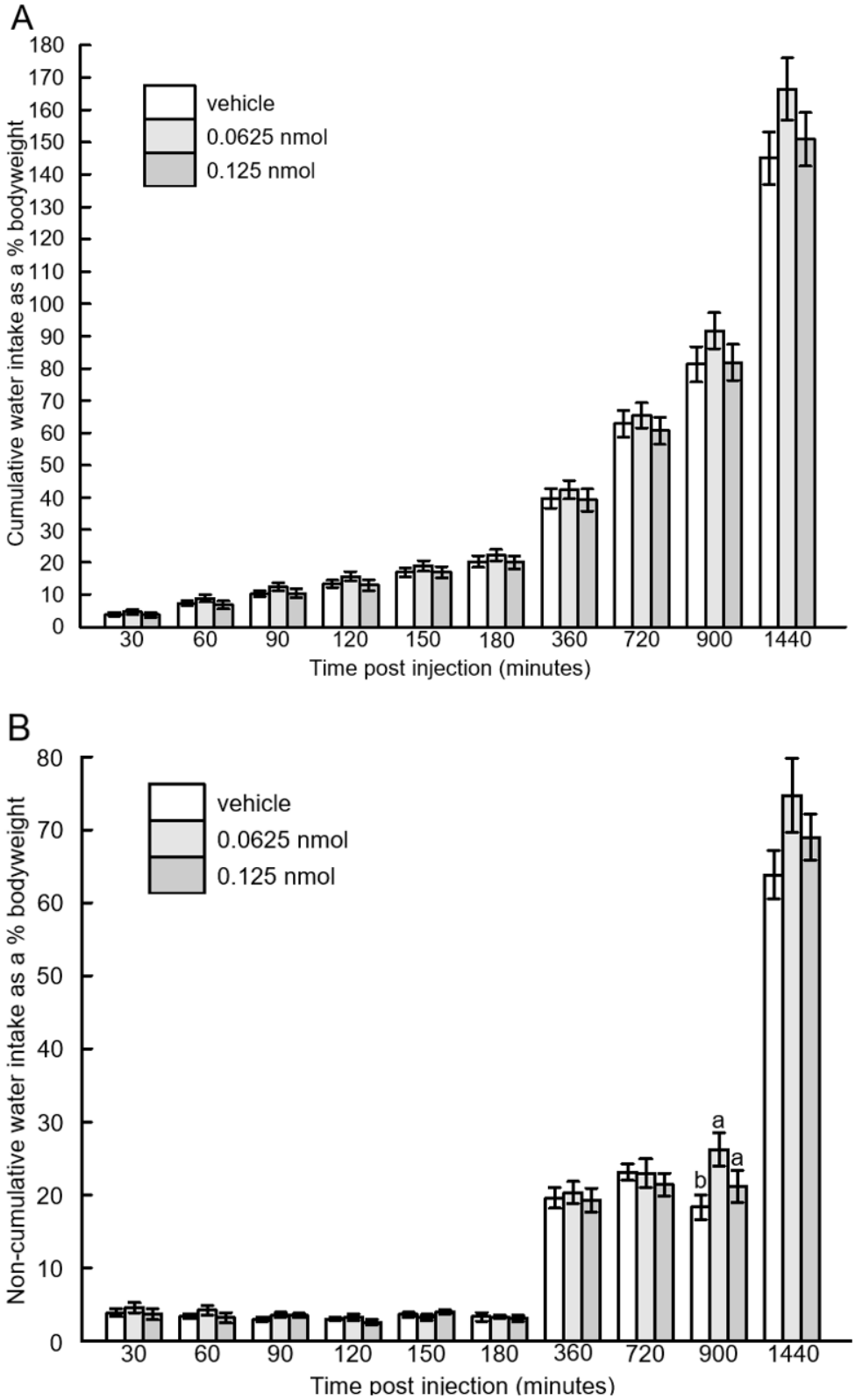

Fig 6. Experiment 3. Cumulative (a) and non-cumulative (b) water intake as a percentage of body weight following intracerebroventricular injection of AgRP in 7-day post-hatch Japanese quail. Values are means ± standard errors. For this experiment, there were 10-12 quail per treatment for analysis.

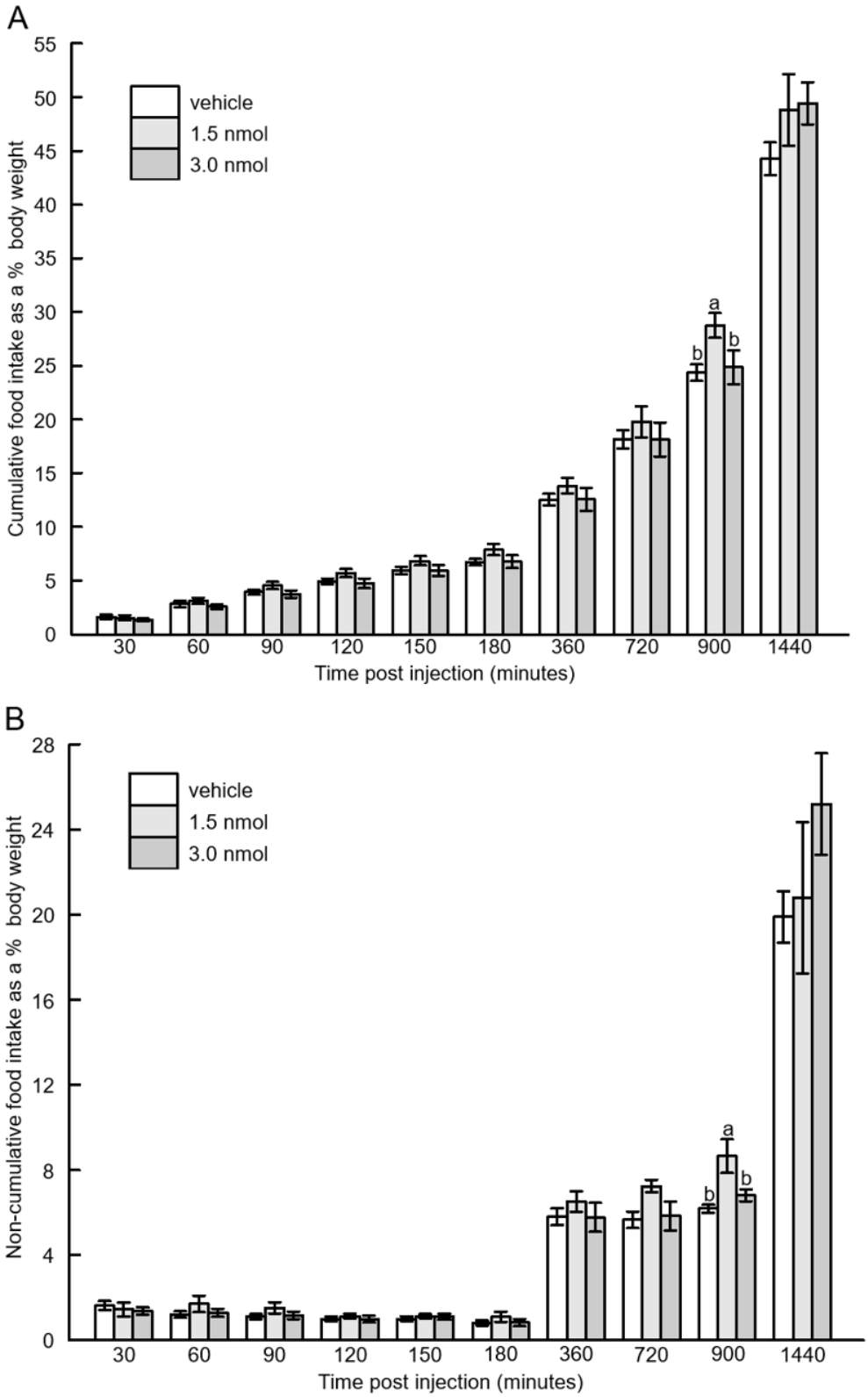

Fig 7. Experiment 4. Cumulative (a) and non-cumulative (b) food intake as a percentage of body weight following intracerebroventricular injection of AgRP in 7-day post-hatch Japanese quail. Values are means ± standard errors. For this experiment, there were 10-12 quail per treatment for analysis

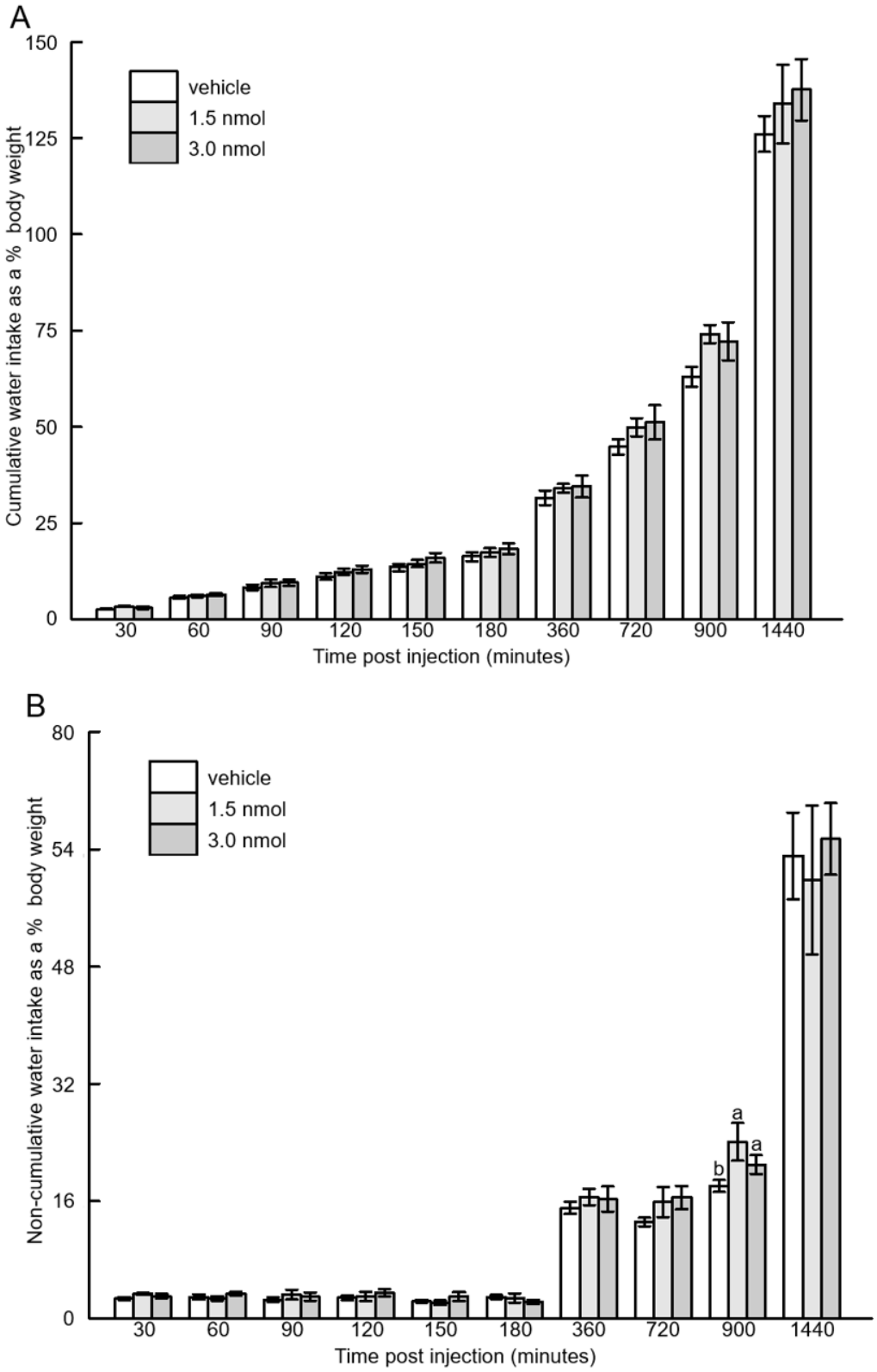

Fig 8. Experiment 4. Cumulative (a) and non-cumulative (b) water intake as a percentage of body weight following intracerebroventricular injection of AgRP in 7-day post-hatch Japanese quail. Values are means ± standard errors. For this experiment, there were 10-12 quail per treatment for analysis.

TABLES (Experiment 5): The effect of intracerebroventricular injection of AgRP on appetite and mobility related behaviors in Japanese quail.

Table 1:

Count-type behaviors[1].

Behaviors (n)	Treatment	Time post injection (min)					
		5	10	15	20	25	30
Locomotion (m)	Control	3.4± 0.6	7.6± 1.7	11.5± 2.5	15.7± 3.3	20.59± 4.37	24.37± 5.11
	AgRP	5.0± 1.1	10.3± 1.8	15.2± 2.2	21.2± 3.1	26.8± 3.9	31.6± 4.7
Steps	Control	176.0± 36.8	330.6± 57.6	494.6± 88.3	665.5± 123.8	848.3± 156.4	1007.6±193.8
	AgRP	207.7± 35.6	412.5± 59.4	601.5± 85.1	811.7± 105.7	1009.6±129.6	1170.0±152.7
Feed Pecks	Control	10.0± 7.2	40.0± 16.3	88.3± 24.1	125.3± 30.1	154.6± 29.8	189.4± 34.4
	AgRP	27.9± 21.8	89.9± 32.7	169.1± 44.4	229.0± 52.4	295.0± 68.3	371.7± 82.0
Jumps	Control	2.6± 1.2	6.1± 3.6	6.8± 3.7	9.9± 4.6	10.3± 4.6	10.5± 4.6
	AgRP	6.2± 5.6	9.4± 5.7	12.4± 5.6	14.6± 6.1	15.3± 6.7	16.2± 7.0
Defecations	Control	0	0.3± 0.3	0.6± 0.4	0.7± 0.4	0.8± 0.4	1.0± 0.4
	AgRP	0.4± 0.2	0.6± 0.3	0.9± 0.5	1.4± 0.6	1.4± 0.6	1.5± 0.7
Exploratory Pecks	Control	41.9± 14.3	86.6± 26.7	125.0±36.4	166.2± 3.1	213.2±51.9	251.6±58.8
	AgRP	32.8± 11.2	80.2± 22.0	122.8±27.2	165.8±35.7	206.2±44.0	244.1±48.8
Escape Attempts	Control	0	0	0	0	0	0
	AgRP	0	0	0	0	0	0
Water Pecks	Control	0	0	0	0	0	1.3± 1.3
	AgRP	0	0	0.3± 0.3	0.8± 0.6	0.8± 0.6	0.80± 0.6

[1]Data are expressed as means ± standard errors. For this experiment, there were 10 chicks per treatment for analysis.

Table 2:

Timed behaviors[1].

Behaviors (s)	Treatment	Time post injection (min)					
		5	10	15	20	25	30
Preen	Control	2.4± 1.0	8.2± 3.2	10.1± 3.6	15.6± 5.70	20.1± 6.7	23.2± 6.8
	AgRP	0.7± 0.4	1.7± 0.5	7.9± 4.6	11.8± 4.9	16.1± 6.1	19.3± 6.3
Deep Rest	Control	0	6.1± 6.1	9.2± 7.3	9.2± 7.3	11.2± 7.1	11.2± 7.1
	AgRP	0	0	0	0	0	0
Stand	Control	274.1±12.6	547.4±26.0	836.±30.0	1104.7±45.0	1381.5±54.7	1659.4±53.1
	AgRP	284.4±10.1	576.7±14.2	853.7±29.4	1148.4±32.8	1439.8±34.5	1714.0±37.5
Sit	Control	24.8±12.4	51.6± 25.8	63.1± 29.9	94.6± 45.2	118.2±54.9	134.7± 54.1
	AgRP	14.1±10.1	22.0±14.2	45.0±29.5	50.5±33.2	59.3±34.8	74.0± 36.4

[1]Data are expressed as means ± standard errors. For this experiment, there were 10 chicks per treatment for analysis.

DISCUSSION

Similar to the ring tail dove (Strader, Schiöth et al. 2003), ICV injection of AgRP was associated with an increase in food intake in Japanese quail. However, while the dove responded to 1 nmol AgRP 10 hours following injection, the quail responded only to 1.5 nmol AgRP 15 hours following injection. It is interesting to note the differences in threshold and response latency across avian species. ICV injection of 0.48 and 0.96 nmol AgRP increased food intake in layer type chicks, but not broilers (Tachibana, Sugahara et al. 2001). While this dose is relatively close to that used in the dove study, layer type chickens increased food intake within an hour post-injection. This response latency is more similar to that of the rat (Hagan, Rushing et al. 2000, Hagan, Benoit et al. 2001), mouse (Joppa, Ling et al. 2005) and hamster (Day and Bartness 2004). Following ICV AgRP, rodents exhibit elevated food intake for up to a week following injection. In rats (Hagan, Rushing et al. 2000), mice (Joppa, Ling et al. 2005), and Siberian hamsters (Day and Bartness 2004), 0.1 nmol AgRP increased food intake for up to 24 hours following injection. Also in rats, 0.01 nmol and 1 nmol increased food intake for up to seven days following injection (Hagan, Rushing et al. 2000). While a response duration has yet to be determined in any avian species, our data suggest that AgRP increases food intake for less than nine hours in Japanese quail. In the present study, we observed an increase in water intake 900 minutes following injection of low (0.0625 and 0.125 nmol) and high (1.5 nmol) doses of AgRP. To our knowledge, AgRP has yet to be associated with water intake. While the increase in water intake observed following administration of 1.5 nmol AgRP may be prandial, that 0.0625 and

0.125 nmol increased water intake suggests a possible dose-dependent role for AgRP in mediating water intake in birds. That only 1.5 nmol worked in the present study suggests that there is likely a tight threshold for AgRP stimulation of food intake in birds; too little is not sufficient for stimulating food intake, while too much may initiate anorexigenic tone, blocking the effect of AgRP. AgRP is also associated with increased food hoarding in hamsters (Day and Bartness 2004) and compulsive behaviors such as pacing, grooming, and digging in mice (Dietrich, Zimmer et al. 2015). In the present study, AgRP did not influence behaviors that could be competitive with food intake. To our knowledge, this is the first report of the effects of AgRP on avian behaviors other than food intake.

While it is puzzling that AgRP did not affect food or water intake until 900 minutes following injection, it is known that AgRP can have lasting effects on neuronal activity up to a day following injection. Rats which received 1nmol ICV AgRP (83-132) ate more chow than control animals for nine hours following injection (Zheng, Corkern et al. 2002). In addition to having elevated food consumption, rats injected with 1 nmol AgRP (83-132) had increased c-Fos immunoreactivity in the lateral hypothalamus (LH), ARC, and the PVN (Zheng, Corkern et al. 2002). Looking deeper, the c-Fos immunoreactivity was most abundant in orexin neurons in the LH, which is hypothesized to be responsible for the long-term increase in food consumption (Zheng, Corkern et al. 2002). However, the orexins do not affect food intake in birds (Furuse, Ando et al. 1999). Rats which received AgRP also had increased c-Fos in CART neurons in the ARC, which is hypothesized to be a potential mechanism responsible for increased anorexigenic tone (Zheng, Corkern et al. 2002). That orexin does not affect food intake and birds, yet quail consumed more food than controls 900 minutes following injection, might suggest that AgRP stimulates food intake in birds via a different mechanism than in mammals.

The different response between chickens, doves, and quail may be related to the amino acid sequence of AgRP. In our study, we used a partial sequence of human AgRP; AgRP (83-132). This segment includes the c-terminal end of the AgRP protein which forms a loop necessary for melanocortin receptor binding and antagonism (Yang, Thompson et al. 1999, McNulty, Thompson et al. 2001, Kim, Yumkham et al. 2005). Also within this segment lies a β hairpin across residues 106-121 which encompasses an RFF triplet (Arg-Phe-Phe) which has also been deemed necessary for melanocortin receptor binding and antagonism (McNulty, Thompson et al. 2001). Human and Japanese quail AgRP share 44.7% identity. However, the c-terminal and β hairpin are relatively conserved with only three residues differing in the binding site. On the other hand, The AgRP protein in chicken and Japanese quail share 90.6% identity, and the sequences housing the loops for receptor binding only differ by a few residues. This is interesting, as human AgRP had a relatively quick effect in chicken compared to quail and doves. The AgRP sequence has not been sequenced for the ring tail dove, and so we were unable to compare with this species.

In the present study, ICV injection of AgRP was associated with an increase in food intake 900 minutes following injection. While the bioactive segment of AgRP is relatively conserved across mammals and birds, the reasoning as to why quail, doves and chickens respond to varying degrees is not known. These are the first reports of the effect of AgRP on Japanese quail food intake and behavior.

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All authors contributed equally to this work.

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